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New Thermosensitive Plasmid for Gram-Positive Bacteria

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We isolated a replication-thermosensitive mutant of the broad-host-range replicon pWV01. The mutant pVE6002 is fully thermosensitive above 35°C in both gram-negative and gram-positive bacteria. Four clustered mutations were identified in the gene encoding the replication protein of pVE6002. The thermosensitive derivative of the related plasmid pE194 carries a mutation in the analogous region but not in the same position. Derivatives of the thermosensitive plasmid convenient for cloning purposes have been constructed. The low shut-off temperature of pVE6002 makes it a useful suicide vector for bacteria which are limited in their own temperature growth range. Using pVE6002 as the delivery vector for a transposon Tn*10* derivative in *Bacillus subtilis*, we observed transposition frequencies of about 1%.

Lactic acid bacteria represent an important group of organisms which are used extensively in the food industry (27). Despite their widespread use and the potential interest in modifying these bacteria, no efficient genetic system for transposon mutagenesis or recombinational inactivation of the chromosomal genes exists (8). For either procedure, the delivery vector carrying a gene of interest (either a homologous gene or a transposon) should be lost.

Nonreplicative plasmids have been used previously as delivery vectors (6, 23, 24). However, high transformation efficiencies are required to allow the detection of low-frequency events such as transposition and recombination into the chromosome. Since the majority of lactic bacteria are poorly transformable (28), these techniques cannot be used.

The need for an efficient transformation system could be circumvented by using a thermosensitive (Ts) replicon. Even if the transformation frequency is low, a Ts replicon could first be established and propagated in a large bacterial population; its subsequent shut-off at elevated temperature would allow selection of low-frequency events like transposition and recombination in a large cell population.

In this report, we describe the isolation of a Ts replicon which can be used as a delivery vector in lactic acid and other gram-positive bacteria. The plasmid pGK12, derived from pWV01 (17), was chosen to perform the work. Originally isolated from *Lactococcus lactis* subsp. *cremoris* (35), pWV01 also replicates in a large number of gram-positive bacteria (e.g., bacilli, lactococci, lactobacilli, and streptococci) as well as in *Escherichia coli* (17). Sequence analysis (25) revealed that pWV01 is a member of a family of plasmids which replicate by a rolling-circle mechanism, via a single-stranded DNA intermediate (11, 25, 44). Among its close relatives are streptococcal plasmid pLS1 (19, 39) and staphylococcal plasmid pE194 (4, 15), which does not replicate in lactococci (6). Plasmid pE194 is naturally Ts at 51°C (43), and a derivative was obtained which is Ts at 39 to 42°C (10a, 48); this example suggested the feasibility of our project.

The Ts derivative of pWV01 was isolated after in vitro mutagenesis. We describe its characterization, localization of the mutation, the construction of derivatives convenient for cloning purposes, and an example of its uses.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. The constructions of pVE6043 and pVE6044 are detailed in Fig. 2; plasmids pVE6004, pVE6006, and pVE6007 are presented in Fig. 4. The plasmid used for transposition, pVE6060, is composed of the blunt-ended *Kpn*I fragment of pVE6004 joined to the blunt-ended *Ava*I-*Alw*NI fragment of pBR322 (containing the replicon). The *Sac*I-*Apa*II fragment of this plasmid (missing the Em^r gene) has been joined to a mini-Tn*10* Em^r transposition cassette (to be described elsewhere). The mini-Tn*10* is the Outside Ends derivative of Tn*10* in which the erythromycin resistance gene of pAMβ1 has been cloned. The transposase gene is expressed from an *L. lactis* subsp. *cremoris* promoter (p59) characterized by van der Vossen et al. (47). *Escherichia coli* and *Bacillus subtilis* were cultured in LB medium (29). *L. lactis* subsp. *lactis* (*L. lactis*) was grown in M17 medium (45) in which lactose was replaced by glucose. Chloramphenicol was used at 5 μg/ml for *L. lactis* and *B. subtilis*, and erythromycin was used at 5 and 0.5 μg/ml, respectively. Chloramphenicol, azaerythromycin, and erythromycin were used at final concentrations of 15, 100, and 150 μg/ml, respectively, for *E. coli*.

Molecular cloning, competence, and transformation procedure. Commercially prepared enzymes were used as specified by the suppliers. Whole-cell minilysates (38) and plasmid DNA (42) were prepared as described previously. Competence induction and transformation of *E. coli* and *B. subtilis* cells were performed by standard procedures (13, 31). *L. lactis* strains were electrotransformed as described before (20) and modified according to Holo and Nes (14).

Plasmid mutagenesis. Hydroxylamine mutagenesis was performed on DNA from plasmid pGK12 under the conditions described by Thomas (46). After 110 and 120 min of treatment at 70°C, hydroxylamine was eliminated by DNA-isopropanol precipitation.

DNA sequencing. For DNA sequencing, the *Tha*I (bp 756)-*Rsa*I (bp 1620) fragment of pVE6002 was cloned in the pBluescript plasmid. A series of overlapping clones were generated by using exonuclease III and mung bean nuclease

TABLE 1. List of strains and plasmids

Strain or plasmid	Genetic markers or description	Source or reference
L. lactis		
IL1403	Plasmid-free, $r^- m^-$, 2 prophages (bI285 and bI286)	5
MG1363	Plasmid-free	9, 10, 22
B. subtilis SB202	*trpC2 tyrA1 aroB2 hisH2 thyA*	Lab strain
E. coli DH5	F^- *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ^- *gyrA96 relA1*	13
Plasmids		
pBluescript	Ap^r, M13 *ori*, pBR322 *ori*	Stratagene
pGK12	Em^r Cm^r	17
pVE6004	3,340-bp *Cla*I-*Hpa*II fragment of pVE6002 joined to 445-bp fragment of pBluescript; Em^r	This work
pVE6006	*Cla*I site of pVE6002 joined to 445-bp fragment of pBluescript; Em^r Cm^r	This work (Fig. 4)
pVE6007	1,175-bp *Sca*I deletion of pVE6006; Cm^r	
pVE6043	*Sst*I-*Tha*I fragment (*ori*$^+$) of pGK12 joined to *Tha*I-*Sst*I fragment (ORF A) of pVE6002; Em^r Cm^r	This work (Fig. 2)
pVE6044	*Sst*I-*Tha*I fragment (*ori*$^+$) of pVE6002 joined to *Tha*I-*Sst*I fragment (ORF A) of pGK12; Em^r Cm^r	This work (Fig. 2)
pVE6060	*Kpn*I blunt-ended pVE6004 fragment joined to *Ava*I-*Alw*NI blunt-ended pBR322 fragment (containing replicon); the *Sac*I-*Apa*II fragment of the resultant plasmid (missing the Em^r gene) is joined to mini-Tn*10* Em^r transposon cassette	This work (26a)

(Stratagene). The *Tha*I (bp 756)-*Nde*I (bp 1140) fragment of our preparation of plasmid pGK12 was also sequenced by the same procedure.

The dideoxy chain termination method of DNA sequencing was carried out on double-stranded DNA plasmids with the *Taq* Dye Primer Cycle Sequencing Kit (Applied Biosystem) on a Perkin-Elmer polymerase chain reaction apparatus. Sequencing reactions were primed with fluorescent oligonucleotides (Applied Biosystem) and analyzed on an automatic sequencer (370 A DNA sequencer; Applied Biosystem). The reported sequences were determined for both strands.

Transposition test. The transposition test was performed as described previously (36). *Hin*dIII digestion of pVE6060 generates two fragments, one of which is 1.3 kb long and carries the transposon. *B. subtilis* cells containing pVE6060 (Table 1 and above) were grown overnight at 28°C with erythromycin selection in LB medium, diluted 100-fold, and grown in the same medium for 2 h. The culture was shifted to 37.5°C for 3 h, and dilutions were plated at 37°C on selective and nonselective media. Transposition frequency was determined as the proportion of colonies on selective versus nonselective plates. Whole-cell DNA was prepared from eight colonies grown in selective medium at 28°C. Southern hybridization analysis was performed with transposon DNA and pVE6004 DNA separately as probes to confirm that transposition and not nonspecific plasmid integration had occurred.

RESULTS

Mutant isolation. The plasmid used in these experiments, pGK12, is a derivative of pWV01 containing two drug resistance markers (17). Ten micrograms of plasmid DNA was mutagenized in vitro by hydroxylamine (Materials and Methods) and introduced by electroporation into lactococcal strain IL1403 after elimination of the mutagenic agent. The efficiency of mutagenesis was estimated by the decrease in plasmid viability and by the appearance of Em^s or Cm^s mutants. After 110 to 120 min of treatment, plasmid viability dropped to less than 0.1%, and antibiotic-sensitive plasmids appeared in about 10% of the transformants. These conditions of mutagenesis were chosen to search for Ts plasmids, which were identified by replica plating of transformants obtained at 28°C on erythromycin plates incubated at 37.5°C. Two Ts candidates, pVE6001 and pVE6002, were obtained after screening about 5,000 clones. Their plasmid copy numbers were compared, and their rates of loss at 37.5°C were determined.

Mutant characterization. (i) pVE6001 is a copy number mutant. Plasmid pVE6001 was more unstable than pGK12 at 28°C, and the defect became more pronounced at 37.5°C. However, 7% of the bacteria still contained the plasmid after 8 h of nonselective growth at 37.5°C, suggesting that some replication occurs under restrictive conditions (Fig. 1A, left).

Compared with pGK12, the copy number of pVE6001 appeared to be decreased at 28 and 37.5°C with or without selection (Fig. 1A, right), which might explain its lower stability. Possibly, plasmid loss at elevated temperature is due to the physiological changes in the host at the higher temperature and not to plasmid thermosensitivity.

(ii) pVE6002 is thermosensitive above 35°C. Measurements of plasmid stability during growth without antibiotics reveal that pVE6002 is as stable as pGK12 at 28°C but is drastically lost at 37.5°C (Fig. 1B, left). The rapid loss of pVE6002 at 37.5°C suggests that replication is blocked immediately after the temperature shift. After 8 h of growth, only about 0.1% of the cells remained Em^r. The copy numbers of pGK12 and pVE6002 were similar at 28°C with and without selection; however, after 5 h at 37.5°C, pVE6002 is undetectable, whereas the pGK12 copy number was about the same (Fig. 1B, right). We conclude from these experiments that the mutation in pVE6002 constitutes a true Ts replication defect.

The minimum nonpermissive replication temperature of pVE6002 was determined. Strain IL1403 containing pVE6002 was tested for plasmid loss during 8 h of nonselective growth at 28, 30, 33, 35, and 37.5°C (Table 2). We found that plasmid loss was equivalent at 37.5 and 35°C. Partial plasmid loss was already observed at 33°C, whereas at 28 and 30°C, the plasmid was stable. Thus, cells containing pVE6002 can be cured of this plasmid by a temperature shift to 35°C or above.

Host range and Ts phenotype of pVE6002. A highly controlled Ts plasmid could be a useful cloning vehicle for other strains and organisms. Thus, the thermosensitive behavior of pVE6002 was examined in *L. lactis* subsp. *cremoris*, *B. subtilis*, and *E. coli* (Table 1). These organisms were chosen as representative of the broad-host-range capacity of the original pWV01 replicon. Plasmid DNA was readily introduced in these species by transformation and selection at 28°C. Our results show that pVE6002 is thermosensitive in

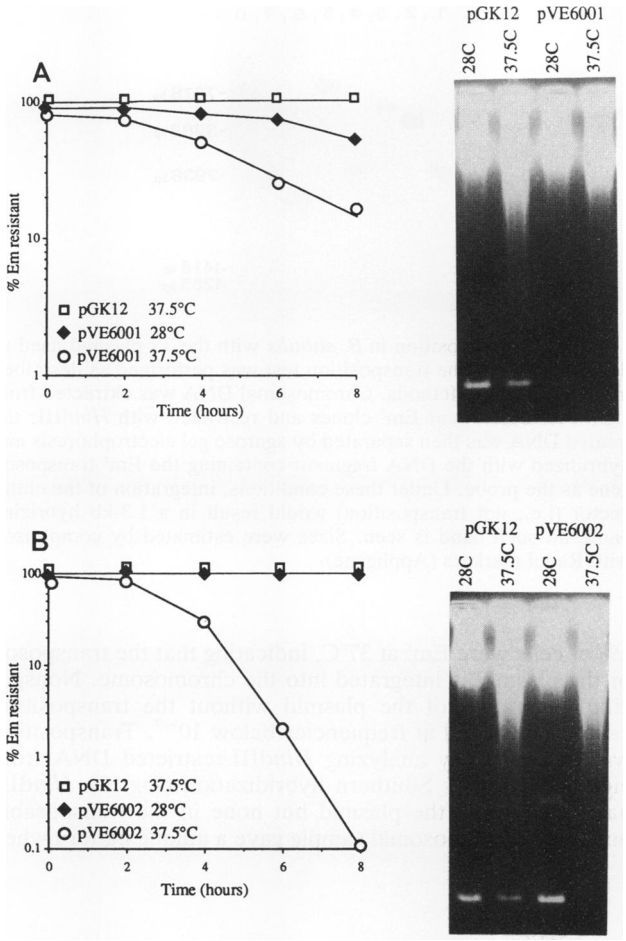

FIG. 1. Kinetics of loss and copy number analysis of (A) pVE6001 and (B) pVE6002. *L. lactis* IL1403 cells carrying plasmid pGK12, pVE6001, or pVE6002 were grown at 28 or 37.5°C. Samples were removed at 2-h intervals for plating at 28°C on selective and nonselective media. One hundred colonies were toothpicked from the nonselective to selective medium (erythromycin, 5 μg/ml) to estimate the proportion of plasmid-containing cells in the population (left side). Values below 1% were calculated by the ratio of CFU at 28°C on selective medium versus nonselective medium. Total DNA extractions were done on cultures grown at 28 or 37.5°C without selection for 5.5 h (right side). Samples were loaded and run overnight on a 0.7% agarose gel.

these hosts (data not shown). It is likely that pVE6002 retains its Ts properties in the broad spectrum of hosts in which it can be established.

Mapping and DNA sequencing of the Ts mutation. The pWV01 DNA sequence reveals the presence of a plus origin and four open reading frames (ORFs) (25). Similarities with better-characterized single-stranded DNA plasmids indicated that ORF A encodes the replication protein (RepA) responsible for nicking of one DNA strand at the plus origin and that ORF C might regulate *repA* expression. ORF B and ORF D have no known functions, although the latter is dispensable for replication (25).

To localize the Ts mutation in pVE6002, we constructed hybrid plasmids associating parts of the Ts and the unmutated replicons. pVE6043 consists of a pGK12 fragment containing the plus origin, ORF B, and ORF C and a pVE6002(Ts) fragment containing ORF A (RepA) missing its

TABLE 2. Loss of pVE6002 at different temperatures[a]

Time (h)	% Em^r cells in the population at growth temp:				
	28°C	30°C	33°C	35°C	37.5°C
0	100	100	100	100	100
2	100	100	99	97	98
4	100	100	48	47	38
6	100	100	9	3	4
8	100	99	5	<1	1

[a] An overnight culture of IL1403 carrying pVE6002 grown in M17 with erythromycin (5 μg/ml) at 28°C was diluted into fresh selective medium and incubated for 3 h at 28°C. At that time, the culture was diluted 1,000-fold in nonselective medium and incubated at different temperatures. At various times, samples were removed and plated on M17 agar at 28°C. For each time and temperature point, loss of the plasmid was estimated by picking 100 colonies to selective (erythromycin) plates at 28°C.

promoter, ORF D, and the Em^r and Cm^r markers (restriction sites *Sac*I and *Tha*I were used; Fig. 2). This hybrid is lost at 37.5°C at the same rate as pVE6002, whereas the reciprocal hybrid (pVE6044) is as stably maintained as pGK12 (Fig. 2). Thus, the mutation conferring thermosensitivity on pVE6002 is in the DNA fragment encoding RepA, ORF D, and the drug resistance markers. Since ORF D is dispensable and the drug resistance markers are not candidates, we conclude that the Ts function lies in RepA.

The 864-bp fragment encoding the RepA protein of pVE6002 was sequenced. Four mutations were identified, located at positions 972, 977, 980, and 987 (Fig. 3). The corresponding region of our parental plasmid pGK12, which was used for the mutagenesis, was confirmed to be identical to the published pWV01 sequence (25). The four mutations are transitions from G to A, corresponding to the known mutagenic effect of hydroxylamine (46). Each base change results in an amino acid alteration (Fig. 3), one of which, Val to Ile, is conservative. The contribution of all or some of these alterations may be involved in the Ts phenotype.

Ts plasmid derivatives. For cloning purposes, we devel-

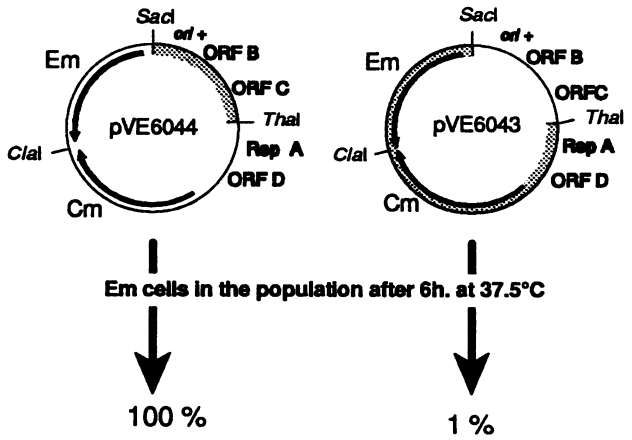

FIG. 2. Hybrid plasmids of pGK12 and pVE6002. pVE6043 is composed of the 994-bp *Sac*I-*Tha*I fragment of pGK12 ligated with the 3,384-bp *Tha*I-*Sac*I fragment of pVE6002. pVE6044 contains the reciprocal pair, the 994-bp *Sac*I-*Tha*I fragment of pVE6002 ligated with the 3,384-bp *Tha*I-*Sac*I fragment of pGK12. Thin lines, pGK12 DNA; stippled boxes, pVE6002 DNA; arrows, direction of transcription of the Em^r and Cm^r genes. The thermosensitivity of the hybrid plasmids was determined as described in Table 2, footnote *a*, after 0 or 6.5 h of incubation at 37.5°C.

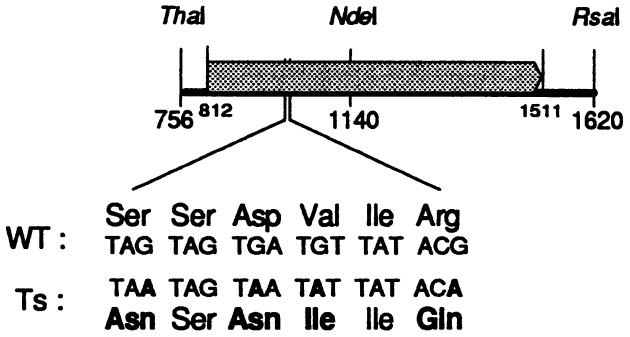

FIG. 3. Location of the Ts mutation in the pVE6002 *repA* gene. The *Tha*I-*Rsa*I fragment of pVE6002 containing the *repA* gene (represented by a stippled box) was sequenced on both strands. The sequence exhibits four changes, at positions 972, 977, 980, and 987, from the wild type (WT), whereas the rest of the sequence does not differ from the published sequence of the parental pWV01 replicon (25). The sequence of the *Tha*I-*Nde*I fragment of the parental pGK12 isolate used for mutagenesis was determined on both strands and is identical with the published sequence (25).

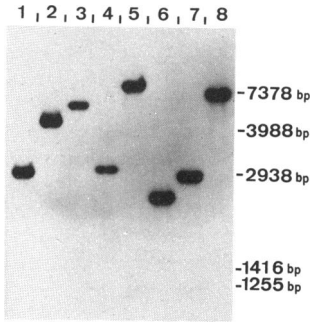

FIG. 5. Transposition in *B. subtilis* with the Ts plasmid used as delivery vector. The transposition test was performed as described in Materials and Methods. Chromosomal DNA was extracted from eight thermoresistant Em^r clones and restricted with *Hin*dIII; the treated DNA was then separated by agarose gel electrophoresis and hybridized with the DNA fragment containing the Em^r transposon gene as the probe. Under these conditions, integration of the entire vector (i.e., not transposition) would result in a 1.3-kb hybrizing band; no such band is seen. Sizes were estimated by comparison with Raoul markers (Appligene).

oped derivatives of the initial Ts plasmid pVE6002 (Fig. 4). These derivatives were modified to contain either two drug resistances (Em^r and Cm^r) or only one, and they all have a multicloning site derived from the pBluescript SK+ plasmid.

Ts plasmid is an efficient transposon delivery vector. Cm^r mini-Tn*10* derivatives have been shown to be active in *B. subtilis* (36). A transposition test was carried out in *B. subtilis* with a Tn*10* transposition cassette cloned on a derivative of pVE6004 (see Materials and Methods). About 1% of cells were Em^r at 37°C, indicating that the transposon or the plasmid is integrated into the chromosome. Nonspecific integration of the plasmid without the transposition cassette occurred at frequencies below 10^{-7}. Transposition was evaluated by analyzing *Hin*dIII-restricted DNA from eight colonies by Southern hybridization (Fig. 5). *Hin*dIII has two sites in the plasmid but none in the transposable unit. Each chromosomal sample gave a unique pattern when

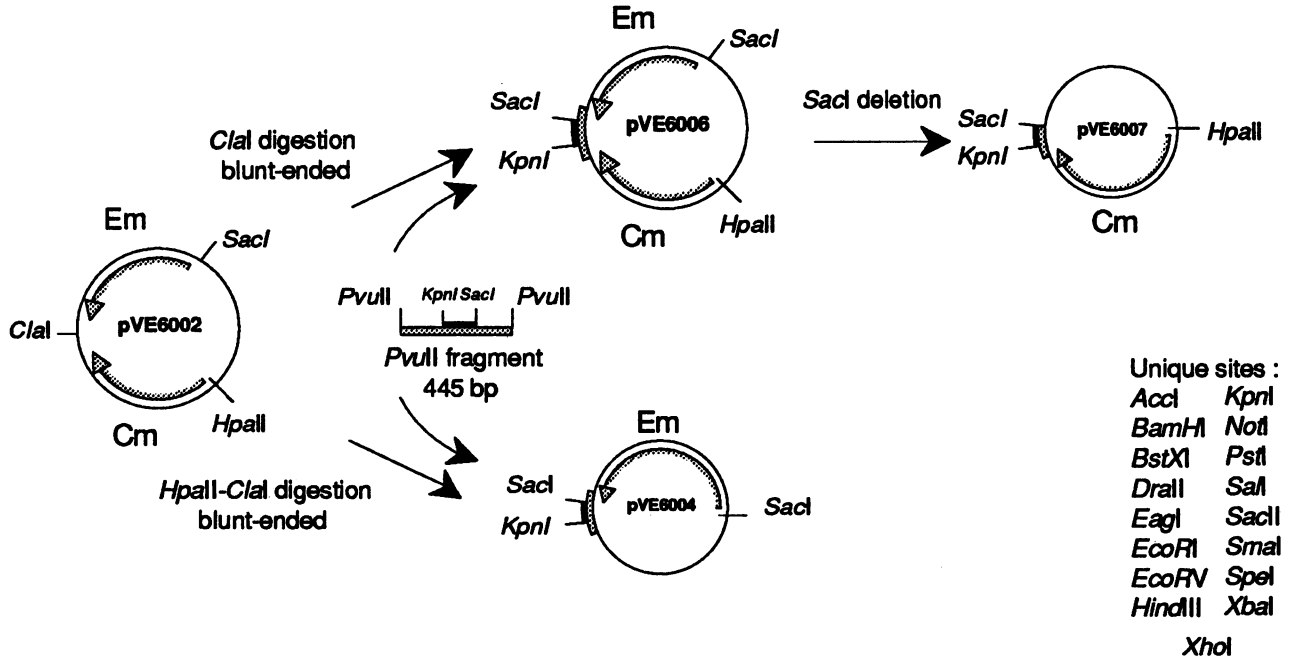

FIG. 4. Description of the Ts derivatives. pVE6006 was constructed by insertion of the 445-bp *Pvu*II fragment of pBluescript SK+ (stippled box; see Materials and Methods) in the blunt-ended *Cla*I site of pVE6002. pVE6007 results from a *Sac*I deletion of pVE6006, which removes the Em^r gene. pVE6004 was constructed by insertion of the 445-bp *Pvu*II fragment of pBluescript SK+ into the 3,340-bp blunt-ended *Cla*I-*Hpa*II fragment of pVE6002 missing the Cm^r gene. The 445-bp *Pvu*II fragment from pBluescript (stippled box) contains a multicloning site (black box limited by the *Kpn*I and *Sac*I sites), the T7 and T3 promoters, and sites for the M13 −20, T7, T3, and reverse primers (Stratagene), which allow direct sequencing from the vector. Unique sites listed in the figure are available in the multicloning site of all pVE6002 derivatives. Dotted arrows indicate the direction of transcription of the Em^r and Cm^r genes.

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          50                        60        70        80
PE.194    LHDRDTDTEGRM.............KKEHYHILVMYEGNKSYEQI
          *** *    *                ** ***    * **** ***
PLB.4     LHDKDVNPDGEK.............KKSHYHLVLNYKGNKSFEQI
          ***** * **                ** * *
PHPK.255  LHDKDLNEDGSH.............KKPHFHAIIVFDKKQRPAAV
          ***** * **                **** *           *
PADB.201  LHDKDVNPDGTI.............KKPHYHIVLAYSGPTTFNNV
          *****                     ** ***      * *   *
PLS.1     LHDKDKSSIKGQKY...........KKAHYHVLYIAKNPVTADSV
          *** *    *                ** **** * * ****  **
PWV.01    LHDMDEKLDKDTWNSSDVIRNGKH.YKKPHYHVIYLARNPVTIESV
          ******* ********************** ********** **********
PSH.71    LHDMDEKKDKDTWNSSDVIRNGKH.YKKPHYHVIYIARNPVTIESV
          ******** *                *  * *****      ********
PFX.2     LHDMDEKKIKIHGIVVMLYEMEMHVIKNPHYHVYILHGNPVTIESV
                    50        60        70        80

consensus LHD D   D                   KKPHYH       P T E V
                   70        80        90       100
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FIG. 6. Comparison of the pE194-like Rep proteins. The MULTALIN Multiple Sequence Alignments software of F. Carpet (7) was used for Rep protein comparison. Only perfect matches of amino acids (stars) are shown. Dots designate gaps generated by the program to optimize the alignment. Wild-type Rep sequences are displayed for all plasmids, including pE194 and pWV01; the amino acids which are mutated in the Ts derivatives are shown in boldface.

the transposon-containing DNA fragment was used as the probe. None of the hybridizing bands were 1.3 kb, the size expected if the entire plasmid had integrated through a site on the vector. Furthermore, no hybridization was observed when the Ts carrier plasmid was used as the probe. Together, these results indicate that transposition took place at different sites and that plasmid DNA does not integrate into the chromosome together with the transposon. These results show that the Ts plasmid can thus be readily employed as a delivery vector.

DISCUSSION

In vitro mutagenesis of plasmid DNA has been used successfully for the isolation of a Ts mutant of the pWV01 replicon, pVE6002. Plasmid loss at temperatures above 35°C is due to a lesion in the pVE6002 Rep protein.

pVE6002 Ts mutations and a Ts mutation of the related plasmid pE194 lie in neighboring regions. Single-stranded DNA replicons are classified into families of highly related plasmids isolated from diverse bacteria (11). The plus origin (plus-*ori*) and the Rep protein of pWV01 (the parent of the Ts plasmid [25]) have homologies with members of the pE194 family, including pADB201 (2), pLB4 (1), pLS1 (19, 30), pSH71 (7a), pFX2 (49), and pHPK255 (16). A Ts derivative of pE194 has already been characterized; two mutations were found, one immediately upstream of its start codon, and the other within the Rep ORF (48).

The Ts lesions of pVE6002 and pE194 map in neighboring regions on the Rep sequences, separated by about 20 amino acids (Fig. 6). However, while the pE194 lesion is in a relatively conserved stretch of the protein sequence, the lesions in pVE6002 are clustered within a hypervariable domain. This domain is flanked by conserved sequences and is absent from plasmids pE194, pLS1, pADB201, pHPK255, and pLB4 of the pE194 group (Fig. 6).

Use of pVE6002 Ts character in different gram-positive hosts. The thermosensitivity of pVE6002 replication was confirmed in *L. lactis*, *B. subtilis*, and *E. coli*. This indicates that a wide range of bacteria can make use of this Ts delivery vector. The low temperature of plasmid shut-off, 35°C, makes it particularly useful for the mesophilic lactic acid bacteria, as certain members of this group do not grow well above 37°C. One potential limitation is the ability of this plasmid to become established in the species of interest. However, transfer of pGK12 by electroporation into *Lactobacillus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus*, and *Propionibacterium* spp. has already been demonstrated (26). Plasmid transfer by other methods may also be feasible, especially when the strains of interest are nontransformable. For example, high-frequency plasmid transfer can be achieved by transduction after insertion of fragments of the transducing bacteriophage into the plasmid (3, 33, 34, 40). Conjugation can also be used (18, 41); we are constructing a pVE6002 derivative which can be mobilized in *trans* by the conjugative plasmid pIP501 (19a) in several gram-positive bacteria, including two species of lactobacilli (*L. delbruckii* subsp. *bulgaricus* and *L. helveticus*), which are presently poorly or nontransformable (21).

Use of the Ts vector to obtain chromosomal insertions. Heterologous nonreplicative vectors have been used previously to mutagenize the *L. lactis* chromosome via single or double crossover integration events (6, 23, 24). The Ts plasmid should be readily adaptable for mutagenesis in a variety of hosts, using both recombinational inactivation and transposition as methods of chromosome targeting. We showed here that pVE6004 is an efficient delivery vector for a Tn*10* derivative in *B. subtilis*; transposition occurred in about 1% of cells. We are presently developing a transposition system for use in the lactic bacteria.

pVE6002 and derivatives containing a polylinker have already been used to obtain chromosomal single crossover integrants through 1 kb of homology by selection at 37°C; integration frequencies are varying from 10^{-2} to 10^{-4} integrants per cell (2a). Double crossover events were also obtained; a strategy was adopted in which shift-down of Campbell-like integrants to 28°C allows plasmid replication and stimulates a second recombination event (12, 32, 37). The Ts plasmid can be used in this way to introduce and stabilize heterologous or homologous genes of interest.

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