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Detection of Streptococcal Mutants Presumed To Be Defective in Sugar Catabolism

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The tetrazolium method for detection of bacterial mutants defective in sugar catabolism was modified for use with streptococci. The critical factors were (i) the concentration of tetrazolium, which must be titrated to determine the optimum concentration for each species or even strain, and (ii) anaerobic incubation of tetrazolium-containing agar plates. When used with standard mutagenesis protocols, this method yielded lactose-negative mutants of nine streptococcal strains representing six species. A collection of lactose-negative mutants of *Streptococcus sanguis* Challis was characterized and contained phospho-beta-galactosidase, lactose phosphotransferase, and general phosphotransferase mutants.

Streptococcus sanguis Challis is amenable to genetic analysis by transformation (2, 21), has been reported to be useful as a recipient for cloned DNA from other species of oral streptococci (11), and can serve as a recipient in streptococcal conjugation experiments (8). Genetically marked variants of this strain would be useful in the eventual construction of a chromosomal map and in the characterization of foreign DNA introduced by cloning or conjugation techniques. However, little has been published on the genetics of metabolic pathways (including carbohydrate catabolism) in this organism. Furthermore, some previously described mutants have been obtained by strenuous mutagenesis procedures (10, 14). Other workers, studying oral streptococci other than *S. sanguis*, have looked at only a few genes and used collections of only a few mutants (4, 7, 12, 18). We are interested in the genetics of *S. sanguis* and other oral streptococci, and accordingly, we devised methods for obtaining mutants of *S. sanguis* that are defective in carbohydrate catabolism. These procedures use accepted genetic techniques (15), including a modification of the tetrazolium plating technique of Lederberg for easy detection of mutant colonies (9), and they allow the rapid development of large collections of mutants defective in carbohydrate catabolism. In addition, these methods can be applied to other streptococci.

The bacterial strains used and their sources are listed in Table 1. Static liquid cultures were grown at 37°C in brain heart infusion (BHI) broth or tryptone broth (19). Solid media used included BHI agar, tryptone agar, and Difco antibiotic medium no. 2 supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) agar (9, 15). Ethyl methanesulfonate (EMS), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (NNG), TTC, *o*-nitrophenyl-beta-D-galactoside phosphate (ONPGP), and lactate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo.

For NNG mutagenesis, bacteria were grown overnight in BHI broth, diluted 1:10 into fresh BHI broth prewarmed to 37°C, and grown to mid-log phase. Mid-log-phase cultures were diluted 1:2 into prewarmed BHI broth containing freshly prepared NNG at 100 μg/ml to give a final NNG concentration of 50 μg/ml, mixed, and incubated at 37°C for 10 min. The mutagen-treated cells were washed twice by centrifugation with sterile saline, and the washed cells were suspended in BHI broth, distributed into sterile tubes, and incubated overnight at 37°C. For EMS mutagenesis, bacteria were grown as for NNG mutagenesis. EMS was added to mid-log-phase cultures to a concentration of 1.5%, and cultures were incubated with EMS for 30 min at 37°C. EMS-treated cells then were washed and grown as described for NNG mutagenesis.

Presumptive *lac* mutants were detected by two methods, the first of which was modified replica plating. Appropriate dilutions of an overnight growth of mutagenized bacteria were plated on BHI agar and incubated anaerobically (Gas-Pak) for 48 h at 37°C. Colonies with relatively large, wild-type morphology were picked and stabbed in a grid pattern into two tryptone agar plates, one with 20 mM glucose and the other with 10 mM lactose; these plates also were incubated anaerobically for 48 h at 37°C. Clones able to form colonies on glucose but not on lactose were repurified by streaking onto BHI agar and were then grown overnight in BHI broth and stored at −80°C in 30% glycerol.

The second mutant detection method made use of TTC. Appropriate dilutions of an overnight growth of mutagenized cells were plated onto TTC agar containing 1% lactose and the concentration of TTC optimal for the strain or species under study (see below). The plates were incubated anaerobically (Gas-Pak) at 37°C for 48 h and examined for occasional red colonies among more numerous white wild-type colonies. Red colonies were picked with a sterile wire, suspended in 0.5 ml of BHI broth, and purified by streaking onto the same medium from which they were isolated. This streaking was repeated twice. Purified presumptive mutants were grown on BHI broth and stored at −80°C in 30% glycerol.

Presumptive *lac* mutants were confirmed by comparative growth on lactose or glucose. Presumptive mutants were grown overnight at 37°C in 10 ml of BHI broth in tubes. Cells were collected by centrifugation, washed twice in sterile saline, and suspended in 10 ml of sterile saline. Washed cells were diluted 100-fold into tubes of tryptone broth containing either 10 mM glucose or 5 mM lactose. These tubes were incubated at 37°C for up to 72 h. Strains showing wild-type total growth on glucose at 24 h but no more than 20% of wild-

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TABLE 1. Optimal TTC concentrations for detection of presumptive Lac⁻ mutants in streptococcal species

Streptococcal species	Strain	Source	Optimal TTC concn (μg/ml)[a]
S. pyogenes	ATCC 12363	ATTC[b]	2.5
S. faecalis	ATCC 19433	ATTC	5
S. faecalis	JH2	D. Clewell	5
S. sanguis	Challis	H. Gooder	10
S. sanguis	0502 (β)	D. LeBlanc	2.5
S. salivarius	ATCC 9222	ATTC	25
S. salivarius	0251 (β)	D. LeBlanc	25
S. mutans	10449	C. F. Schachtele	15
S. lactis	C_{10}	J. Thompson	25

[a] Difco Antibiotic Medium no. 2 with 1% lactose and TTC as indicated.
[b] ATCC, American Type Culture Collection.

type growth on lactose by 72 h were considered to be confirmed as *lac* mutants.

Confirmed *lac* mutants were characterized with regard to glucose and lactose phosphoenolpyruvate phosphotransferase (PTS) activities and for phospho-beta-galactosidase. Mutant strains were grown to late-log or early-stationary phase on tryptone broth containing a mixture of 5 mM glucose and 5 mM lactose. Cells were collected by centrifugation, washed twice with cold MP buffer (0.05 M potassium phosphate [pH 7.0] containing 1 mM magnesium sulfate) (19), suspended in a 0.1-culture volume of the same buffer, and decryptified by addition of toluene to 1% (vol/vol) followed by blending with a Vortex mixer for 1 min (3, 6). Decryptified cells were stored in an ice bath and assayed for PTS and phospho-beta-galactosidase activities.

PTS assays were set up as previously described (19), with a volume of 1.0 ml. Control reactions contained no sugar. Reaction mixtures were incubated at 37°C for 15 min, and the reaction was terminated by adding 1.0 ml of cold 0.6 N perchloric acid. After the addition of 1.0 ml of cold 2.4 N trisodium phosphate, reaction mixtures were centrifuged to remove potassium perchlorate. A portion of the neutral supernatant fluid was removed for spectrophotometric determination of pyruvate with lactate dehydrogenase and NADH (3, 6). It was presumed that 1 mol of pyruvate corresponded to 1 mol of sugar phosphate (17). Phospho-beta-galactosidase activity was assayed by measuring hydrolysis of ONPGP by toluene-decryptified cells, as described by Calmes (1). Results of PTS and ONPGP hydrolysis assays were expressed as nanomoles or micromoles of product formed per minute per milligram of protein and were normalized to the value obtained with the wild-type strain.

In preliminary experiments, we found that 50% reductions in CFU of *S. sanguis* CT1-7 were produced by exposure to NNG (50 μg/ml) for 10 min or to EMS (1.5%) for 30 min (data not shown). Such results are difficult to interpret for streptococci grown in broth cultures, since each CFU may consist of a chain of several cells. However, this treatment was mutagenic, since *lac* mutants frequently were found among the survivors (Table 2). Such mutants were not found in control cultures, which received no treatment with mutagen. Furthermore, after development of the TTC plating method for detection of presumptive *lac* mutants, we found that these mutagenesis protocols gave 1 to 10 red colonies per 100 wild-type white colonies with NNG and 1 to 5 red colonies per 100 white colonies with EMS. After streaking onto TTC-lactose agar and subculturing in tryptone-lactose broth approximately 1% of the red colonies were confirmed as lactose mutants, giving a frequency of about 10^{-4} per surviving CFU.

When wild-type and *lac* mutants of *S. sanguis* CT1-7 were plated separately onto standard TTC agar (containing 1% lactose and 50 μg of TTC per ml) (9, 15) and incubated aerobically or in candle extinction jars at 37°C for 48 h, no reduction of TTC was seen, and both wild-type and mutant colonies were white. When a set of the same plates was incubated anaerobically (Gas-Pak) at 37°C for 48 h, TTC reduction (red colonies) was seen for both wild-type and mutant colonies. Thus, standard TTC agar could not distinguish between wild-type and *lac* mutants of *S. sanguis* CT1-7. However, when this organism was plated anaerobically onto TTC-lactose agar containing lesser concentrations of TTC, differences between wild-type and *lac* strains appeared. When plated anaerobically onto TTC agar containing 1% lactose and 2.5, 5, 10, 25, 50, or 100 μg of TTC per ml, white wild-type colonies were differentiated clearly from red *lac* colonies at TTC concentrations ranging from 2.5 to 10 μg/ml (Fig. 1, *S. sanguis* Challis). No such differences were seen when similar series were incubated aerobically or in candle jars. A concentration of 10 μg of TTC per ml was adopted for routine use in anaerobic detection of presumptive carbohydrate mutants of *S. sanguis* CT1-7.

Similar results were obtained with the other streptococci listed in Table 1. When appropriate dilutions of mutagenesis survivors were plated onto TTC agar with 1% lactose and TTC concentrations ranging from 2.5 to 100 μg/ml and incubated anaerobically at 37°C for 48 h, differentiation of the presumptive mutant from wild-type colonies was observed with TTC concentrations up to 25 μg/ml. TTC concentrations greater than 25 μg/ml did not permit differentiation of the mutant from wild-type colonies for any of the streptococcal strains examined. Representative results are shown for *Streptococcus mutans* 10449, *S. sanguis* 0502, and *Streptococcus salivarius* 0251 (Fig. 1).

The *lac* mutants obtained by replica plating and used in development of the TTC plating method have been partially characterized (Table 2). Four strains (0013, 0016, 0018, and

TABLE 2. Characterization of NNG-induced Lac mutants of *S. sanguis* Challis CT1.7

Strain	Relative growth on 5 mM[a]:		Relative activity of[b]:		Relative ONPGP hydrolysis[c]	Phenotype
	Glucose	Lactose	Glucose PTS	Lactose PTS		
0001	1.00	1.00	1.00	1.00	1.00	Wild type
0012	0.63	0.18	0	0	0.25	General PTS
0013	1.03	0.18	1.26	0.04	0.32	Lactose PTS
0014	0.88	0.16	0.07	0	0.11	General PTS
0015	0.74	0.20	2.39	0.12	0.26	?
0016	0.80	0.18	0.33	0	0.42	Lactose PTS
0017	0.69	0.16	0.32	0.72	0.21	?
0018	1.00	0.16	0.19	0.02	0.23	Lactose PTS
0019	0.80	0.04	0	0.15	0.06	Glucose PTS, phoso-beta-galactosidase
0020	0.91	0.16	1.66	0.04	1.14	Lactose PTS

[a] Strain 0001 growth: absorbance at 650 nm ≈ 0.7, final cell density.
[b] Strain 0001 PTS activity: rate ≈ 25 nmol/min per mg of protein.
[c] Strain 0001 ONPGP hydrolysis: rate ≈ 30 μmol/min per mg of protein.

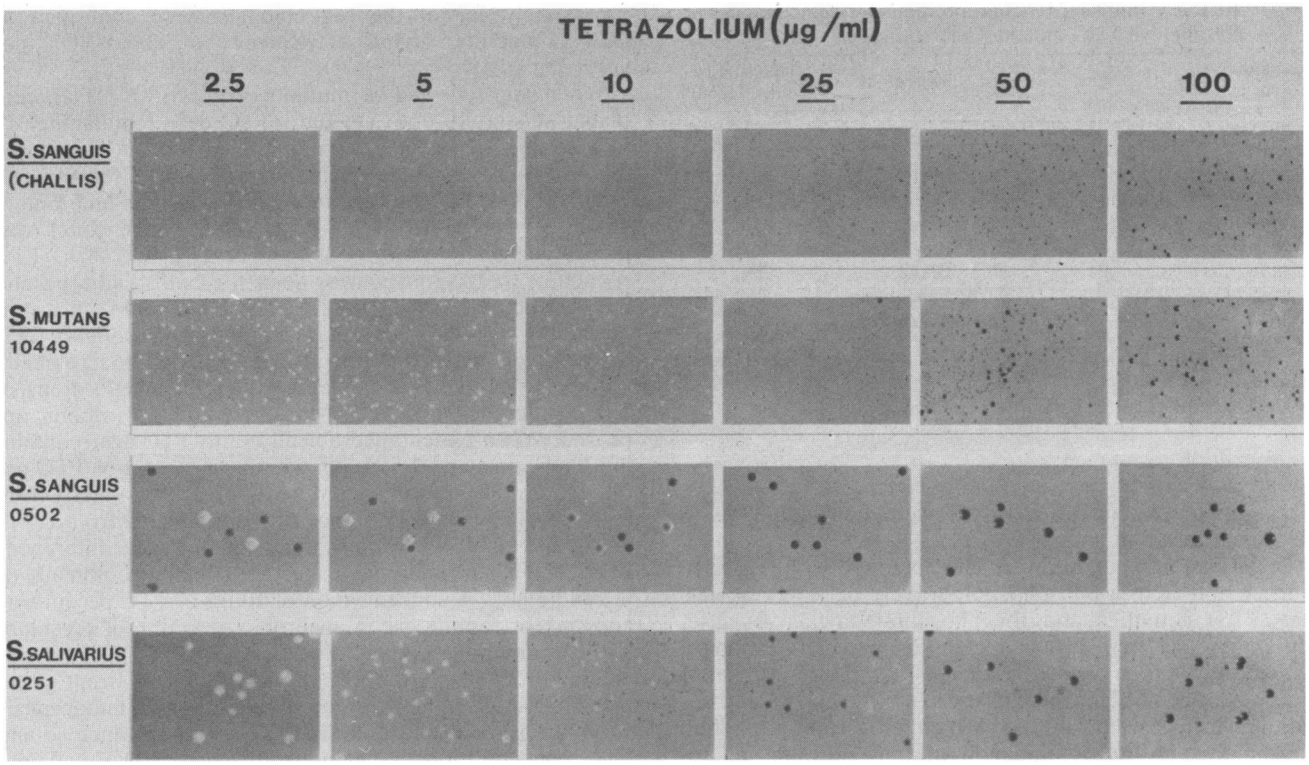

FIG. 1. Plating of wild-type and Lac$^-$ mutants of streptococci on TCC-lactose agar. At the optimum TTC concentration, wild-type colonies are white and Lac$^-$ colonies are red.

0020) are lactose PTS mutants and two (0012 and 0014) are general PTS mutants. Strain 0019 is a double mutant deficient in both glucose PTS and phospho-beta-galactosidase. The defects in two strains (0015 and 0017) were not revealed by this analysis. The existence of strains that lack general PTS or glucose PTS activity and that still can grow on glucose is of particular interest and will be discussed later.

Earlier attempts at obtaining carbohydrate mutants of oral streptococci have been fraught with difficulty. Using *S. sanguis* Challis, Luginbuhl and Gooder (10) have reported the isolation of a small collection of galactose mutants after three cycles of mutagenesis with NNG at 500 μg/ml and subsequent replica plating of the surviving colonies. Only 0.2% of the replicated colonies were stable *gal* mutants, and attempts to select nonfermenting mutants on indicator plates were unsuccessful. Similarly, with *S. mutans*, prolonged cultivation on plates containing excessive amounts of NNG has been used to isolate mutants (14).

In contrast, the results presented here, taken in conjunction with the work of others (6, 7, 16, 18, 20), indicate that standard genetic methods can be used to obtain mutants from a variety of streptococcal species and strains. Specifically, it is possible to obtain carbohydrate mutants after brief exposure to relatively low concentrations of mutagens and to use mutagens other than NNG, which may produce multiple lesions on a single chromosome (15). Furthermore, with appropriate manipulation of experimental conditions, it is possible to use indicator plates to detect mutants.

Indicator media are of great value in detecting relatively rare mutant colonies among a background of numerous wild-type colonies (15). Medium containing tetrazolium and a sugar has been used widely for isolation of mutants with altered fermentative abilities (7, 9, 15). In this report, we described modifications of the tetrazolium method which render it of general use in the isolation of streptococcal mutants defective in sugar catabolism. The modifications include anaerobic incubation and lowering of the TTC concentration.

When nonfermenting bacteria are grown on TTC-sugar agar, it is presumed that the resulting colonies are red as a consequence of the reduction of tetrazolium to formazan at alkaline pH, whereas fermenters are white because no reduction occurs at acid pH (13). Reduction of tetrazolium by bacteria suggests the transfer of electrons to tetrazolium during metabolism. The requirement of anaerobic incubation to see reduction of tetrazolium by these streptococci raises the possibility that they may be able to reduce oxygen and that TTC and oxygen compete for the reductant. Noteworthy in this context is the work of Whittenbury, who has summarized the evidence that implies the existence of an electron transport chain in streptococci (22).

In an earlier report, Hillman has described the detection of lactate dehydrogenase mutants of *S. mutans* BHT-2 by use of a standard TTC agar, containing 1% lactose and TTC at 50 μg/ml, and incubation of plates in candle extinction jars (7). In our survey of nine streptococcal strains from six species, we found that use of standard TTC-lactose agar did not permit distinction of wild-type and known *lac* mutants of *S. sanguis* Challis. Furthermore, we found that standard TTC-lactose agar did not detect presumptive *lac* colonies of the other streptococci listed in Table 1, regardless of incubation conditions. However, our study did not include the Hillman strain.

The results in Table 1 and Fig. 1 demonstrate that for a particular streptococcal species (or even strain within a single species) a TTC concentration can be found which will

differentiate the presumptive mutant from wild-type colonies. If the standard TTC method (9, 15) does not yield mutant colonies, then it may be necessary to adjust the TTC concentration and to use different incubation conditions. We found that anaerobic incubation and TTC concentrations of no more than 25 μg/ml provide a general method for detection of carbohydrate-negative mutants in streptococci. In addition, such medium is useful for detecting spontaneous reversion of mutants to relatively large white wild-type colonies against a confluent background of red *lac* growth (unpublished data).

In view of the difficulties in distinguishing mutant from wild-type colonies on TTC plates during the early phases of this investigation, we thought it necessary to obtain *lac* mutants of *S. sanguis* Challis CT1-7 by another method and to use these authentic mutants to define the TTC methodology. Such mutants were obtained by a modified replica plating procedure and were characterized as *lac* mutants by their patterns of growth on glucose and lactose (Table 2). Further characterization of these mutants led to several interesting results.

First, both lactose PTS and phospho-beta-galactosidase mutants were obtained. This was expected since in *S. sanguis* lactose PTS is the major pathway for lactose catabolism (5), and mutants unable to grow on lactose therefore would be expected to be deficient in at least one of these activities. We have not reported beta-galactosidase activities in this study; it has been shown that negligible activity for this enzyme can be detected in lactose-grown *S. sanguis* (5). Furthermore, in a comparison of two serotypes of *S. mutans*, Calmes has reported that a strain which exhibited phopho-beta-galactosidase activity lacked beta-galactosidase, and the converse is also true (1). Thus, we would predict that beta-galactosidase would be negligible or absent in *S. sanguis* Challis.

Second, general PTS mutants were found. These mutants lacked both glucose and lactose PTS activity and probably have lesions in one or both of the nonspecific components of the PTS, namely Enzyme I and the histidine-containing phosphocarrier protein HPr (17).

Third, we found one double mutant which lacked both phospho-beta-galactosidase and glucose PTS activities. Thus, a selection for a lactose mutant yielded a glucose mutant. This result probably is fortuitous.

Fourth, the general PTS and glucose PTS mutants are of particular interest. Despite their lack of PTS activity for glucose, their total growth on that sugar is essentially the same as for the wild type (Table 2). This result implies that *S. sanguis* Challis has at least two transport systems for glucose. This notion has been inferred from continuous culture studies in which the glucose PTS has been repressed or induced (3) and is supported here by genetic evidence. Hamilton and St. Martin have presented evidence that a glucose PTS mutant of *S. mutans* can transport glucose with a system which involves proton motive force (6).

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