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High-Efficiency Gene Inactivation and Replacement System for Gram-Positive Bacteria

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A system for high-efficiency single- and double-crossover homologous integration in gram-positive bacteria has been developed, with *Lactococcus lactis* as a model system. The system is based on a thermosensitive broad-host-range rolling-circle plasmid, pG$^+$host5, which contains a pBR322 replicon for propagation in *Escherichia coli* at 37°C. A nested set of *L. lactis* chromosomal fragments cloned onto pG$^+$host5 were used to show that the single-crossover integration frequency was logarithmically proportional to the length of homology for DNA fragments between 0.35 and 2.5 kb. Using random chromosomal 1-kb fragments, we showed that homologous integration can occur along the entire chromosome. We made use of the reported stimulatory effect of rolling-circle replication on intramolecular recombination to develop a protocol for gene replacement. Cultures were first maintained at 37°C to select for a bacterial population enriched for plasmid integrants; activation of the integrated rolling-circle plasmid by a temperature shift to 28°C resulted in efficient plasmid excision by homologous recombination and replacement of a chromosomal gene by the plasmid-carried modified copy. More than 50% of cells underwent replacement recombination when selection was applied for the replacing gene. Between 1 and 40% of cells underwent replacement recombination when no selection was applied. Chromosomal insertions and deletions were obtained in this way. These results show that gene replacement can be obtained at an extremely high efficiency by making use of the thermosensitive rolling-circle nature of the delivery vector. This procedure is applicable to numerous gram-positive bacteria.

Numerous gram-positive bacteria are targets of study as biological models (e.g., *Bacillus subtilis*), industrially important fermenter strains (the lactic acid bacteria), or pathogens (e.g., clostridia, listeria, staphylococci, and streptococci). Many strains of industrial or medical importance have been characterized physiologically, but relatively few have been studied or modified genetically. The study or modification of strains could be facilitated by the use of delivery vectors for the introduction of directed or nonspecific insertions in the bacterial chromosome. Delivery systems that rely on nonreplicative vectors are limited to bacteria that can be transformed at a high frequency, and those with conditionally active replicons are often restricted in their host range. Thus, the construction of recombinant strains requires substantial effort and can be applied efficiently only to particular organisms.

We previously described a broad-host-range thermosensitive (Ts) plasmid, pVE6002 (22), which was isolated from pGK12 (15). Such plasmids replicate by a rolling-circle (rc) mechanism (rc plasmids) (21). pVE6002 was shown to have potential use as a delivery vector in numerous gram-positive bacteria (22). Ts plasmids are nonreplicative at 37°C, making them particularly useful in bacteria with a low-temperature growth range or when a drastic thermal shock is undesirable. Here, we use a Ts plasmid derivative to characterize gene inactivation or replacement by homologous recombination, with *Lactococcus lactis* as the model system.

We examined single-crossover (sco) integration at several random locations on the *L. lactis* chromosome and at a defined region (*ilv* operon; 7). Integration frequencies varied between 10^{-5} and 10^{-2} and were affected by both the length and the nature or position of the recombining sequences. We also developed a system of high-efficiency gene replacement by double-crossover (dco) integration and tested it in a poorly transformable strain. With two different constructs, gene replacement in the chromosome was obtained at frequencies of between 50 and 98%. Furthermore, replacement or deletion of a chromosomal gene could be obtained without selection for the replacing gene at frequencies of between 1 and 40%. These frequencies are significantly higher than those previously reported for other gene replacement strategies. This high efficiency is attributed to the stimulatory effect of rc replication on intramolecular recombination. Since pG$^+$host plasmids can be established in numerous gram-positive organisms, this methodology may facilitate the construction of recombinant food-grade "safe" strains.

MATERIALS AND METHODS

Bacterial strains and plasmids and transformation and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani broth. *L. lactis* was cultured in and plated on M17-glucose broth (35) or minimal medium (32) when tested for the *ilv* phenotype. Erythromycin was added at 5 μg/ml for *L. lactis* and 150 μg/ml for *E. coli*, and tetracycline was used at 12.5 μg/ml for *L. lactis*. Electroporation of *L. lactis* by published methods (13) yielded between 10^5 and 10^6 transformants per μg of plasmid DNA for IL1403 and about 10^2 transformants per μg of plasmid DNA for NCDO2118, the *ilv*$^+$ strain used for the gene replacement experiments. *E. coli* was transformed as described before (11).

Construction of plasmids for integration. (i) Construction of the vector. pG$^+$host4 (previously published as pVE6004; 22) is a Ts derivative of pWV01 carrying the Em^r gene of pE194 and a multicloning site originating from pBluescript (Strata-

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TABLE 1. List of strains and plasmids

Strain or plasmid	Genetic markers and/or description	Source or reference
Strains		
L. lactis		
IL1403	Plasmid free; r^- m^-; prophages bI285 and bI286	2
NCDO2118	Natural isolate	National Collection of Dairy Organisms
E. coli TG1	*supE* Δ*hsd-5 thi* Δ(*lac-proAB*) F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	31
Plasmids		
pG$^+$host4	Ts derivative of pGK12; Em^r	22
pG$^+$host5	*Nsi*I-blunt-ended pG$^+$host4 fragment joined to the 1.46-kb *Ava*I-*Alw*NI-blunt-ended pBR322 fragment; Em^r	This work
pVE7021 to pVE7034	*Sma*I-*Hin*dIII-restricted pG$^+$host5 joined to an *Eco*RV-*Hin*dIII random chromosomal fragment of IL1403; Em^r	This work
pIL515	3.9-kb *Eco*RI *ilv* fragment of IL1403 in pBluescript; Amp^r	6
pVE7009	3.9-kb *Eco*RI fragment of pIL515 joined to *Eco*RI-restricted pG$^+$host5	This work (see Fig. 2A)
pVE7009R	Same construct as pVE7009, but with the insert in the opposite orientation	This work (see Fig. 2A)
pVE7015	*Sph*I-*Eco*RV deletion of pVE7009R, leaving a 3,362-bp *ilv* fragment	This work (see Fig. 2A)
pVE7014	*Sty*I-*Eco*RV deletion of pVE7009R, leaving a 2,904-bp *ilv* fragment	This work (see Fig. 2A)
pVE7010	*Cla*I deletion of pVE7009R, leaving a 2,552-bp *ilv* fragment	This work (see Fig. 2A)
pVE7016	*Xcm*I-*Eco*RV deletion of pVE7009R, leaving a 1,912-bp *ilv* fragment	This work (see Fig. 2A)
pVE7013	*Aat*II-*Eco*RV deletion of pVE7009R, leaving a 1,206-bp *ilv* fragment	This work (see Fig. 2A)
pVE7011	*Hin*dIII deletion of pVE7009R, leaving a 497-bp *ilv* fragment	This work (see Fig. 2A)
pVE7012	*Pst*I deletion of pVE7009R, leaving a 356-bp *ilv* fragment	This work (see Fig. 2A)
pVE7017	*Pfl*MI-*Eco*RV deletion of pVE7009R, leaving a 330-bp *ilv* fragment	This work (see Fig. 2A)
pIL500	18.5-kb *Xba*I *ilv* fragment of the NCDO2118 chromosome in pIL253	7
pIL1202	*Xba*I-restricted pG$^+$host4 containing the 1.1-kb *Xba*I-*Bgl*II and the 2.5-kb *Eco*RI-*Xba*I extremities of the 18.5-kb fragment of pIL500 joined together by the 4-kb *Bam*HI *tetM* gene; for insertion of the *tetM* gene, the *Bgl*II, *Eco*RI, and *Bam*HI sites were blunt ended	6; see Fig. 4B
pIL1261	2.3-kb *Xba*I-*Eco*RI fragment from pIL500 disrupted by a 4-kb *Bam*HI *tetM* gene cloned in its *Bgl*II site and joined to *Xba*I-*Eco*RI-restricted pBluescript	8
pIL1263	*Xba*I-*Eco*RI-restricted pG$^+$host4 joined to the 6.3-kb *Xba*I-*Eco*RI fragment of pIL1261	8; see Fig. 4A

gene, La Jolla, Calif.). For facilitation of cloning in *E. coli*, the 1.4-kb fragment containing the pBR322 origin was inserted into pG$^+$host4 (Table 1). The resulting plasmid was designated pG$^+$host5 (Appligene, Illkirch, France). The pBR322 origin activity allowed maintenance of the plasmid at 37°C in *E. coli*, and the Ts origin maintained pG$^+$host5 at 28°C in gram-positive bacteria.

(ii) Cloning of random *L. lactis* chromosomal fragments into pG$^+$host5. Chromosomal DNA of IL1403 was restricted to completion with *Eco*RV and *Hin*dIII. Chromosomal fragments of between 0.9 and 1.4 kb were purified from agarose gels with Geneclean (Bio 101, Inc., La Jolla, Calif.) and ligated with *Sma*I-*Hin*dIII-restricted pG$^+$host5. Recombinant plasmids were established in *E. coli* and then electroporated into *L. lactis*. DNA isolated from the latter was used to verify plasmid structures and insert sizes (Table 2). Restriction enzymes *Hpa*I (a single site in the vector moiety only) and *Hin*dIII (a single site between the insert and the vector) were used to analyze the integrants.

(iii) Cloning and deletion of an *ilv* operon fragment. A 3,949-bp *Eco*RI fragment of the IL1403 *ilv* operon (7) was cloned in either orientation into the *Eco*RI site of pG$^+$ host5; the plasmids were named pVE7009 and pVE7009R. A set of nested deletions in the DNA insert of pVE7009R was generated by restriction digestion. For plasmid constructions and insert sizes, see Table 1 and Fig. 2A.

sco integration into the *L. lactis* chromosome. Lactococcal strains containing the test plasmids were grown overnight at 28°C in the presence of erythromycin and then diluted 100-fold in the same medium and grown at 28°C for 2 to 2.5 h (log phase). Cultures were shifted to 37.5°C for 3 h (between six and nine generations) to lower the plasmid copy number per cell. Samples were then diluted and plated at

TABLE 2. Frequency of ipc at different locations on the *L. lactis* chromosome

Group	Plasmid	Insert size (kb)	Frequency of ipc (avg ± SD)[a]
I	pVE7025	1.29	$(3.0 \pm 0.3) \times 10^{-2}$
	pVE7034[b]	1.05	$(3.8 \pm 0.5) \times 10^{-3}$
	pVE7021	1.29	$(3.4 \pm 2.6) \times 10^{-3}$
	pVE7024	0.96	$(2.5 \pm 1.3) \times 10^{-3}$
	pVE7030	1.42	$(2.3 \pm 0.8) \times 10^{-3}$
	pVE7028[b]	1.46	$(7.2 \pm 0.7) \times 10^{-4}$
	pVE7023	1.29	$(6.6 \pm 3.9) \times 10^{-4}$
	pVE7022	1.08	$(5.7 \pm 0.3) \times 10^{-4}$
	pVE7027	1.05	$(5.2 \pm 1.3) \times 10^{-4}$
	pVE7026	0.96	$(4.0 \pm 0.5) \times 10^{-4}$
II	pVE7029	1.02	$(1.1 \pm 0.4) \times 10^{-5}$
	pVE7031	0.96	$(9.9 \pm 3.9) \times 10^{-6}$
	pVE7032	1.37	$(8.6 \pm 5.0) \times 10^{-7}$
	pVE7033	1.25	$(3.9 \pm 0.9) \times 10^{-7}$

[a] Determined as described in Materials and Methods. For each plasmid, at least two independent measurements were made. The frequency of nonspecific background integration of pG$^+$host5 was between 10^{-6} and 10^{-7}.
[b] HMW-producing plasmid.

37°C on M17-erythromycin plates to detect integration events and at 28°C on nonselective plates to determine the viable cell count. The frequency of integrations per cell (ipc) was estimated as the ratio of the number of Em^r cells at 37°C to the number of viable cells at 28°C. For subsequent use, the integrants isolated at 37°C were routinely maintained at 37.5°C in M17 medium containing erythromycin.

dco integration into the *L. lactis* chromosome. Plasmids pIL1263 and pIL1202, constructed by N. Goupil (8) and J. J. Godon (6), are composed of the Ts vector (pG^+host4; Em^r) and of 2.3- and 3.6-kb chromosomal regions, respectively, interrupted by the *tet* gene of Tn*1545* (23). A strain carrying pIL1202 or pIL1263 was grown overnight at 37.5°C in M17 medium with tetracycline or erythromycin to obtain a population of integrants. The culture was then diluted $1:10^5$ in M17 medium without antibiotic and shifted to 28°C to allow the stimulation of recombination by plasmid replication. We determined that growth for 12 h or more at 28°C yielded maximal frequencies of gene replacement. Routinely, an overnight saturated 28°C culture was plated at various cell concentrations at 37°C with or without tetracycline selection (as indicated in Results). Colonies were transferred by use of toothpicks to erythromycin- and tetracycline-containing plates. Colonies in which gene replacement had occurred were phenotypically Tet^r and Em^s.

DNA isolation and electrophoretic analysis. Chromosomal DNA was prepared as previously described (9). For detection of integration by homologous recombination, purified DNA was treated with restriction enzymes, separated by agarose gel electrophoresis (31), and analyzed by Southern hybridization (33) with nick-translated (31) plasmid DNA probes.

RESULTS

sco integration. Random *L. lactis* chromosomal fragments were cloned into pG^+host5 in *E. coli*. Fourteen different plasmids, each containing a distinct chromosomal insertion ranging from 0.9 to 1.4 kb, were isolated. These plasmids were established in IL1403 at 28°C and used to measure integration frequencies along the *L. lactis* chromosome. Integrants were selected as Em^r colonies at 37°C (see Materials and Methods), and respective integration frequencies were determined (Table 2). The ipc frequency was between 10^{-2} and 10^{-7}. In comparison, the ipc frequency obtained with vector pG^+host5 without a chromosomal insert was between 10^{-6} and 10^{-7}. Nonspecific vector integration was observed with pE194Ts (12) in *B. subtilis* and with a nonreplicative vector in *L. lactis* (20).

The 14 plasmids bearing chromosomal inserts could be classified into two groups on the basis of their ipc frequencies. In group I (10 plasmids), the ipc frequency varied from 3×10^{-2} to 4×10^{-4}. Since the lengths of homology were nearly the same in all cases, we supposed that the variations in integration frequencies were due to the location or the nature of the insert rather than to its size. In group II (four plasmids), the ipc frequency was between 1×10^{-5} and 3×10^{-7}. These frequencies were very close to the background level, and integration of these plasmids was found to be nonspecific (see below).

It was reported that insertions of certain DNA fragments into rc plasmids led to the accumulation of high-molecular-weight molecules (HMW), i.e., linear head-to-tail repetitions of the plasmid (9). As HMW alter the conformation and copy number of the plasmid, they could have an effect on recombination frequency. We therefore examined all our plasmid derivatives for HMW production. Two plasmids (pVE7028 and pVE7034) of 14 produced HMW; integration frequencies were in the range of the first group of integrants, a result suggesting that this plasmid form does not strongly affect intermolecular recombination frequency.

Physical analysis of integrants. Chromosomal DNA prepared from integrant strains maintained at 37°C was submitted to restriction enzyme analysis and Southern hybridization with the plasmid vector (pG^+host5) as a probe. Preliminary analysis of independent isolates of each integrant indicated single- and multiple-copy integrations for eight plasmids (three independent colonies were tested) and multiple-copy integration for two plasmids, pVE7034 and pVE7028 (six colonies were tested), which generated HMW. Eight single-copy integrants were characterized further. To confirm that plasmid integration occurred by sco, we treated integrant DNAs with *Hin*dIII. Each plasmid contains a single *Hin*dIII site at the vector-insert junction, and digestion should liberate a single linear plasmid band. Indeed, *Hin*dIII digestion of the eight characterized single-copy integrants of group I gave rise to a monomeric plasmid band (Fig. 1A). Southern hybridization of nondigested total DNA did not reveal free plasmids in any of the group I plasmids, a result indicating that the plasmid copy was integrated (Fig. 1B). Taken together, these results show that sco homologous recombination occurred in the eight group I plasmids examined. A similar analysis of the two HMW-producing plasmids, pVE7028 and pVE7034 (data not shown), confirmed that these plasmids were also integrated by sco. Using *Hpa*I, which recognizes a single site within the vector, we determined that each plasmid was integrated at a distinct position, as each junction fragment DNA pattern was different (Fig. 1C).

The four group II plasmids (low ipc frequency) appeared to be integrated at random, as *Hin*dIII digestion did not release a monomeric plasmid band, and *Hpa*I digestion of three integrants of the same plasmid did not yield the same gel pattern (data not shown). The lack of integration of these plasmids by homologous recombination may indicate that such an event would be lethal.

The megabase restriction map of the *L. lactis* chromosome developed with *Sma*I and *Apa*I (16–18) allowed us to localize the site of integration of the sco integrants on the chromosomal map. The eight single-copy sco integrants were mapped by *Sma*I and *Apa*I digestion; each integrant was present on a different segment (data not shown). Taken together with the results of the *Hpa*I analysis, these results indicated that the chromosomal insertions were randomly positioned on the chromosome, thus excluding any bias in our procedure.

ipc depends on the length of homology. A 3.9-kb segment of the sequenced IL1403 *ilv* operon (7) was cloned in pG^+host5, and a nested set of deletions of the fragment were generated on the same vector (Fig. 2A). While plasmids carrying the entire 3.9-kb insert in either orientation (pVE7009 and pVE7009R) showed some structural instability in *L. lactis*, all eight deletion derivatives of pVE7009R were stable. These clones were used to examine the relationship between length of homology and ipc (Fig. 2A).

We observed a log-linear relationship between ipc and length of homology for lengths of between 356 and 2,552 bp, with a slope of 1.12×10^{-3} per base pair (Fig. 2B). The ipc frequency for one smaller fragment (330 bp; pVE7017) fell below this proportion, a result suggesting that we may have reached a threshold length for efficient homologous integration. Nevertheless, the pVE7017 ipc frequency was higher

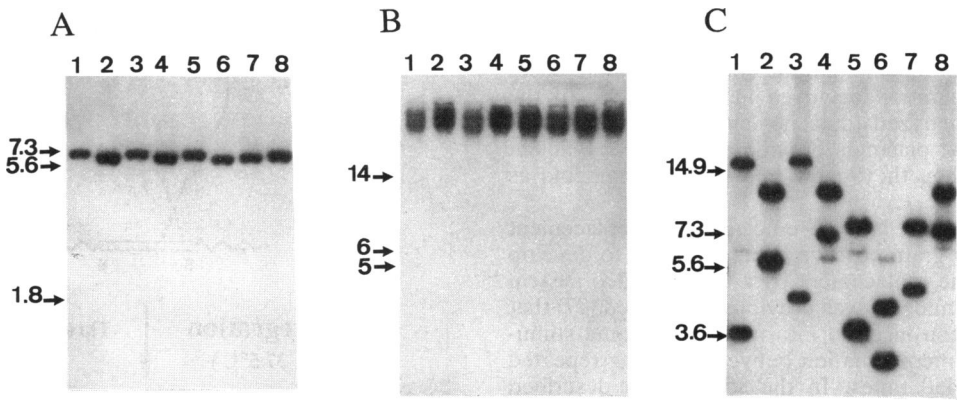

FIG. 1. sco integration of group I plasmids. Chromosomal DNA of single-copy plasmid integrants was treated with restriction enzymes and analyzed by agarose gel electrophoresis and Southern hybridization with pG$^+$host5 as a probe. The integrants analyzed were obtained with pVE7021 (lane 1), pVE7022 (lane 2), pVE7023 (lane 3), pVE7024 (lane 4), pVE7025 (lane 5), pVE7026 (lane 6), pVE7027 (lane 7), and pVE7030 (lane 8). The Raoul marker and a supercoiled ladder were used as size references in panels A and C and panel B, respectively. DNA lengths are indicated in kilobases. (A) *Hin*dIII recognizes a single site between the vector and the chromosomal DNA insert. In each case, the hybridizing band corresponds to the linear plasmid, a result indicating that integration occurred through sco. No secondary bands were visible. (B) Undigested chromosomal DNA shows hybridization only at the chromosomal level, a result indicating the absence of free plasmid DNA in integrants. (C) *Hpa*I recognizes a single site within the vector, and digestion generated two bands, corresponding to the chromosomal junction fragments. A faint band corresponding to the linear plasmid was detected in lanes 1, 4, 5, 6, and 8. Since we did not find any free plasmid in the integrants (panel B), we concluded that the faint band was due to low levels of plasmid amplification in the population (29).

than the nonspecific background ipc frequency for the vector. For fragments larger than 2,552 bp, recombination frequencies appeared to reach a plateau, as ipc frequencies for the 2.5-, 3.3-, and 3.9-kb homologous segments were not significantly different. Factors other than length (i.e., HMW production, structural instability, and copy number) also appear to be important, as the ipc frequencies for the same 3.9-kb segment in the two orientations on the vector differed by 18-fold.

DNAs of integrant strains were examined biochemically for homologous integration. For each plasmid used, three integrants were analyzed with restriction enzymes recognizing a single site in the vector, in the insert, or in the vector-insert junction. After separation on agarose gels,

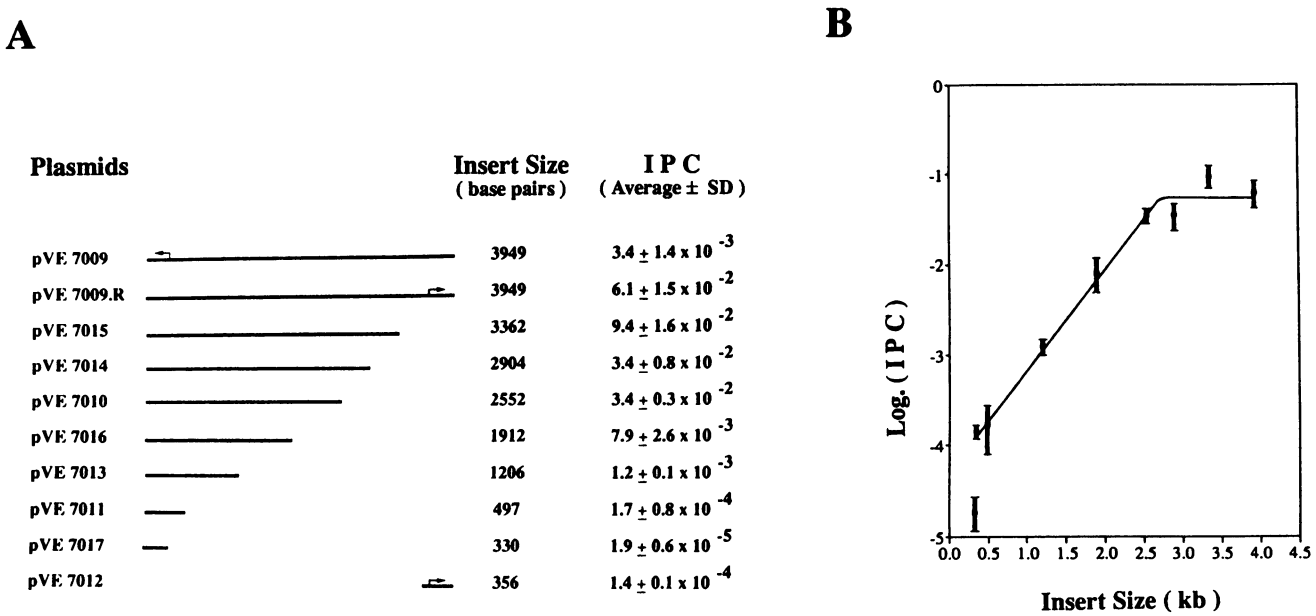

FIG. 2. Length of homology and relative integration frequencies. (A) A 3,949-bp *Eco*RI fragment of the IL1403 *ilv* operon was cloned into pG$^+$host5 in both orientations, generating pVE7009 and pVE7009R. A nested set of eight deletion derivatives were made from pVE7009R with different enzymes (Table 1). Plasmid names and insert sizes are indicated. An arrow indicates the presence of a putative promoter in the segment. For each plasmid, at least three independent measurements of ipc were performed as described in Materials and Methods; the averages and standard deviations (SD) are given at the right. (B) Relationship between insert size (x axis) and ipc (y axis). For inserts of between 356 and 2,552 bp, a log-linear relationship was observed. There was no significant variation in ipc above a 2,552-bp insert length.

Southern hybridization was performed with pIL515 (Table 1) as a probe. The results confirmed that integration occurred by sco homologous recombination. For each plasmid used, multiple-copy integration occurred in at least one of the three integrants analyzed (data not shown). These results show that pG$^+$host plasmids provide a means for efficient sco integration when they carry homologous segments as small as 330 bp.

dco integration. The lack of efficient gene replacement strategies in many gram-positive bacteria led us to develop efficient direct gene replacement by a two-step dco system with pG$^+$host plasmids. It was previously reported (27) that an active rc replicon inserted into the chromosome stimulates homologous recombination between flanking repeated sequences 20 to 450 times. In the sco system described above, the integrated plasmid is flanked by repeated sequences. Thus, when integrant strains generated at 37°C are shifted to 28°C, replication of the plasmid should strongly stimulate a second recombination event. This event will result in high-frequency excision of the replicon, giving rise to either parental or dco chromosomal structure. We developed a gene replacement protocol based on this principle (Fig. 3).

To study gene replacement, we used a poorly transformable strain of *L. lactis*, NCDO2118, which is a prototroph for the branched-chain amino acids (Ilv, Leu, and Val). It is notable that no genetic modification in this strain was previously feasible. Two pG$^+$host4 derivatives, which carry either a contiguous or a noncontiguous chromosomal segment, were used in these experiments. Plasmid pIL1263 contains a contiguous 2.3-kb chromosomal fragment upstream of the *ilv* operon interrupted by a 4-kb DNA segment containing a Tet^r marker (8) (Fig. 4A). Gene replacement would generate insertion of the Tet^r marker in the chromosome and leave the *ilv* operon intact. Plasmid pIL1202 contains noncontiguous segments of 1.1 and 2.5 kb, corresponding to the extremities of an 18.5-kb region, including the *ilv* operon, joined by the 4-kb Tet^r marker (6) (Fig. 4B). Gene replacement would generate a 14.9-kb chromosomal deletion, including the *ilv* operon, and lead to an *ilv* phenotype.

Selection for the replacing gene. A strain containing either pIL1202 or pIL1263 was submitted to the growth conditions described for dco in Materials and Methods, with Tet^r as a selective marker. In independent experiments with pIL1263, 69 and 98% of Tet^r colonies were Em^s; with pIL1202, 50 and 91% of Tet^r colonies were Em^s. In control cultures (kept at 37°C to block rc replication during the same time period), of 400 Tet^r colonies tested, all were also Em^r. This result indicates that rc plasmid replication stimulates excision from the chromosome. Five Em^s colonies obtained upon integration of pIL1202 were grown on minimal medium without branched-chain amino acids and found to be *ilv*, a result confirming that replacement recombination had occurred. The structure of the relevant chromosomal region of five Tet^r Em^s isolates was examined by Southern hybridization (Fig. 4); gene replacement was confirmed in all cases.

No selection for the replacing gene. To mimic the case in which the chromosomal fragment carried by the plasmid has no selective marker, we used the same protocol as that described above but without Tet^r selection. In three experiments with pIL1263 (gene insertion), 10 to 40% of colonies obtained at 37°C without selection were Tet^r Em^s, a result indicating that a gene replacement event had occurred. With pIL1202 (chromosomal deletion), 1 to 7% of colonies were Tet^r Em^s, a result indicative of gene replacement. Of four

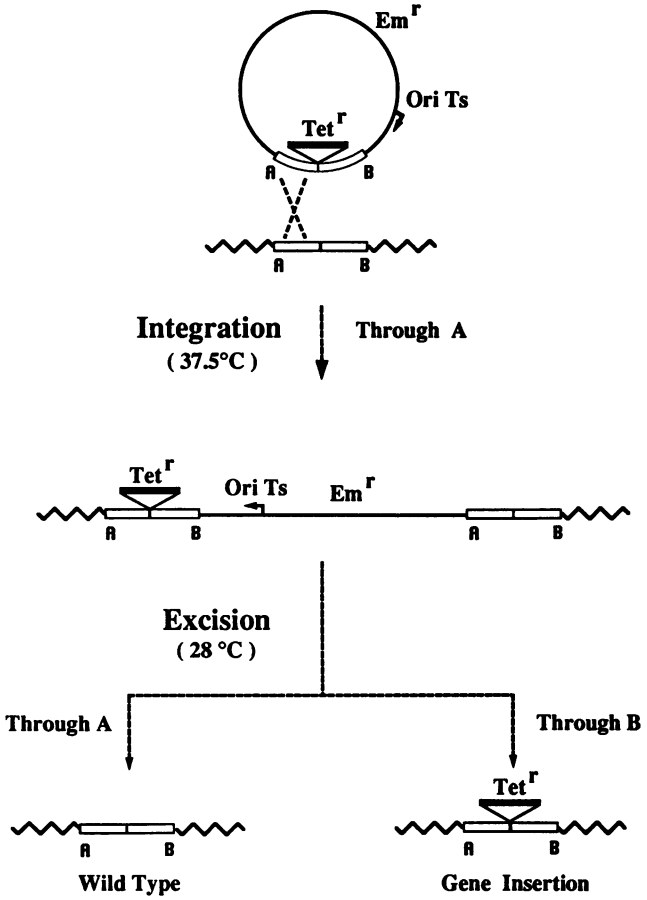

FIG. 3. General strategy for dco integration. The first recombination event (integration), selected at 37.5°C with antibiotic, occurs by sco through region A. A shift of the sco integrants to 28°C activates replication of the integrated plasmid (Ori Ts), which stimulates a second recombination event between the duplication created by the first recombination event and leads to plasmid excision. Excision through region A restores the parental chromosomal structure, while excision through region B gives rise to a gene replacement. Alternatively, an initial sco recombination event at B and excision at A also result in gene replacement. Symbols: straight line, plasmid backbone; wavy line, chromosome; open boxes, regions of homology between the chromosome and the plasmid; closed bars, Tet^r gene.

Tet^r Em^s colonies tested, all were *ilv*. Chromosomal structure analysis of four dco integrants of each type, performed as described above, confirmed that replacement had occurred without selection for the newly inserted fragment (data not shown). These results demonstrate the feasibility of gene replacement without an antibiotic marker being left in the chromosome. This protocol is thus suited for chromosomal modification without the use of selective markers.

Use of pG$^+$host plasmids in other gram-positive bacteria. The efficiencies of intermolecular recombination at 12 different locations on the *B. subtilis* chromosome were previously determined by transforming competent cells with a nonreplicative plasmid (1); in those experiments, the homologous segment was invariant (a 3.7-kb inserted pBR322 fragment). Efficiencies varied by only about threefold and were dependent on position of integration. Using the pG$^+$host sco system, rather than a nonreplicative vector, we performed

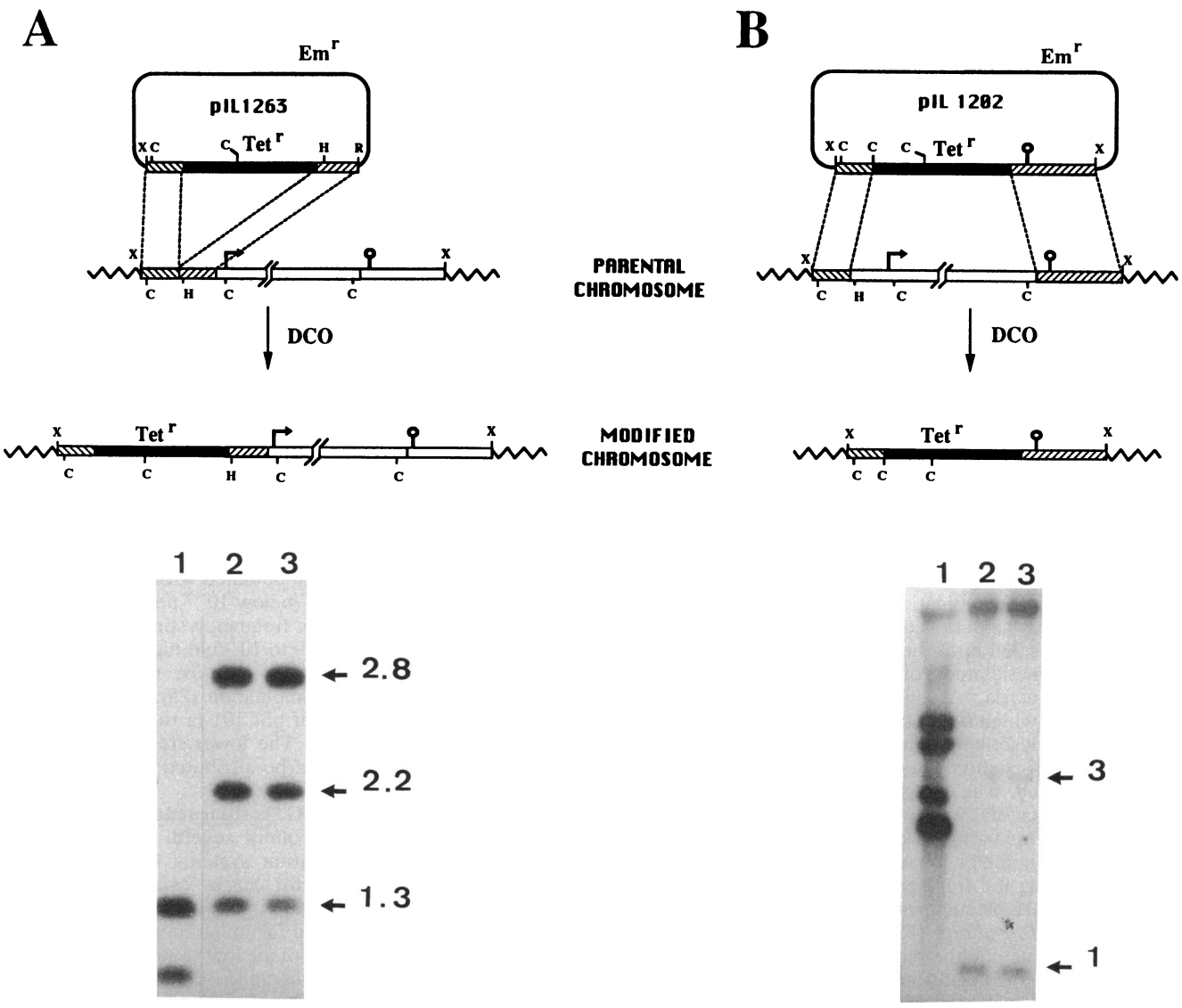

FIG. 4. dco integration analysis. (A) dco with pIL1263. (Upper panel) Maps of pIL1263, wild-type chromosomal, and dco-modified chromosomal structures. pIL1263 is a pG$^+$host derivative that contains a 2.3-kb contiguous chromosomal fragment interrupted by the Tet^r gene (Table 1). Symbols: solid line, plasmid backbone; closed boxes, *tetM* gene; hatched boxes, regions of homology between the plasmid and the chromosome; wavy line, chromosome; open boxes, regions of the 17.4-kb *Xba*I fragment with no homologous counterpart on the plasmid. The *ilv* operon is delimited by the bent arrow, symbolizing a promoter, and the lollipop, representing a transcriptional terminator. Restriction enzymes are indicated as follows: C, *Cla*I; H, *Hin*dII; R, *Eco*RI; X, *Xba*I. (Lower panel) Southern analysis of wild-type and modified chromosomal structures. Two of the five Tet^r Em^s recombinants analyzed are shown. Chromosomal DNAs were digested with *Cla*I-*Hin*dII and probed with pIL1261 (Table 1). In lane 1, NCDO2118 (parental strain) exhibits the expected 1- and 1.3-kb bands. In lanes 2 and 3, the two independent dco integrants exhibit the 1.3-kb band and two additional bands, of 2.2 and 2.8 kb, revealing insertion of the Tet^r gene. The amounts of DNA loaded in lanes 2 and 3 were smaller than that loaded in lane 1, explaining the difference in intensities of the 1.3-kb band. (B) dco with pIL1202. (Upper panel) Maps of pIL1202, wild-type chromosomal, and dco-modified chromosomal structures. pIL1202 is a pG$^+$host derivative that contains 1.1- and 2.5-kb extremities of the 18.5-kb *Xba*I fragment flanking the 4-kb Tet^r gene. During cloning, a *Cla*I site was generated between the 1.1-kb left-hand chromosomal fragment and the Tet^r gene. In the wild-type chromosomal structure, the whole *ilv* region is not shown; as a consequence, three *Cla*I sites are not indicated. Symbols are the same as in panel A. (Lower panel) Southern analysis of wild-type and modified chromosomal structures. Two of the five Tet^r Em^s recombinants analyzed are shown. Chromosomal DNAs were digested with *Cla*I and probed with pIL500 (containing the entire 18.5-kb *Xba*I fragment; Table 1). In lane 1, the restricted wild-type structure shows seven bands, of about 10, 4.2, 3.7, 3, and 2.7 kb and a doublet of 2.2 kb. The bands of 10 and 3 kb correspond to the right and left chromosomal junctions, respectively. The size of the left junction would be intact while the size of the right junction would be increased by 3 kb (part of the Tet^r gene) in a strain in which dco occurred. In lanes 2 and 3, the restricted dco-modified structures show the two junction bands of expected sizes (13- and 3-kb faint bands), a 1-kb band (due to the plasmidic *Cla*I site), and the disappearance of the internal *ilv* fragments present in the NCDO2118 pattern (lane 1). The 3-kb band corresponding to the left chromosomal junction is hardly visible, since it has only 300 bp of homology with the probe. Fragment sizes estimated with the Raoul marker are indicated in kilobases at the right side of each autoradiograph.

identical recombination experiments on the two *B. subtilis* strains showing the threefold variation in integration frequencies. pG$^+$host5, which carries a 1.4-kb fragment of pBR322, was introduced into the *B. subtilis* strains of interest (carrying the pBR322 chromosomal insertion). When the sco procedure was used (see Materials and Methods), ipc frequencies varied from $(1.8 \pm 0.6) \times 10^{-3}$ to $(6.1 \pm 0.9) \times 10^{-4}$. We found the same threefold maximal variation between the two different locations as that observed with the nonreplicative vector. This result demonstrates the applicability of the system.

With a Ts plasmid joined to a pSC101 replicon, sco gene disruption was recently obtained in the pathogen *Streptococcus pyogenes* for construction of a strain altered in M-protein production (28). We have also used pG$^+$host plasmids to obtain gene replacement in *Listeria monocytogenes* (3). These results show that the use of pG$^+$host plasmids can be extended to gram-positive bacteria other than *L. lactis*. However, plasmid replication, thermosensitivity, background integration, and level of drug resistance must be determined for each new host.

DISCUSSION

We have used thermosensitive pG$^+$host delivery vectors, which are nonreplicative above 37°C, to obtain integration by homologous recombination in the *L. lactis* chromosome. sco integration was obtained all along the chromosome and with as little as 330 bp of homology. This work constitutes the first systematic study of homologous recombination in lactic acid bacteria. A highly efficient protocol for dco integration, in which selection for the replacing DNA is not necessary, is described. The methodology is applicable to numerous gram-positive bacteria.

sco integration. The integration frequencies for random cloned segments (of 0.9 to 1.4 kb) varied by a factor of about 75 (Table 2). This variation could be due to specific signals present on the homologous fragment or to a local conformation surrounding the fragment. Location-specific effects on intra- and intermolecular recombination were previously described for *B. subtilis* (1, 5, 36). The disruption of an essential gene may be the reason for which only illegitimate (nonhomologous) integration of the group II plasmids was observed. The small size of the *L. lactis* chromosome (2.4 megabases; 16) may explain the high frequency (4 of 14) of putative essential gene segments found by random cloning.

We examined integration at a single region as a function of length of homology (Fig. 2). A log-linear relationship between insert size and the frequency of integration was observed for inserts of between 0.35 and 2.5 kb, a result suggesting a constant average probability of recombination per nucleotide pair. Previous studies of integration by recombination in *B. subtilis* (24) and *E. coli* (24, 34) did not reveal a similar log-linear correspondence. However, as the experimental systems used (interplasmid recombination [24] and suicide [34] and Ts plasmid delivery vectors) were all different, direct comparisons are not meaningful. In a recent study of sco integration in *B. subtilis* with a Ts derivative of rc plasmid pE194, integration frequency showed a linear dependence on substrate length (14). However, the homologous lengths were shorter than in this study (fragments of 77 to 165 bp), and it is likely that the starting vector (an rc plasmid containing *E. coli* DNA) produced HMW (9).

We observed that the stability of the integrated plasmid was greater than 99% after 75 generations at 37.5°C without selection, a result indicating that such a structure can be stably maintained at this temperature. Thus, sco integration is useful for genetic studies and for the characterization of a genetic region. As expected, none of the sco structures appeared to be stable at 28°C (data not shown).

dco integration in gram-positive bacteria. A limited number of gram-positive bacteria, such as *Streptococcus pneumoniae* (30) and *B. subtilis* (4, 26), can achieve highly competent states and thus allow efficient assimilation of foreign DNA into the genome. Even in these cases, selection is required. The majority of bacteria, however, particularly those of medical or industrial use, require extensive manipulations to integrate foreign DNA. Nonselective systems of integration have not been available for such gram-positive bacteria. The strategy that we adopted with the Ts plasmid (Fig. 3) constitutes, to the best of our knowledge, the first demonstration of dco modification based on the stimulation of recombination by an integrated rc plasmid. The reported effects of rc plasmids on excision (27) and amplification (29) motivated this approach. In this work, we used rc replication to stimulate the second step of the dco procedure (plasmid excision; Fig. 3). This procedure allowed us to obtain 50 to 98% gene replacement when selection for the replaced gene was available and 1 to 40% replacement when no selection was applied. In the absence of replication, dco integration was not detected (less than 0.25%). This result is consistent with the excision frequency obtained with a nonreplicative delivery vector in *L. lactis* (below 10^{-5} per generation; 19). In our system, the excision frequency stimulated by the rc plasmid appeared to be 10^2- to 10^3-fold higher.

It is of interest that in *B. subtilis*, rc but not theta replication stimulates recombination (25). A dco protocol based on a Ts derivative of pSC101 (a theta replicon) was described for *E. coli* (10). The lower frequencies of gene replacement reported might be attributed to the use of a θ replicon delivery vector.

General applications of pG$^+$host-mediated homologous integration. The Ts vector offers several advantages over presently available integration systems for gram-positive bacteria. Since the Ts vector can be established at a low temperature in a large bacterial population before selection of integrants, it can be used for integration in poorly transformable bacteria. Because of the low shutoff temperature of pG$^+$host plasmids, this integration system can be used in bacteria that have a limited temperature growth range. As shown here, adaption of the plasmids with an *E. coli* replicon facilitates constructions. The procedure that we used to obtain dco integration is highly efficient and does not require a selective marker in the replaced gene. The construction of food-grade recombinant strains may have considerable applications in medical and industrial domains.

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