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Insertional Mutagenesis and Recovery of Interrupted Genes of *Streptococcus mutans* by Using Transposon Tn*917*: Preliminary Characterization of Mutants Displaying Acid Sensitivity and Nutritional Requirements

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New vectors were constructed for efficient transposon Tn*917*-mediated mutagenesis of poorly transformable strains of *Streptococcus mutans*(pTV1-OK) and subsequent recovery of interrupted genes in *Escherichia coli*(pTV21Δ2TetM). In this report, we demonstrate the utility of Tn*917* mutagenesis of a poorly transformable strain of *S. mutans* (JH1005) by showing (i) the conditional replication of pTV1-OK, a *repA*(Ts) derivative of the broad-host-range plasmid pWVO1 harboring Tn*917*, in JH1005 at the permissive temperature (30°C) versus that at the nonpermissive temperature (45°C); (ii) transposition frequencies similar to those reported for *Bacillus subtilis* (10^{-5} to 10^{-4}) with efficient plasmid curing in 90 to 97% of the erythromycin-resistant survivors following a temperature shift to 42 to 45°C; and (iii) the apparent randomness of Tn*917* insertion as determined by Southern hybridization analysis and the ability to isolate nutritional mutants, mutants in acid tolerance, and mutants in bacteriocin production, at frequencies ranging from 0.1 to 0.7%. Recovery of transposon-interrupted genes was achieved by two methods: (i) marker rescue in *E. coli* with the recovery vector pTV21Δ2TetM, a tetracycline-resistant and ampicillin-sensitive Tn*917*-pBR322 hybrid, and (ii) "shotgun" cloning of genomic libraries of Tn*917* mutants into pUC19. Sequence analyses revealed insertions at five different genetic loci in sequences displaying homologies to *Clostridium* spp. *fhs* (66% identity), *E. coli dfp* (43% identity), and *B. subtilis ylxM-ffh* (58% identity), *icd* (*citC* [69% identity]), and *argD* (61% identity). Insertions in *icd* and *argD* caused nutritional requirements; the one in *ylxM-ffh* caused acid sensitivity, while those in *fhs* and *dfp* caused both acid sensitivity and nutritional requirements. This paper describes the construction of pTV1-OK and demonstrates that it can be efficiently employed to deliver Tn*917* into *S. mutans* for genetic analyses with some degree of randomness and that insertions in the chromosome can be easily recovered for subsequent characterization. This represents the first published report of successful Tn*917* mutagenesis in the genus *Streptococcus*.

Transposon mutagenesis has proven to be a powerful tool for genetic analysis of several medically important bacteria. In the case of *Streptococcus mutans*, the oral pathogen responsible for dental caries, current methodologies have been limited to the introduction of Tn*916* on suicide vectors into readily transformable strains (2, 44, 55). There are several limitations to this approach. Introduction of this transposon requires both transformation and transposition, two low-frequency events. Once a mutant of interest has been isolated, there is difficulty in recovering this large (18-kb), unstable element for molecular characterization. Therefore, genetic characterization by Tn*916*-mediated mutagenesis has been of limited use with the genus *Streptococcus*. A recent report by Lunsford (30) indicates that the small staphylococcal transposon Tn*4001* (4.5 kb) can be used for mutagenesis of two oral *Streptococcus* species, *S. gordonii* and *S. mutans*. As in the case of mutagenesis with Tn*916*, this transposon is delivered by transformation on a suicide vector, which significantly lowers the overall frequency of transposition and the efficacy of these transposons in mutagenesis.

Advances in transposon mutagenesis in *Bacillus subtilis* (4, 56, 57) have centered around delivery of smaller transposable elements, such as Tn*917*, for in vivo, transformation-independent mutagenesis. This approach is particularly useful in poorly transformable strains. This small (5.2-kb) transposon is delivered on replication-conditional (temperature-sensitive) vectors, pTV1, pTV1Ts, and derivatives (4, 56, 57), which are based on the staphylococcal replicons pE194 (22) and pE194Ts (53). Exploiting the conditional function of this replicon, transposon mutants can be selected after inducing the loss of the delivery system. By this approach, transposon Tn*917* and its derivatives have been successfully used in other gram-positive organisms, including species in the genera *Lactococcus* (23), *Clostridium* (1), *Listeria* (4, 9), *Staphylococcus* (6, 48), and *Enterococcus* (8) (in which it was first discovered [51]). However, success in the generation of mutants of *S. mutans* and other streptococcal species with pTV1Ts and derivatives have not been reported, indicating that the pE194 origin of replication may not promote efficient propagation and thus mutagenesis in this genus.

To circumvent these limitations, we constructed vectors and developed methods for efficient Tn*917* mutagenesis in *S. mutans* and recovery in *Escherichia coli*. The transposon was delivered in plasmid pTV1-OK, analogous to pTV1 but containing the *repA*(Ts) gene from pWVO1, a broad-host-range *Lactococcus lactis* plasmid (31, 36). pWVO1 is among a small group of plasmids which are capable of replication in gram-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
Streptococcus mutans		
JH1005	Wild type; BLIS$^+$	19
NG8	Wild type; BLIS$^-$	K. Knox
AS5	JH1005 *fhs*::Tn*917*, pH5^s Ade$^-$ BLIS$^-$	This work
AS5N	NG8 *fhs*::Tn*917*, pH5^s Ade$^-$	This work
AS17	JH1005 *sat*::Tn*917*, pH5^s	This work
AS17N	NG8 *sat*::Tn*917*, pH5^s	This work
AS25	JH1005 *dfp*::Tn*917*, partial pH5^s TDM$^-$	This work
AS25N	NG8 *dfp*::Tn*917*, partial pH5^s TDM$^-$	This work
AX1	JH1005 *icd*::Tn*917*, Glu$^-$	This work
AX3	JH1005 *argD1*::Tn*917*, Arg$^-$	This work
DM25	JH1005 *bls*::Tn*917*, BLIS$^-$	This work
Streptococcus rattus		
BHT-2	Wild type; Str^r (1 mg/ml), BLIS indicator strain	19
Escherichia coli		
DH5α	F′ ϕ80*dlac*Δ(*lacZ*) *M15 endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lacZYA-argF*)*ul69*	Bethesda Research Laboratories, Gaithersburg, Md.
HB101	*hsd-520 recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 leu mtl-11 mcrB*	J. Hillman
MC1061	Δ(*araA-leu*)*7697 araD139* Δ(*codB-lac*)*3 galE15 galK16 mcrA0 relA1 rpsL150 spoT1 mcrB9999 hsdR2*	J. Hillman
RR1	HB101 *recA*$^+$	P. Youngman
Plasmids		
pJRS233	*repA*(Ts)-pWV01Ts *rep*-pSC101 *erm*	36
pKD102	pUC18 *cat aphA3*	W. Haldenwang
pOTS	*repA*(Ts)-pWV01Ts *rep*-pUC19 *bla*	This work
pOTSKan	*repA*(Ts)-pWV01Ts *rep*-pUC19 *aphA3 bla*	This work
pPC1917	pUC19 harboring the 3.6-kb *Pst*I-*Kpn*I *erm*$^+$ fragment of pTV1-OK	This work
pTV1	*repA*-pE194 *cat* Tn*917*(*erm*)	56
pTV1-OK	*repA*(Ts)-pWV01Ts *aphA3* Tn*917*(*erm*)	This work
pTV21Δ2	*repA*-pE194 Tn*917*Δ*erm*::pHW9 (*rep*-pBR322*cat bla*)	58
pTV21Δ2TetM	*repA*-pE194 Tn*917*Δ*erm*::pVA981 (*rep*-pBR322*tetM*)	This work
pUC19	*lacZ*α *bla*	Pharmacia, Piscataway, N.J.
pUC-AS17	pUC19 harboring a 9.0-kb *Eco*RI fragment from strain AS17 containing *sat*::Tn*917* (Em^r in *E. coli* MC1061)	This work
pVA981	*rep*-pBR322*tetM*	50
pAS5NCE, pAS25NCE, pAX1CE, and pAX3CE	*fhs*, *dfp*, *icd*, and *argD*::Tn*917*Δ*erm*::pVA981 recovery with *Eco*RI digests of chromosomal DNA from convertant strains AS5N, AS25N, AX1, and AX3, respectively	This work

[a] BLIS$^+$ or BLIS$^-$ denotes proficiency or deficiency in production of a BLIS, respectively; pH5^s denotes inability to grow in media at pH 5; Glu$^-$, Arg$^-$, and Ade$^-$ indicate dependency on L-glutamate, L-arginine, and adenine for growth on CMM, respectively; TDM$^-$ indicates dependency on a uncharacterized nutrient present in THYE for growth on TDM or CMM; and Str^r indicates streptomycin resistance.

positive and -negative bacteria (26), including *Streptococcus suis* type 2 (43). The plasmid also harbors a selectable antibiotic resistance gene, *aphA3*, which expresses kanamycin resistance in both *E. coli* and gram-positive hosts (52).

Beginning with the studies presented in this paper, we are undertaking a comprehensive study of the genetic basis of acidurance, bacteriocin production, and other physiological properties of the major etiological agent of human dental caries.

(Parts of this work have been presented previously [10, 14].)

MATERIALS AND METHODS

Bacteria, plasmids, and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown either in liquid or on solid (2% agar) Luria-Bertani (LB) medium. *S. mutans* NG8 and JH1005 and the streptomycin-resistant *Streptococcus rattus* BHT-2 were grown in Todd-Hewitt broth or on 2% agar containing 0.3% yeast extract with 10% sucrose (THYES) or without it (THYE), brain heart infusion (BHI) agar, a chemically defined medium (TDM [47]), or Carlsson's minimal medium (CMM [5]) at pH 5 or 7, with the pH adjusted by addition of NaOH or HCl. *E. coli* was cultivated at 37°C or, when harboring pTV1-OK, at 28°C. All *S. mutans* incubations were performed at 37°C, except for strains bearing the plasmid pTV1-OK, which were grown at 28 to 30°C. In liquid media, cultures of *S. mutans* were incubated aerobically without agitation, while those in solid media were incubated in either candle jars or GasPak anaerobic chambers (BBL, Cockeysville, Md.). Antibiotics included ampicillin (50 μg/ml), streptomycin (1 mg/ml), kanamycin (75 μg/ml for *E. coli* and 500 μg/ml for *S. mutans*), erythromycin (300 μg/ml for *E. coli* and 10 μg/ml for *S. mutans*), and tetracycline (15 μg/ml for *E. coli*, 10 μg/ml for *S. mutans* derivatives of strain NG8, or 20 μg/ml for derivatives of *S. mutans* JH1005).

General genetic manipulations. Transformation of *S. mutans* was performed as described by Perry and Kuramitsu (37) with some modifications. Competent cells were obtained by cultivation in THYE broth, and transformants were selected on THYES or BHI agar containing an antibiotic(s). For transformation with pTV1-OK, competent cells grown at 37°C were kept at 25°C for 10 min prior to the addition of plasmid DNA. After its addition, all subsequent incubations were carried out at 30°C. *S. mutans* chromosomal DNA was obtained by a modification of the method of Marmur (33). DNA was extracted from exponentially growing cultures in THYE broth containing 20 mM D,L-threonine which

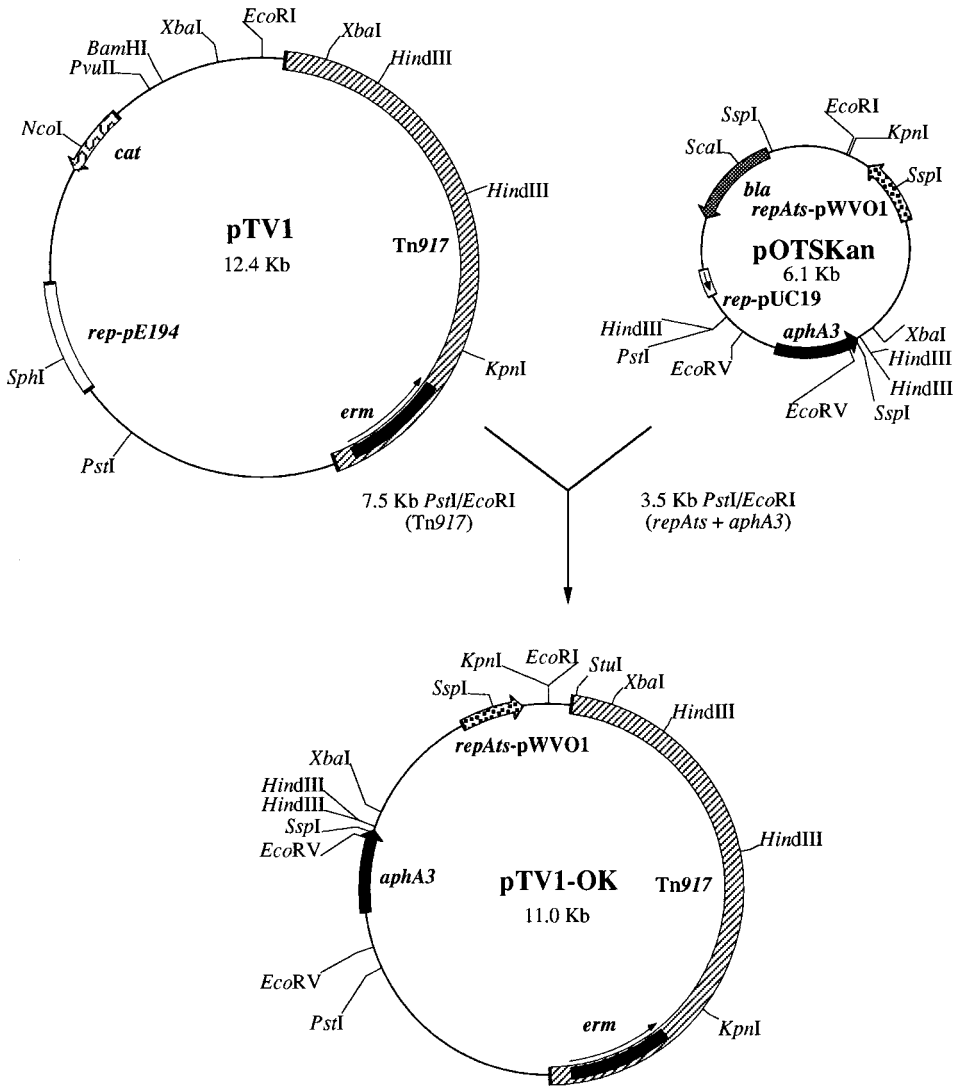

FIG. 1. Construction of pTV1-OK, a Tn*917* delivery vector that uses the broad-host-range replicon of the *L. lactis* plasmid pWVO1. Gene designations: *rep-pE194*, replicon from plasmid pE194; *repA*(Ts)-pWVO1, temperature-sensitive replicon from pWVO1; *rep*-pUC19, replicon from plasmid pUC19 (ColEI); *aphA3*, kanamycin resistance; *cat*, chloramphenicol resistance; Tn*917*, transposon Tn*917*; *erm*, erythromycin resistance associated with Tn*917*.

were treated with 5% glycine for 1 h prior to harvesting. Other DNA manipulations were as described by Maniatis et al. (32). Southern hybridizations were done by the PhotoGene system (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions with biotinylated pTV1-OK as the probe.

Construction of pTV1-OK and pTV21Δ2TetM. Plasmid pTV1-OK (Fig. 1) is a *repA*(Ts) derivative of the *L. lactis* plasmid pWVO1 (31, 36) for conditional replication in both gram-positive bacteria and *E. coli*. It harbors the *aphA3* gene (52), which expresses kanamycin resistance (Km^r) in *E. coli* and *S. mutans*, and the transposon Tn*917* (51), which confers erythromycin resistance (Em^r) to gram-positive organisms. pTV1-OK was constructed by ligation of the 3.5-kb *Eco*RI-*Pst*I backbone containing the *repA*(Ts) and *aphA3* genes of pOTSKan (see below) to the 7.5-kb *Eco*RI-*Pst*I fragment of pTV1 containing Tn*917* (56). Recombinant plasmids were obtained by transformation of *E. coli* HB101 with selection at 30°C on LB-kanamycin agar. For *S. mutans* transformation experiments, the plasmid was propagated in *E. coli* RR1 ($recA^+$) to obtain multimers thought to improve transformation efficiency by plasmid DNA (42).

Plasmid pOTSKan was constructed in two steps. First, the *repA*(Ts) pWVO1 gene, as a 2-kb *Sau*IIIA fragment from pJRS233 (36), was cloned into the *Bam*HI site of pUC19, which yielded pOTS. The *aphA3* gene, as a 1.5-kb *Pst*I-*Xba*I fragment of pKD102 (14a), was then cloned into *Pst*I-*Xba*I-cut pOTS, yielding pOTSKan. pKD102 is a derivative of pUC18 harboring the antibiotic resistance markers *aphA3* (52) and *cat* (21).

The *Bacillus* recovery plasmid pTV21 (58), harboring the recovery construct Tn*917*::pHW9, was prepared by "stuffing" the replication function of pBR322 (*rep*-pBR322) and two antibiotic resistance markers (*cat* and *bla*) from pHW9 (21) into the middle of Tn*917*, rendering it defective in its ability to transpose. pTV21Δ2 (58), a derivative of pTV21, contains a deletion extending into the *erm* gene of Tn*917*. The deletion in this construct, designated Tn*917*Δ*erm*::pHW9, abolishes the toxicity associated with Tn*917* in $rpsL^+$ *E. coli* strains. Plasmid pTV21Δ2TetM (Fig. 2) is a derivative of pTV21Δ2 in which the *S. mutans tetM* (tetracycline-resistance [Tc^r]) gene from plasmid pVA981 (50) replaced the *bla* and *cat* genes from pHW9, which also donated the pBR322 *rep* gene. The resulting recovery construct was designated Tn*917*Δ*erm*::pVA981. To construct pTV21Δ2TetM, the 9.3-kb *Pvu*II-*Sal*I fragment from pTV21Δ2 was purified, blunted with the Klenow fragment, and ligated to pVA981 which had been digested with *Pvu*II and dephosphorylated with calf intestinal phosphatase. We routinely propagated this plasmid in *E. coli* HB101.

Tn*917* mutagenesis with pTV1-OK. Transformants of JH1005 were selected on BHI agar containing kanamycin at 30°C. Independent pools of Tn*917* insertions into host chromosomal DNA were generated by a temperature shift (42 to 45°C), as described by Camilli et al. (4) with some modifications. Saturated cultures grown in THYE broth plus kanamycin at the permissive temperature (28 to 30°C) were subcultured 1/100 or 1/200 into five tubes containing THYE broth plus erythromycin at a sublethal concentration (0.04 μg/ml) previously shown to induce the *erm* and transposase genes of Tn*917* (51). Following a shift to 42 to 45°C and overnight incubation, insertions of Tn*917* into the host chromosome were isolated by plating samples on BHI or THYE agar containing erythromycin

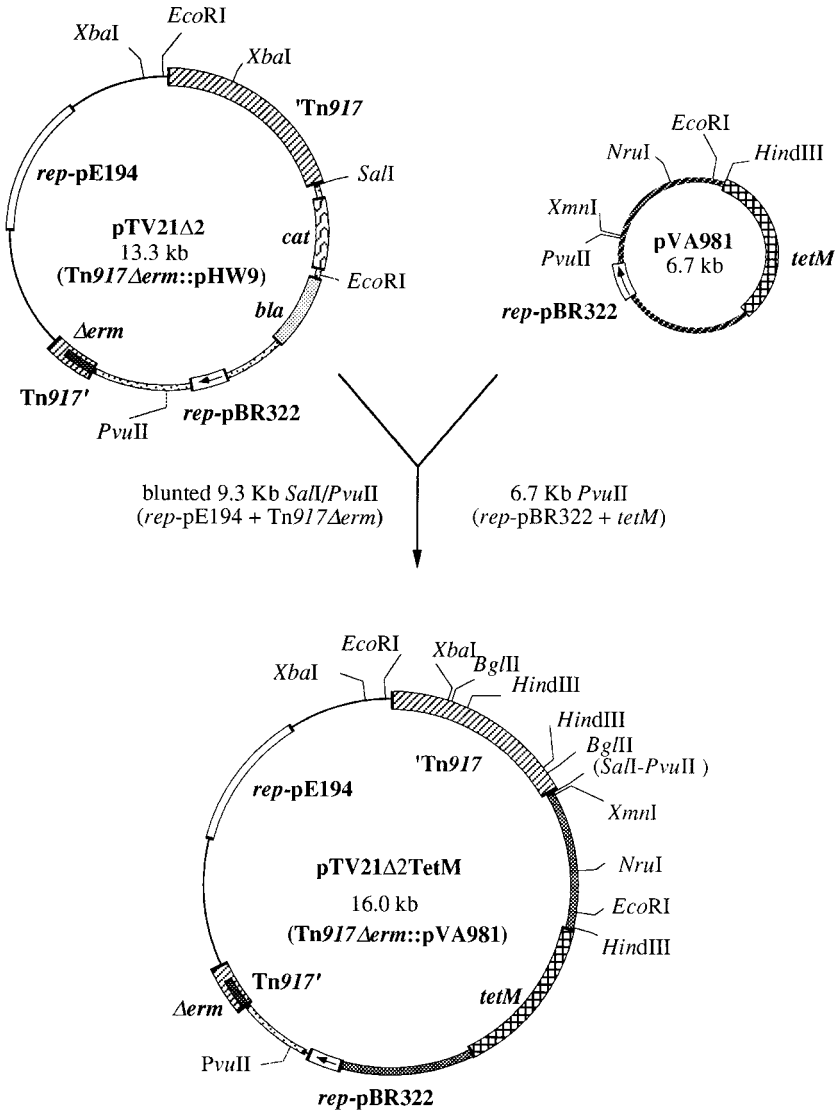

FIG. 2. Construction of pTV21Δ2TetM, a Tn*917*-pBR322 (Tn*917*Δ*erm*::pVA981) hybrid vector derived from pTV21Δ2 and pVA981 for recovery of Tn*917* transpositions in *E. coli*. Gene designations: *rep*-pE194, replicon from plasmid pE194; *rep*-pBR322, replicon from plasmid pBR322 (ColEI); *'*Tn*917* or Tn*917'*, interruption of transposon Tn*917*; Δ*erm*, deletion of part of Tn*917* that inactivates the *erm* determinant associated with Tn*917*; *cat*, chloramphenicol resistance; *bla*, ampicillin resistance; *tetM*, tetracycline resistance.

at the selective concentration (10 μg/ml). Colonies which arose were screened for loss of the plasmid backbone on BHI agar or THYE containing kanamycin. Kanamycin-sensitive clones were then screened for a variety of mutant phenotypes as indicated in Tables 1 and 2.

Recovery of Tn*917* mutations. Two approaches were used to clone Tn*917* insertions. The first is outlined in Fig. 3: Tn*917*-mutagenized strains (ca. 10^8 competent cells) were transformed with ca. 0.5 μg of *Xba*I-linearized or uncut pTV21Δ2TetM. Transformants were selected on THYES agar supplemented with tetracycline after incubation at 37°C for 42 h. Recombinants displaying a Tc^r and Em^s phenotype, called convertants, were isolated. Marker rescue was accomplished as previously described (58). Chromosomal DNAs from convertant strains were isolated; digested with restriction enzyme *Eco*RI, *Hin*dIII, *Nru*I, *Xba*I, *Bgl*II, or *Bam*HI; and self-ligated at low concentrations (ca. 0.1 to 0.2 μg of DNA per 100 μl). The DNAs were ethanol-precipitated with 10 μg of carrier *Saccharomyces cerevisiae* tRNA (Boehringer Mannheim, Indianapolis, Ind.) and used to transform $CaCl_2$-competent *E. coli* DH5α. Clones harboring the Tn*917*Δ*erm*::pVA981 basic replicon and adjacent streptococcal DNAs were obtained by selection on LB-tetracycline agar.

In the second recovery method, libraries were constructed by ligation of *Eco*RI chromosomal digests from the Tn*917* mutants into pUC19, which had been cut with *Eco*RI and dephosphorylated. Libraries were introduced into $CaCl_2$-competent MC1061. To isolate clones harboring Tn*917*, transformants were plated onto LB agar containing ampicillin and replica plated onto LB agar supplemented with erythromycin. Alternatively, transformants were plated onto LB agar containing both antibiotics.

Nucleotide sequencing and sequence analyses. Nucleotide sequencing was carried out at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished with *Taq* DiDeoxy terminators and by the DyePrimer Cycling Sequence protocol developed by Applied Biosystems with fluorescently labeled dideoxynucleotides and primers, respectively. Labeled extension products were analyzed on an Applied Biosystems 373A DNA sequencer. The Tn*917*-based primers used were P1, GCAATAACCGTTACCTG, an *erm*-proximal oligonucleotide; and P2, GAAGCATGTATCTCCTAT, an *erm*-distal oligonucleotide, both synthesized at the DNA Synthesis Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Plasmids recovered with pTV21Δ2TetM (Fig. 3) were sequenced with P1, while the plasmid recovered with pUC19 was sequenced with both P1 and P2. Sequence analyses were carried out with MacVector version 3.5 software and the programs BLASTN and BLASTX (National Center for Biotechnology Information, Los Alamos, N.Mex.), available via the Internet.

Nucleotide sequence accession numbers. The partial sequences described in Table 3 have been deposited in the GenBank-EMBL data bank under the

TABLE 2. Description of JH1005 mutants isolated by Tn*917* mutagenesis

Selected mutant phenotype[a]	Strain	Pool (frequency)[b]	Additional phenotype
Acid sensitivity (pH5^s)	AS5	1 (0.09)	Ade$^-$ BLIS$^-$
	AS17	1 (0.09)	ND[c]
	AS25	1 (0.09)	TDM$^-$
Nutritional requirements			
Glu$^-$	AX1	3 (0.69)	ND
Arg$^-$	AX3	4 (0.69)	ND
BLIS$^-$	DM25	5 (0.69)	ND

[a] The phenotypes for acid sensitivity, nutrient requirement, and loss of bacteriocin production (BLIS$^-$) were scored on the basis, respectively, of their inability to grow on TDM (pH 5) and THYE (pH 5), to grow on CMM, or to produce a zone of inhibition when an overlay of *S. rattus* BHT-2 (10^3 to 10^4 cells) in top agar containing streptomycin was placed on THYE or BHI agar plates that had been stab inoculated and incubated for 24 to 48 h prior to the overlay (19). To determine the nutritional requirements of mutants failing to grow on CMM, we used the approach described in reference 11. The requirement(s) of mutant AS25, which also failed to grow on the complete but defined medium TDM (TDM$^-$), could not be determined by this method.

[b] Mutational frequencies were estimated as the percentages of independent clones displaying a particular phenotype in a given pool. Pool 1 consisted of 3,500 clones, while pools 3 to 5 consisted of 170 clones each.

[c] ND, none detected.

following accession numbers: U48882 for *fhs*, U48883 for *ffh*, U48884 for *ylxM*, U48885 for *dfp*, U48886 for *icd*, and U48887 for *argD.*

RESULTS

pTV1-OK replicates conditionally in *S. mutans*. Plasmid pTV1-OK (Fig. 1) is a novel delivery vector for Tn*917* mutagenesis that uses the conditional replicative functions of a *repA*(Ts) derivative of the *Lactococcus* broad-host-range plasmid pWVO1. Transformation of JH1005 (ca. 10^8 cells) with 1.0 μg of pTV1-OK DNA carried out at 30°C yielded four Km^r transformants, which is in agreement with earlier experiments that suggested a low transformation frequency associated with this strain (17). When incubated at 42°C on solid media containing kanamycin, however, all transformants failed to grow, presumably because the *aphA3* backbone marker was lost by a temperature-induced curing of the *repA*(Ts) plasmid. This result indicated that pTV1-OK replicates in *S. mutans* JH1005 at the permissive temperature (30°C) but not at the nonpermissive temperature (42°C).

Transformants were also shown to be resistant to 5 to 10 μg of erythromycin per ml at 30°C, indicating that the *erm* determinant in Tn*917* can express resistance to selectable levels of erythromycin in *S. mutans*. These observations suggested that pTV1-OK could function as a delivery vector for Tn*917* mutagenesis in *S. mutans*. One transformant was chosen for mutagenesis.

Tn*917* mutagenesis of *S. mutans* with pTV1-OK. JH1005/pTV1-OK, grown at 28 to 30°C, was subcultured at 42 to 45°C until late exponential phase (six to seven generations) as described in Materials and Methods. Samples of the cultures were plated on media with and without erythromycin; 10^4 to 10^5 of 10^9 (0.01 to 0.1%) total CFU retained their resistance to this antibiotic. When groups of 100 Em^r colonies obtained in five separate experiments were patched onto kanamycin-containing media, 90 to 97 were Km^s while 3 to 10 were Km^r. This finding indicated that 90 to 97% of the Em^r clones were the result of the coincident loss of pTV1-OK and the transposition of Tn*917* into the JH1005 chromosome, which, therefore, occurred at a frequency of ca. 10^{-4}. The occurrence of Km^r in the remaining 3 to 10% of the Em^r clones could be explained if the plasmid pTV1-OK integrated into their chromosomes, resulting in replicon fusions, or if spontaneous temperature-resistant pTV1-OK-containing derivatives were selected. To study integration of the transposon into the chromosome of *S. mutans*, we performed Southern blot analysis of chromosomal DNAs derived from 10 randomly chosen Em^r mutants (Fig. 4) and digested with *Stu*I, an enzyme that cuts once within pTV1-OK (74 bases into the *erm*-distal end of Tn*917* [Fig. 1]), and whole pTV1-OK as the probe. Analysis of the blot suggested that Tn*917* transposition was random and resulted in single chromosomal insertions (Fig. 4). Two Em^r Km^r clones revealed two fragments of chromosomal DNA able to react with the vector probe, indicating the occurrence of replicon fusions (see the legend to Fig. 4.)

Mutant screening and initial characterization. By screening five independent pools of Tn*917* insertions in JH1005, we isolated mutants (Table 2) displaying acid sensitivity, designated pH5^s (strains AS5, AS17, and AS25), requirements for the amino acids L-arginine or L-glutamate, designated Arg$^-$ or Glu$^-$, respectively (strains AX1 and AX3), and loss of production of a bacteriocin-like inhibitory substance, designated BLIS$^-$ (strain DM25).

Comparisons of the growth characteristics of the pH5^s mutants on solid media at pH 5 showed that the degree of acid sensitivity ranged from weak, in AS25, to strong, in AS17, with strain AS5 showing an intermediate level of sensitivity. In addition, AS5 required adenine for growth on the minimal medium CMM and displayed a BLIS$^-$ phenotype. AS25 failed to grow on CMM and TDM (TDM$^-$), a defined medium containing 18 amino acids, 3 nitrogenous bases, and several vitamins, but grew normally on THYE (pH 7). We were unable to identify the nutrient(s) required for growth of AS25 on TDM or CMM in standard auxotrophy experiments (11).

Southern hybridization analyses of pH5^s mutants AS5, AS17, and AS25 indicated that Tn*917* insertions occurred in different loci (data not shown). To determine whether the lesions induced by Tn*917* are responsible for the mutant phenotypes, we transformed the highly competent *S. mutans* wild-type strain NG8 with chromosomal DNAs from these strains, selecting for Em^r on THYES. Examination of the phenotypes of 38 transformants derived from each of the mutants suggested that the transposons (Em^r) were indeed coinherited with their respective mutant phenotypes at frequencies ranging from 82 to 100%. Representative transformants, designated AS5N, AS17N, and AS25N, were chosen for further studies.

Cloning of chromosomal sequences bordering Tn*917* insertion sites. We cloned DNAs of six different loci adjacent to Tn*917* insertions in strains AS5N, AS17, AS25N, AX1, and AX3 (Table 3). Two methods were used to clone these DNA sequences. The principal method exploited Tn*917* homology-mediated recombination into Tn*917*-mutagenized strains to introduce the recovery construct Tn*917*Δ*erm*::pVA981 harbored in plasmid pTV21Δ2TetM (Fig. 2 and 3). Allelic replacement of the wild-type Tn*917* by the Tn*917*Δ*erm*::pVA981 hybrid stabilized transposon insertions and allowed for the cloning of DNAs adjacent to Tn*917* insertion sites by marker rescue (Fig. 3). The five aforementioned mutant strains were transformed with *Xba*I-linearized pTV21Δ2TetM, selecting for Tc^r derivatives. Transformation efficiencies ranged from 5 to 500 Tc^r transformants per μg of linearized DNA. As expected, 100% of the transformants were Em^s and retained the phenotypes of the original mutant strains, indicating the isolation of convertant strains. When pTV21Δ2TetM was introduced as a

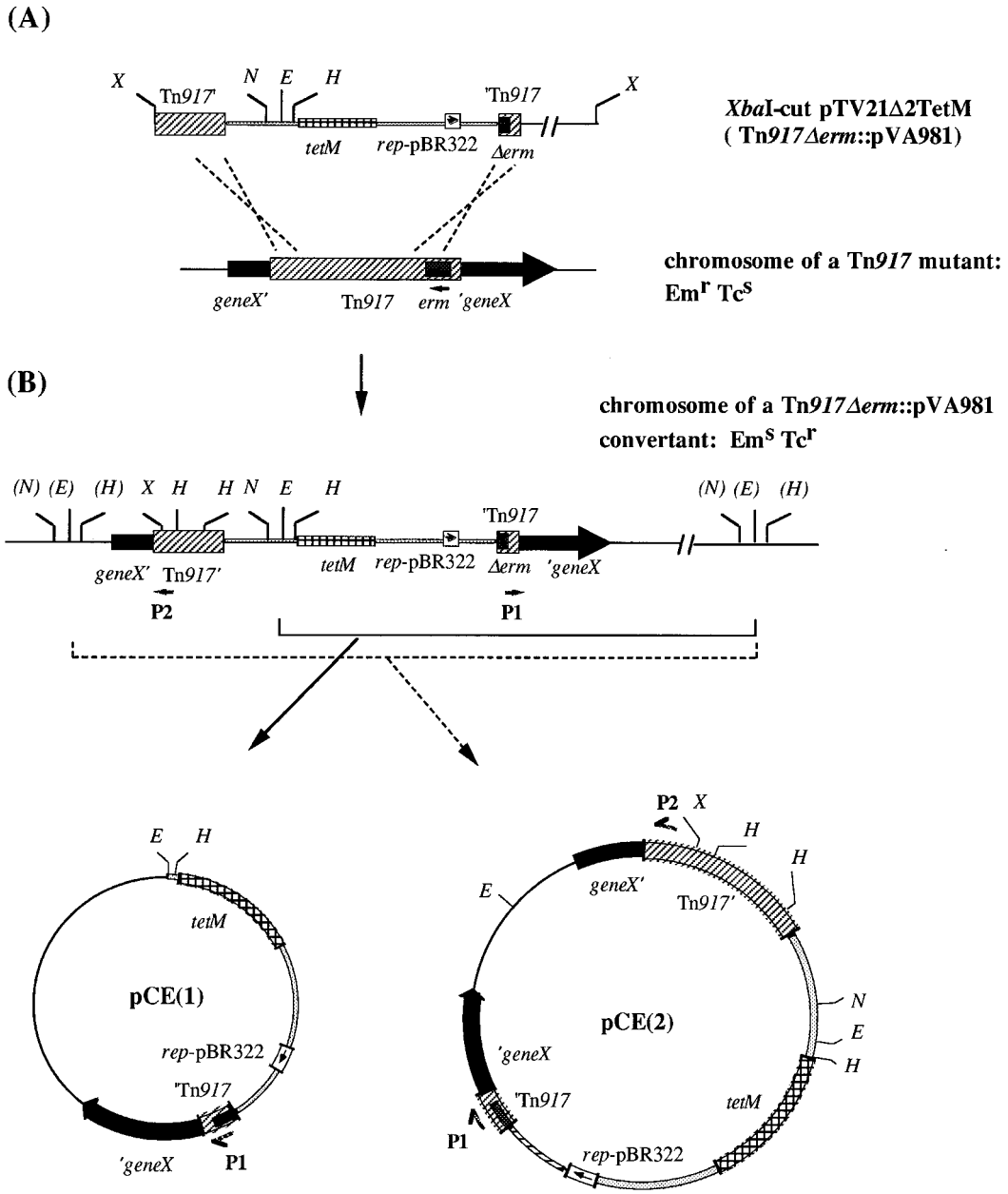

FIG. 3. Cloning streptococcal DNA sequences adjacent to a Tn*917* insertion with the recovery vector pTV21Δ2TetM. The two-step process requires, first, allelic replacement of wild-type Tn*917* by Tn*917*Δ*erm*::pVA981 (A), followed by marker rescue in *E. coli* (B). To clone the DNA bordering the *erm*-proximal end of the transposon insertion, chromosomal DNA from the *S. mutans* convertant strain is digested with restriction enzymes that cut outside of the selectable marker *tetM* and the pBR322 *rep*, such as *Bgl*II, *Eco*RI, *Hin*dIII, *Nru*I, or *Xba*I (e.g., *Eco*RI). The digests are then ligated at concentrations that promote self-ligation. Finally, ligation products are precipitated and used to transform *E. coli*, selecting for clones containing plasmids expressing tetracycline resistance [e.g., pCE(1)]. Digests with restriction enzymes that do not cut within the Tn*917*Δ*erm*::pVA981 element, such as *Bam*HI, *Xho*I, and others, as well as partial digests with the above-mentioned enzymes can also be used for marker rescue (e.g., partial digest with *Eco*RI). In this case, DNA sequences adjacent to the two ends of the transposon insertion could be cloned [e.g., pEC(2)]. Gene designations are those described in the legends to Fig. 1 and 2. *geneX*, a Tn*917*-inactivated gene; P1 and P2, sequencing primers based on homology to the *erm*-proximal sequence and to the *erm*-distal sequence, respectively (see Materials and Methods). *E*, *Eco*RI; *H*, *Hin*dIII; *N*, *Nru*I; *X*, *Xba*I.

supercoiled molecule rather than as a linear molecule, we found that ca. 50% of the Tc^r transformants were Em^s, which suggests that in addition to Campbell-type integrants expected to retain the original Em^r marker, allelic exchange occurred at high frequencies. Although the transformation efficiencies of JH1005 derivatives (AX1 and AX3) were ca. 20- to 100-fold lower when compared with those obtained with NG8 derivatives (AS5N, AS17N, and AS25N), we nonetheless were able to obtain convertants to carry out marker rescue. *Eco*RI digests of chromosomal DNA preparations of the five convertant strains were subjected to marker rescue in *E. coli*, resulting in the isolation of recombinant plasmids for four of the five strains (pAS5NCE, pAS25NCE, pAX1CE, and pAX3CE) (Table 1). We found that chromosomal digests with enzymes *Hin*dIII and *Nru*I also yielded rescued plasmids from the same four convertants (data not shown), but we were unable to

TABLE 3. Homology analyses of nucleotide sequences of plasmids derived from five Tn*917*-mutagenized strains using BLASTN and BLASTX

Plasmid/strain/phenotype	Primer/no. of bp sequenced/ accession no.	Schematic representation of gene(s) and organism(s)[a]	Homology	Reference, accession no.
pAS5NCE/ AS5/ pH5^S, Ade$^-$, BLIS$^-$	P1/ 477 bp U48882	P1 → *fhs* (143–299); 1–556; *Clostridium* spp.	143-299 aa of 556-559 aa 64-67% Identity 78-83% Similarity	54, P21164 27, P13419 40, Q07064
pUC-AS17/ AS17/ pH5^S	P1/ 459 bp U48883 P2/ 435 bp U48884	P2 P1 → *ylxM* (1–86); *ffh* (1–142); 1–110; 1–446; *Bacillus subtilis*	*ffh* 1-142 aa of 446 aa 57% Identity 77% Similarity *ylxM* 1-86 aa of 110 aa 59% Identity 75% Similarity	20, D14356 3, P07019
pAS25NCE/ AS25/ partial pH5^S, TDM$^-$	P1/ 437 bp U48885	P1 → *dfp* (30–169); 1–430; *Echerichia coli*	30-169 aa of 430 aa 43% Identity 61% Similarity	29, P24285 12, L10328 5
pAX1CE/ AX1/ Glu$^-$	P1/ 477 bp U48886	P1 → *citZ* — *citC (icd)* (68–227) — *citH*; 1–423; *Bacillus subtilis*	68-227 aa of 423 aa 69% Identity 84% Similarity	25, P39126 49, 1IKA
pAX3CE/ AX3/ Arg$^-$	P1/ 212 bp U48887	P1 → *argCJB* — *argD* (93–163) — *carABargF*; 1–385; *Bacillus subtilis*	93-163 aa of 385 aa 61% Identity 75% Similarity	34, P36839 16, P18335

[a] Gene designations and encoded proteins are as follows: *fhs*, encoding formyl-tetrahydrofolate synthetase (EC 6.3.4.3); *ylxM*, also referred to as *orf1*, and *ffh* of the *B. subtilis ylxM-ffh* operon, encoding a hypothetical protein, YlxM, and the homolog of the mammalian 54-kDa subunit, Ffh, of the signal recognition particle; *dfp*, encoding a DNA flavoprotein; *citC* (*icd*), encoding isocitrate dehydrogenase (EC 1.1.1.42), of the *B. subtilis citZCH* operon; and *argD*, encoding *N*-acetylornithine transaminase (EC 2.6.1.11), of the *B. subtilis argCJBDcarABargF* operon. The triangles with superimposed arrows represent the orientations and approximate locations of Tn*917* in the various genes. The arrows beneath the schematic diagrams represent the *erm* gene associated with Tn*917* and indicate its orientation. The symbol ▨ represents the extent of homology found with the predicted amino acid sequences derived from nucleotide sequences of the various clones. Other designations are as described in the legend to Fig. 3.

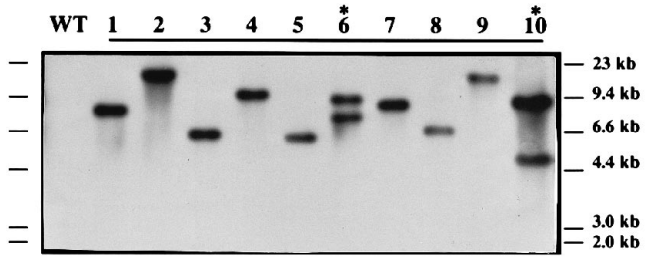

FIG. 4. Randomness and multiplicity of chromosomal insertions of Tn*917* in JH1005 mutagenized with pTV1-OK. Southern blot analysis was performed with *Stu*I chromosomal digests of JH1005 (the wild type [WT]) and 10 randomly chosen Em^r mutants with pTV1-OK as the probe. Mutants 6 and 10 (marked with an asterisk) were also Km^r, indicating the occurrence of replicon fusions. The restriction enzyme *Stu*I cuts once within pTV1-OK, 74 bases into the *erm*-distal end of Tn*917* (Fig. 1). Even though *Stu*I cuts within Tn*917*, it does so at the end of the transposon so that only one fragment is visible by Southern hybridization with the chromosomes of mutants harboring single transposon insertions (mutants 1 to 5 and 7 to 8). The fragment is too short to yield detectable hybridization. However, in the case of a replicon fusion, an event in which the whole delivery vector integrates into the chromosome, two fragments are detected with the probe (mutants 6 and 10). Molecular size markers are noted at the right.

recover plasmids from the AS17N convertant strain, even after repeated attempts using other restriction enzymes (*Hin*dIII, *Nru*I, *Xba*I, *Bgl*II, and *Bam*HI).

To clone the transposon insertion in the chromosome of the AS17 mutant, we used an alternative method. Customarily, cloning of transposon-linked sequences is achieved by a "shotgun" approach in which recombinant clones are selected by their ability to express the antibiotic resistance associated with the transposon in a suitable host. This method, however, has not been used for cloning of Tn*917* insertions into *E. coli*, because it has been reported that the Tn*917 erm* gene could not express Em^r at selectable levels in this host (7a). In addition, in *rpsL*$^+$ strains, the Tn*917 erm* gene or sequences proximal to it are toxic (58). We found that strain MC1061 (*rpsL150*) could be transformed with pPC1917, a pUC19 derivative harboring the 3.6-kb *Pst*I-*Kpn*I *erm*$^+$ fragment of pTV1-OK, by selecting for ampicillin resistance (Ap^r). More importantly, we found that Ap^r transformants expressed resistance to 300 μg of erythromycin per ml. Also, direct Em^r selection yielded pPC1917 transformants but only after 48 h of incubation. This observation suggested that MC1061 could be used as a suitable host for direct cloning of the transposon and adjacent chromosomal sequences in *E. coli*. Using this host, we cloned a library of *Eco*RI fragments in pUC19 from strain

AS17. By selecting for Ap^r clones able to express Em^r to 300 μg/ml upon screening, we isolated plasmid pUC-AS17. Interestingly, direct selection of transformants on LB agar containing ampicillin and erythromycin also allowed for the isolation of plasmid pUC-AS17 in MC1061, although transformants took an additional 24 h to arise and were 10-fold less numerous than were found in the 2-step process. *Eco*RI does not cut within the transposon sequences, allowing the rescue of chromosomal DNAs flanking both sides of the insertion site. Restriction enzyme mapping verified that pUC-AS17 carried the entire Tn*917* element (data not shown).

Nucleotide sequence analyses. To identify the Tn*917*-interrupted genes, we sequenced the first 300 to 700 bases (including ca. 200 bases of Tn*917* DNA) of the recovered plasmids by using Tn*917*-specific primers. The results were analyzed with BLASTN and BLASTX for homology comparisons at the DNA and amino acid levels, respectively, with gene sequences in databases. Results of these comparisons are summarized in Table 3.

In the case of the chromosomal DNA recovered from mutant AS5 ($pH5^s$ Ade^- $BLIS^-$), we found homology to the clostridial *fhs* genes (64 to 67% identity [27, 40, 54]), encoding formyl-tetrahydrofolate synthetase (EC 6.3.4.3), which is involved in several biosynthetic pathways requiring transfer of one-carbon units. The Tn*917*-containing fragment cloned from mutant AS17 ($pH5^s$) yielded homology to the *ylxM* and *ffh* genes of the *B. subtilis ylxM-ffh* operon (57 and 59% identity, respectively [20]), which encodes a protein of unknown function, YlxM, and the homolog of the mammalian 54-kDa subunit of the signal recognition particle, Ffh, involved in protein secretion. Henceforth, the *S. mutans ylmX-ffh* locus will be called *sat* for protein secretion and acid tolerance. We also found homology to the *E. coli ffh* gene (45% identity [3, 41]). With mutant AS25 ($pH5^s$ TDM^-), we found homology to the *E. coli* (43% identity [29]) and *Haemophilus influenzae* (38% identity [12]) *dfp* genes, encoding a flavoprotein presumed to be involved in pantothenate metabolism and DNA biosynthesis (45, 46). In the case of mutant AX1 (Glu^-), we found homology to the *B. subtilis citC* gene (69% identity [25]) and the *E. coli icd* gene product (69% identity [49]), encoding isocitrate dehydrogenase (EC 1.1.1.42), which is involved in regeneration of 2-ketoglutarate pools for nitrogen assimilation. Finally, with mutant AX3 (Arg^-), we found homology to the *B. subtilis* (61% identity [34]) and *E. coli* (42% identity [16]) *argD* genes, encoding *N*-acetylornithine transaminase (EC 2.6.1.11), which is involved in L-arginine biosynthesis.

DISCUSSION

Our laboratories are involved in studies concerning the genetic bases of acidurance in *S. mutans*, the production of a BLIS, and other physiological characteristics. To this end, we sought to isolate and characterize genes responsible for encoding a number of diverse proteins by transposon mutagenesis. Because the BLIS producer strain (JH1005) is poorly transformable and the available methods for mutagenesis (Tn*916* and insertional vectors) depend on high efficiencies of transformation, we considered alternative methods based on transposon Tn*917* delivered on a conditionally replicative vector (pTV1-OK). We demonstrated the usefulness of pTV1-OK for Tn*917* mutagenesis of *S. mutans* by isolating mutants with lesions in several genes that cause defects in amino acid biosynthesis, acidurance, and BLIS production (reference 14 and this report). Rubens and collaborators (13) successfully mutagenized group B *Streptococcus* spp. with Tn*917*, using pTV1-OK as the delivery vector. This result suggests that Tn*917* could be employed as a mutagen, in general, in members of the genus *Streptococcus*. Previous failures with constructs, such as pTV1(Ts), and derivatives were attributed to the inability of the pE194(Ts) plasmid backbone to replicate in *S. mutans* (7a). With the replicative functions of the *Lactococcus* replicon pWVO1Ts, pTV1-OK was found to be stably maintained in *S. mutans* at the permissive temperature, thereby providing sufficient opportunity for Tn*917* transposition to occur. We found that in *S. mutans* JH1005, pTV1-OK was efficiently cured (99.9 to 99.99%) in a single passage when incubated at 42 to 45°C. At present, it is not clear why in this strain only 6 to 7 generations were sufficient to achieve these levels of plasmid curing, since for related systems (13, 31), it has been reported that 12 to 13 generations are required to get similar results. With another *S. mutans* strain (NG8) studied under the above-described conditions, we have observed decreased levels of efficiency in plasmid loss (40 to 70%) (13a). This finding indicates that with other strains, 12 to 13 generations (achievable with two serial passages at the nonpermissive temperature) may be required to achieve satisfactory levels of plasmid loss.

Plasmid pTV21Δ2TetM is an Ap^s derivative of pTV21Δ2 (58) that can be used for marker rescue of genes mutagenized with Tn*917* in pathogenic streptococci, since it lacks the *bla* gene present in pBR322. Using this vector, we cloned DNA flanking insertions from four different Tn*917* mutant strains. The recovered plasmids described in this work contained 2.5- to 8.5-kb inserts derived from *S. mutans* chromosomal DNAs flanking the *erm*-proximal Tn*917* insertion junctions and 6.5 to 7.0 kb of vector backbone derived from the recovery construct Tn*917*Δ*erm*::pVA981, depending on the restriction enzyme used for marker rescue (Fig. 2 and 3). It should also be possible to recover DNAs adjacent to both sides of the transposon insertion if, for instance, marker rescue is performed with enzymes that do not cut within Tn*917*Δ*erm*::pVA981 or with enzymes that may cut within this segment but under conditions that would result in partial digestion (Fig. 3). We also developed a novel approach to clone streptococcal DNA fragments containing Tn*917* into *E. coli* based on conventional shotgun cloning. This strategy had never been used with this transposon, because no previously tested *E. coli* host was found to express Em^r from the transposon-linked *erm* marker. We found, however, that *E. coli* MC1061, which was shown to tolerate and express the Tn*917* Em^r determinant, could be used as a host to clone Tn*917* insertions from chromosomal libraries by selection of the *erm* gene.

The bacterium *S. mutans* is known to be the principal etiologic agent for dental caries. The virulence of *S. mutans* resides in its ability to adhere to tooth surfaces, to produce lactic acid from dietary sugars, and to withstand the acid environment it creates, leading ultimately to tooth decay. Consequently, acid tolerance is regarded as an important virulence factor. Results presented in this paper suggest that in addition to maintenance of neutral intracellular pH by the F_0F_1-H^+-ATPase (15) and membrane architecture (55), several other aspects of *S. mutans* basic physiology may be important for acidurance. Of particular interest to us are the mutations in the acid-sensitive strains AS5 (*fhs*::Tn*917*) and AS17 (*sat*::Tn*917*), which exhibited a tight $pH5^s$ phenotype. As was predicted from the biochemical role of Fhs, formyl-tetrahydrofolate synthetase, in C-1 transfer reactions (39), we found the *fhs*::Tn*917* mutation of strain AS5 to cause an absolute requirement for adenine on minimal media and that additional methionine supplementation afforded wild-type levels of growth (9a). However, the molecular bases of the $pH5^s$ and $BLIS^-$ phenotypes caused by a lesion in *fhs* are less obvious.

The acid sensitivity of mutant AS17 is caused by the insertion of Tn*917* in the intergenic region of the *sat* (*ylxM-ffh*) locus. In *B. subtilis* (20), this locus constitutes an operon which encodes YlxM, a 13.2-kDa hypothetical protein displaying no significant homology to known proteins in data banks, and Ffh, i.e., the fifty-four-kilodalton subunit homolog of the eucaryotic signal recognition particle involved in protein translocation that is also found in *E. coli* (reviewed in reference 28). It remains to be determined whether the $pH5^s$ phenotype of AS17 stems from a polar effect of this mutation on the expression of the downstream gene *ffh*. Also, if *ylxM* and *ffh* constitute an operon in *S. mutans*, it is not clear why the *sat*::Tn*917* insertion is not lethal, since as mentioned above, *ffh* is essential for viability in *E. coli* (38) and *B. subtilis* (20). As with transposon Tn*10*, with which it was shown that insertions in operons may not be tightly polar on the expression of downstream genes (7), it could be that the Tn*917* insertion in AS17 is only partially polar on *ffh* expression. Conceivably, decreased levels of *ffh* expression in the mutant could cause a defect in protein secretion and ultimately in membrane structure and function, which could be accentuated at low pH. Experiments to study the origin of acid sensitivity in AS17 and the effect of *sat*::Tn*917* on *ffh* expression are being conducted.

Many gram-positive bacteria, including some *S. mutans* strains, are capable of producing bacteriocins (35) or BLIS (24). In general, these molecules have broad spectra of activity against other gram-positive bacteria and are thus the subject of intense research (24). *S. mutans* JH1005 (19) was isolated as a derivative which produces increased levels of BLIS. This strain was able to inhibit the growth of *S. mutans* as well as other gram-positive oral bacteria and was shown to have superior colonization potential in the oral cavity (18). To characterize the nature of this BLIS, we have isolated Tn*917* mutants of JH1005 exhibiting defects in BLIS production. Lesions in these mutants could be caused by an insertion in a BLIS structural, biosynthetic, or regulatory gene(s). Using methods introduced in this paper, we are currently attempting to recover DNA adjacent to the Tn*917* insertions in these mutants for further characterization.

The phenotypes of AX1 (*icd*::Tn*917* Glu^-) and AX3 (*argD*::Tn*917* Arg^-) can easily be understood by analogy to the phenotypes of similar auxotrophic mutants in other bacteria. The role of *icd* in the nitrogen assimilation of *S. mutans* is being studied (10a).

Our ability to obtain Tn*917*-induced mutations affecting several different phenotypes together with Southern analyses suggests that transposition of this element may be essentially random in *S. mutans*. Hence, pTV1-OK, a replication-conditional (temperature-sensitive) derivative of the pWVO1 replicon, allows for transformation-independent mutagenesis of *S. mutans* with the enterococcal transposon Tn*917*. Additional advantages to mutagenesis of *S. mutans* with transposon Tn*917* include (i) its small size; (ii) transposition events that usually result in single chromosomal insertions with apparent randomness; (iii) its stability, even upon transformation of a transposon-free strain with chromosomal DNAs derived from Tn*917*-mutagenized strains; (iv) the existence of Tn*917* derivatives (Tn*917-lac* [57] and Tn*917*-LTV3 [4]) that could be used to study gene expression; and (v) the relative ease of recovery of streptococcal DNA sequences adjacent to the insertion site for molecular characterization. Collectively, these attributes make pTV1-OK and pTV21Δ2TetM powerful tools for the study of the genetic basis of pathogenesis and physiology of *S. mutans* and possibly other members of the genus *Streptococcus*. The unusually broad host range of pWVO1 replication and of Tn*917* transposition, which include many species of gram-positive and gram-negative bacteria, should make vectors such as pTV1-OK tools of wide utility.

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