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Tn*916* Insertional Inactivation of Multiple Genes on the Chromosome of *Streptococcus mutans* GS-5

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***Streptococcus mutans* GS-5 was transformed with the *Escherichia coli* plasmid pAM150 containing the cloned streptococcal transposon Tn*916*. Southern blot analyses with the tetracycline-resistant determinant of Tn*916* showed that Tn*916* was inserted into the chromosome of *S. mutans* at a variety of different sites. Tn*916* insertions resulted in the inactivation of genes that code for various steps in the biosynthesis of several different amino acids. Two auxotrophs which contained a single copy of Tn*916* were shown to revert to prototrophy at frequencies of about 10^{-8}. All of the revertant prototrophs were susceptible to tetracycline, indicating regeneration of the functional gene by excision of Tn*916*.**

Currently, knowledge of the genetics of streptococci in general and of *Streptococcus mutans* in particular is rather limited, primarily because of the dearth of genetic technologies useful for studies of these bacteria. The introduction of a transposon into the chromosome to inactivate a single gene provides a highly useful means of gene manipulation (9). In gram-positive bacteria, the conjugative transposon Tn*916*, which codes for tetracycline (TC) resistance, has been used for transposon mutagenesis (6). (Tn*916* is a 16.4-kb transposon which is capable of conjugative transfer in the absence of plasmid DNA [5].) To this end, Tn*916* has been cloned in an *Escherichia coli* plasmid (6). When the plasmid is introduced into a streptococcus, TC-resistant (Tc^r) clones must contain the transposon inserted into the chromosome, since the plasmid cannot replicate in the streptococcus. Evidence has been obtained that Tn*916* is inserted into several different sites on the chromosomes of *Streptococcus faecalis*, *Streptococcus sanguis*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* (5, 6, 12, 21). However, it has been suggested that Tn*916* is inserted at a specific site (7) on the chromosome of *S. mutans* 6715. The introduction of Tn*916* into *S. mutans* GS-5 by transformation via pAM118 also resulted in insertion at a specific site (7). Therefore, we have attempted to determine whether Tn*916* is inserted into a single site or into a variety of sites on the chromosome of *S. mutans* GS-5.

In the work presented here, *S. mutans* GS-5 was transformed with plasmid pAM150 (Tn*916*), and Tn*916* insertions into the chromosome were generated by transposition. Tn*916* was introduced into the chromosome of *S. mutans* GS-5 by conventional transformation. The Tn*916* used in these experiments was carried on an *E. coli* plasmid (pAM150) that is unable to replicate in *S. mutans*. Thus, all Tc^r transformants were expected to have the transposon inserted into the *S. mutans* chromosome. By Southern hybridization we show that Tn*916* is inserted into a variety of different sites on the *S. mutans* chromosome, thereby generating a variety of different amino acid auxotrophs. The partial characterization of some of these auxotrophs is described. In addition, we show that Tn*916*-inactivated chromosomal genes are restored to full function upon the loss of the transposon.

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MATERIALS AND METHODS

Bacterial strains and culture media. A streptomycin-resistant (Str^r) strain of *S. mutans* GS-5 (serotype c) was obtained from H. K. Kuramitsu (Northwestern University, Chicago, Ill.) (13). *E. coli* DH1 (F^- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44*) containing plasmid pAM150 (Tn*916*) was provided by D. B. Clewell (University of Michigan, Ann Arbor) and grown as described by Gawron-Burke and Clewell (6). Plasmid pAM150 was used as the delivery system for Tn*916* to avoid the problem of introducing an ampicillin resistance gene into streptococci. pAM150 was constructed from pAM120 by a deletion of the 2.4-kilobase (kb) fragment spanning the ampicillin resistance determinant and extending into the *Eco*RI-F sequences adjacent to Tn*916* (6, 7). pAM120 was derived from pGL101 (a derivative of pBR322) by the cloning of Tn*916* into the *Eco*RI site (6, 7). *S. mutans* cultures were routinely grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% glucose and 0.1% L-cysteine (THG) at 37°C. For the isolation and identification of amino acid auxotrophs, cultures were grown in a chemically defined medium (FMC; 19) containing all 20 amino acids or in a minimal medium (MM) consisting of FMC minus all amino acids except L-glutamine, L-glutamate, and L-cystine. These three amino acids are essential for the growth of *S. mutans* GS-5 (19). All L-amino acids were obtained from GIBCO Laboratories, Grand Island, N.Y. Putative precursors of methionine, such as L-homoserine, cystathionine (L- and L-allo stereoisomers), DL-homocysteine, and 5-methyltetrahydrofolate, which were used in nutritional substitution studies, were purchased from Sigma Chemical Co., St. Louis, Mo. Vitamin B_{12} was obtained from Elkins-Sinn, Inc., Cherry Hill, N.J.

Chromosomal and plasmid DNA isolation. *S. mutans* GS-5 was grown overnight at 37°C in THG. The 50-ml cultures were diluted to 200 ml with fresh medium and incubated at 37°C for 1 h. Glycine (final concentration, 3.75%) was added, and the cultures were incubated for 1 h. Cell lysis and chromosomal DNA isolation were performed as described by Reider and Macrina (15). Covalently closed circular plasmid DNA was extracted from *E. coli* by the technique of Birnboim and Doly (2) and purified on a column of Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, N.J.) (16).

Restriction analysis and Southern hybridization. Restric-

tion endonuclease (Bethesda Research Laboratories, Gaithersburg, Md.) digestion was done as described by Maniatis et al. (11). A 4.8-kb *Hin*cII fragment of Tn*916* containing the *tet* determinant (3) was isolated with low-melting point agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine). The Multiprime DNA Labeling System (Amersham Corp., Arlington Heights, Ill.) was used to label the 4.8-kb *Hin*cII fragment with [^{32}P]dCTP. DNA transfer to nitrocellulose and hybridization were done by the method of Southern (17).

Bacterial transformations. *S. mutans* was transformed by a modification of the method described by Perry et al. (13, 14). Cells were grown in THG at 37°C to an A_{675} of 0.5 (exponential phase). Samples of this culture were frozen in THG containing 30% glycerol and stored at −70°C. A frozen glycerol culture of *S. mutans* GS-5 was diluted 1:1,000 in THG supplemented with 10% horse serum and grown for 5 h. Competent cultures were exposed to pAM150 for 2 h, and transformants were scored on THG containing 5 μg of TC per ml. In transformations with *S. mutans* DNA, 50 μl of DNase I (10 mg/ml) and 50 μl of $MgSO_4$ (0.1 M) (20) were added 30 min after the DNA addition; 10-ml cultures were incubated for an additional 2 h to allow for phenotypic expression before plating. For the screening of auxotrophs, cultures were first plated on FMC agar plates and then replica plated on MM agar plates. The agar plates were incubated for 48 h in an anaerobic GasPak system (BBL Microbiology Systems, Cockeysville, Md.). The amino acid(s) required by each of the auxotrophs was identified as follows. Colonies which showed no growth on MM agar were inoculated into FMC, grown at 37°C to an A_{675} of 0.5, washed four times with distilled H_2O, and suspended in MM. Each of the cultures was streaked onto a series of plates containing FMC agar, MM agar, and MM agar plus one or, in one case (serine plus glycine), two amino acids that were present in FMC but absent in MM. Strains that failed to grow on MM agar but that grew on MM agar plus a single amino acid (or serine plus glycine) were retested on MM agar and MM agar plus that supplement. Strains that were positive upon retesting were scored as auxotrophs for that amino acid(s).

Cross-feeding experiments. Cultures of methionine auxotrophs were grown in FMC at 37°C to an A_{675} of 0.5. The bacteria were centrifuged, washed four times with ice-cold water, and suspended in MM. The washed cultures were streaked onto plates of MM agar supplemented with a low concentration of methionine (5 μg/ml). Streaks were arranged at 90° angles to each other so that metabolites diffusing from one mutant strain could supplement the nutritional requirement of another mutant strain. MM agar plates were incubated for 48 h in the anaerobic GasPak system, after which growth of the various mutant strains was assessed.

Penicillin enrichment. Transformed cultures were grown overnight in the presence of TC (5 μg/ml). The bacteria were sedimented by centrifugation, washed twice with ice-cold water, and diluted 1:30 in fresh MM. After incubation at 37°C to an A_{675} of 0.2, 3 μg of benzylpenicillin per ml was added, and incubation was continued for an additional 2.5 h. Bacteria were washed twice with ice-cold water, plated on FMC agar, and replica plated on MM agar.

Determination of reversion frequency. Cultures were streaked on THG agar containing TC at 5 μg/ml for the isolation of single colonies. An overnight culture which had been inoculated from an isolated colony was grown in THG in both the presence and the absence of TC (5 μg/ml). Dilutions of this culture were plated on MM agar to determine the frequency of prototrophs and on THG agar for viable cell counting. After 48 h at 37°C, the numbers of colonies on THG agar and on MM agar were counted, and the reversion frequency was determined. The TC susceptibility of the prototrophic revertants was then determined by picking colonies with sterile toothpicks onto THG agar containing TC at 5 μg/ml.

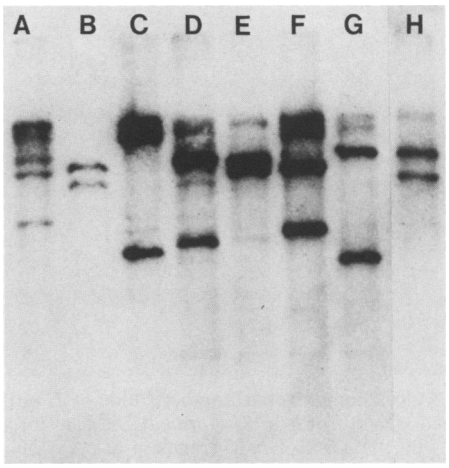

FIG. 1. Autoradiogram of a Southern blot of *Hin*dIII-digested chromosomal DNAs from eight randomly selected *S. mutans* GS-5 (Tn*916*) transformants (lanes A to H) hybridized with a ^{32}P-labeled 4.8-kb *Hin*cII fragment of pAM150.

RESULTS

Introduction of Tn*916* into *S. mutans* GS-5. A frozen glycerol culture of *S. mutans* GS-5 was diluted 1:1,000 in THG and grown in the presence of 10% heat-inactivated serum for 5 h. Previous experiments demonstrated that these growth conditions, which yielded 4×10^7 cells per ml, resulted in maximum competence for transformation. When pAM150 was used at 3 μg/ml, the transformation frequencies of the recipient for TC resistance ranged from 4×10^{-5} to 5×10^{-6}. Cultures (10 ml) transformed with pAM150 were incubated for 2 h, diluted with 250 ml of THG containing TC (5 μg/ml), and grown overnight. Samples of the transformed overnight cultures were frozen in THG containing 30% glycerol and stored at −70°C.

Analysis of Tn*916* insertions in *S. mutans* GS-5. Southern blot analysis (Fig. 1) of *Hin*dIII-digested DNAs from eight Tc^r transformants probed with the 4.8-kb *Hin*cII *tet* determinant of Tn*916* (3) revealed junction fragments of several different sizes. *Hin*dIII cuts once within the *tet* determinant of Tn*916*, yielding two junction fragments for each copy (3, 7). In some instances (e.g., in Fig. 1, lanes A, C, D, and F), more than one pair of junction fragments was present, indicating that there was more than one Tn*916* insertion in the chromosome of that transformant.

Isolation of auxotrophic mutants. An initial screening of 8,700 Tc^r colonies resulted in the isolation of eight amino acid auxotrophs (frequency, 0.09%): five for methionine, one for homoserine, one for serine plus glycine, and one for histidine. As shown below, the five methionine auxotrophs appeared to be due to transposon insertions at different sites rather than at a single site. A second experiment, in which penicillin enrichment of the transformed cultures was used to screen 1,404 colonies, resulted in the isolation of four

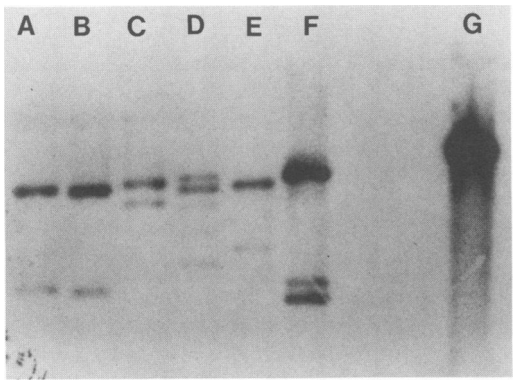

FIG. 2. Autoradiogram of a Southern blot of *Hin*dIII-digested chromosomal DNAs from six *S. mutans* GS-5 (Tn*916*) auxotrophs hybridized with the 4.8-kb *Hin*cII fragment of pAM150. Lanes: A, mutation *his-1*; B, mutation *pro-1*; C, mutation *phe-1*; D, mutation *thr-1*; E, mutation *met-1*; F, mutation *met-2*; G, pAM150.

additional amino acid auxotrophs (frequency, 0.29%): one for proline, one for phenylalanine, one for threonine, and one for lysine. In another experiment with penicillin enrichment, three additional methionine auxotrophs were isolated from a screening of 850 colonies (frequency, 0.35%). As shown below, these last three auxotrophs appeared to be derived from the same Tn*916* insertional event.

Six auxotrophs were used as recipients for transformation with parental GS-5 DNA. The prototrophs obtained were screened for TC susceptibility. When four of the six auxotrophs (strains with mutations *his-1*, *pro-1*, *phe-1*, and *met-1*) were transformed to prototrophy, they concurrently lost TC resistance. Each of these four auxotrophs contained a single pair of Tn*916* junction fragments (Fig. 2, lanes A, B, C, and E). For the other two auxotrophs transformed to prototrophy (strains with mutations *thr-1* and *met-2*), the frequencies of loss of TC resistance were 30 and 25%, respectively. The chromosomes of these two auxotrophs contained several junction fragments (Fig. 2, lanes D and F). The Southern blot shown in Fig. 2 also suggests that the two methionine auxotrophs tested (lanes E and F) contained different junction fragments.

The DNAs of the two auxotrophs that did not result in 100% TC-susceptible (Tc^s) transformants when transformed to prototrophy (strains with mutations *thr-1* and *met-2*) were isolated, purified, and used to transform the parental GS-5 strain, selecting for TC resistance. Each of the Tc^r transformants was plated on MM agar. The Tc^r transformants which were found to be auxotrophs were used as recipients for transformation with parental GS-5 DNA. Those auxotrophs which resulted in 100% Tc^s transformants when transformed to prototrophy were interpreted as containing a single Tn*916* insertion.

TABLE 2. Cross-feeding patterns and nutritional requirements of methionine auxotrophs

Methionine auxotroph number	Class	Cross-fed auxotroph(s)	Growth on[a]:				
			Hom	Cys	Hys	CH_3-H_4F	B_{12}
2	I	None	−	+	+	−	−
4	II	2	−	+	+	−	−
6, 7, or 8	III	2 and 4	−	+	+	−	−
5	IV	2, 4, 6, 7, and 8	−	+	+	−	−
3	V	2, 4, 5, 6, 7, and 8	−	+	+	−	−
1	VI	2, 3, 4, 5, 6, 7, and 8	−	−	−	+	+

[a] Hom, L-Homoserine; Cys, cystathionine; Hys, DL-homocysteine; CH_3-H_4F, 5-methyltetrahydrofolate; B_{12}, vitamin B_{12}. All were used at concentrations of 10 to 100 μg/ml. −, No growth; +, growth.

Each of the methionine auxotrophs was transformed with DNA isolated from each of the other Met^- strains, selecting for prototrophs. The results obtained (Table 1) showed that the eight strains contained six separate insertionally inactivated methionine mutations.

Characterization of methionine auxotrophs. Cross-feeding experiments were performed to characterize the eight methionine auxotrophs. Included were five auxotrophs (mutations *met-1* through *met-5*) isolated from the first screening and three auxotrophs (mutations *met-6*, *met-7*, and *met-8*) isolated from the second penicillin enrichment experiment. Based on the ability of each mutant to support the growth of other strains (Table 2), the Met^- mutants could be classified into six distinct groups. The three methionine auxotrophs (mutations *met-6*, *met-7*, and *met-8*), all isolated from the second penicillin enrichment experiment, produced identical cross-feeding patterns and were therefore all placed in class III (Table 2).

Homoserine, cystathionine, and homocysteine are known precursors in the pathway for methionine biosynthesis (4), and 5-methyltetrahydrofolate and vitamin B_{12} are known cofactors in methionine biosynthesis. Each of these compounds was examined for its ability to support the growth of each mutant (Table 2). The strain with mutation *met-1* grew only on methionine or 5-methyltetrahydrofolate. All the other auxotrophs failed to grow on homoserine, 5-methyltetrahydrofolate, or B_{12} but grew when cystathionine or homocysteine replaced methionine.

Southern blot analysis of the DNA of each of these methionine auxotrophs (Fig. 3) showed that each of the strains that yielded a different cross-feeding pattern (Table 2) contained a different Tn*916* junction fragment. On this basis

TABLE 1. Frequency of transformation of methionine auxotrophs of *S. mutans* GS-5 to prototrophy

Recipient strain	Frequency of transformation to prototrophy (10^{-6}) with indicated donor DNA:							
	met-2	*met-4*	*met-6*	*met-7*	*met-8*	*met-5*	*met-3*	*met-1*
met-2	ND[a]	1.00	1.13	1.18	1.22	1.85	1.55	1.00
met-4	1.29	ND	1.55	1.42	1.35	1.11	1.40	1.46
met-6	1.38	1.87	ND	ND	ND	2.16	2.00	1.55
met-7	1.25	1.29	ND	ND	ND	1.45	1.31	1.43
met-8	1.31	1.21	ND	ND	ND	1.28	1.51	1.39
met-5	1.24	1.10	1.13	1.16	1.11	ND	1.16	1.65
met-3	1.13	1.02	1.27	1.21	1.18	1.07	ND	1.30
met-1	1.60	1.54	1.56	1.49	1.44	1.44	1.65	ND

[a] ND, Not detectable (less than 10^{-8}).

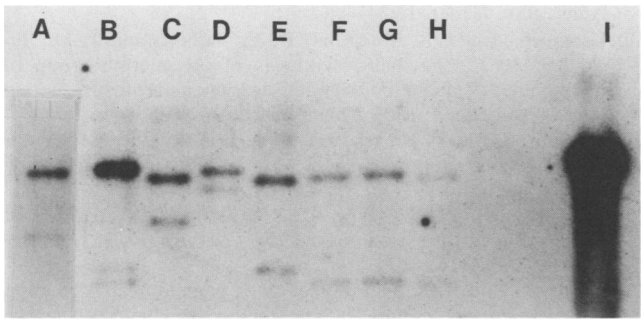

FIG. 3. Autoradiogram of a Southern blot of *Hin*dIII-digested chromosomal DNAs from eight *S. mutans* GS-5 (Tn*916*) methionine auxotrophs hybridized with the 4.8-kb *Hin*cII fragment of pAM150. Lanes A to H represent auxotrophs 1 to 8, respectively; lane I, represents pAM150. Lane A has been enhanced photographically to compensate for the lower DNA concentration used.

each of these strains was put in a separate class (Table 2). The DNAs of the three class III (Table 2) auxotrophs contained identical junction fragments (mutations *met-6*, *met-7*, and *met-8*; Fig. 3, lanes F, G, and H). These data are consistent with the view that each of the six auxotrophic classes is the result of a separate insertional event.

Reversion of auxotrophic mutants. Two auxotrophic mutants (mutations *pro-1* and *phe-1*), each of which contained a single copy of the transposon, were examined for the frequency of reversion to prototrophy in the presence and absence of TC (Table 3). In both the presence and the absence of TC, revertants were obtained at a frequency of about 4×10^{-8} to 6×10^{-8} (Table 3). All revertants were Tc^s. Auxotrophs containing a single Tn*916* insertion and cultured in the presence of TC failed to show any indications of additional transpositions, as indicated by the absence of Tc^r prototrophs (data not shown).

DISCUSSION

Tn*916* was used to mutagenize *S. mutans* GS-5 in the present work. Southern blot analysis (Fig. 1) indicated that Tn*916* was inserted in a variety of locations along the GS-5 chromosome. The finding of auxotrophs in five of six amino acid biosynthetic pathways and of six apparently different classes of methionine auxotrophs is in agreement with this view.

TABLE 3. Reversion of two amino acid auxotrophic strains to prototrophy

Auxotroph	Expt	TC (5 μg/ml)[a]	Total CFU (10^9/ml)	Prototrophic colonies (10^2/ml)	Reversion frequency[b] (10^{-8}/ml)
Mutation *pro-1*	1	+	2.9	1.3	4.0 ± 0.4
	2	+	4.4	1.6	
	1	−	3.3	2.7	6.3 ± 2.0
	2	−	6.1	2.6	
Mutation *phe-1*	1	+	5.1	1.6	4.6 ± 1.4
	2	+	5.5	3.3	
	1	−	4.3	3.2	4.9 ± 2.5
	2	−	5.8	1.4	

[a] +, TC present; −, TC absent.
[b] Number of prototrophic colonies (on MM) divided by total number of CFU (on THG). Reported as the mean ± the standard deviation in two independent experiments.

The data obtained from Southern blot analyses of junction fragments proved useful in the identification of different Tn*916* insertion sites and provided information on the occurrence of multiple insertion sites. Conventional transformation procedures were used to isolate *S. mutans* GS-5 strains carrying single Tn*916* insertions from strains carrying more than one Tn*916* insertion.

The Tn*916* insertions generated in these studies appeared to be relatively stable, with reversion frequencies on the order of 10^{-8} (Table 3). This result is in contrast to the results of Nida and Cleary (12), who found the frequency of beta-hemolytic revertants in cultures of Tn*916*-inactivated streptolysin S mutants of *S. pyogenes* to be quite variable, ranging from 10^{-3} to 10^{-7}. Kathariou et al. (8) found the average frequency of hemolytic colonies in Tn*916*-inactivated nonhemolytic mutants of *Listeria monocytogenes* to be 10^{-4}.

In the present study, Tn*916* appeared to be excised precisely from two inactivated chromosomal genes. These results extend the observations of Gawron-Burke and Clewell (6), who showed the regeneration of a plasmid-borne hemolysin gene.

The transformation (Table 1) and cross-feeding and nutritional (Table 2) data did not permit us to identify the specific defects in the six classes of methionine auxotrophs. The strain with mutation *met-1* stimulated growth of all auxotrophs, and thus the *met-1* mutation appeared to be at the end of the pathway. In contrast, the strain with the *met-2* mutation failed to stimulate growth of any other methionine auxotroph. The *met-2* mutation thus appeared to be blocked in an early gene in the pathway. The other methionine auxotrophs were arranged according to a similar rationale from the cross-feeding results. The results shown in Tables 1 and 2 suggest that three methionine auxotrophs (mutations *met-6*, *met-7*, and *met-8*) contained mutations in the same gene. This observation probably does not indicate a hot spot, as these mutants were all isolated in one screening and so could be derived from a single transformation event. Since all auxotrophs except the strain with mutation *met-1* grew on only cystathionine, homocysteine, or methionine, they appeared to be blocked after the branch point of the pathway but prior to the conversion of cystathionine to homocysteine. The strain with mutation *met-1*, which grew on only 5-methyltetrahydrofolate or methionine, appeared to be defective in 5,10-methylenetetrahydrofolate reductase. Consequently, mutation *met-1* may correspond to *metF* in *E. coli* (10, 18). Moreover, more classes than the three or four expected from the known pathway in *E. coli* (1) were observed. Specifically, five classes of auxotrophs between homoserine and cystathionine were observed, whereas, at most two classes (*metA* and *metB*) were expected (1). Further work is needed to clarify this point.

Transposon Tn*916* was used successfully to mutagenize *S. mutans* GS-5 by insertional inactivation. The isolation of a variety of different amino acid auxotrophs and Southern blot analyses of these auxotrophs indicated that Tn*916* can be inserted stably into a variety of different sites on the chromosome of *S. mutans* GS-5. An advantage of Tn*916*-generated mutations is that one can further identify the gene that has been inactivated by cloning the gene by using the strategy described by Gawron-Burke and Clewell (6). Subsequently, the insertionally inactivated gene can be regenerated upon excision of Tn*916*. The use of Tn*916* to study *S.*

mutans genes such as those for virulence factors will prove very useful for future work.

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