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Inhibition of *Streptococcus mutans* by the Antibiotic Streptozotocin: Mechanisms of Uptake and the Selection of Carbohydrate-Negative Mutants

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The antibiotic streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranoside], an analog of *N*-acetylglucosamine (NAG), has been shown to be useful for the selection of carbohydrate-negative and auxotrophic bacterial mutants. We have adapted this method for use with the oral pathogen *Streptococcus mutans*, a gram-positive, aerotolerant anaerobe that uses predominantly carbohydrates as carbon sources for growth. Streptozotocin selectively kills growing cells of *S. mutans* GS-5, and under appropriate conditions it can reduce the number of viable cells in actively growing cultures by a factor of 10^3 to 10^4. However, unlike in enteric bacteria, which take up this antibiotic by a single NAG-specific transport system, streptozotocin appears to be taken up in *S. mutans* by both a NAG-specific system and a relatively nonspecific system that is also involved in glucose, fructose, and mannose uptake. Combining streptozotocin selection and a screening procedure involving indicator plates containing triphenyl-tetrazolium chloride, we developed a general method for the isolation of carbohydrate-negative and auxotrophic mutants of *S. mutans*. A preliminary characterization of both pleiotropic and specific carbohydrate-negative mutants isolated by using this procedure is presented.

The oral pathogen *Streptococcus mutans* is believed to be the primary etiologic agent of dental caries in humans and other animals, and the metabolism of sucrose appears to play a central role in the cariogenicity of this organism. Sucrose is both a substrate for extracellular and membrane-bound glucosyltransferases, which synthesize extracellular glucans important for adhesion of this organism to tooth surfaces, and an efficient growth substrate for *S. mutans*, leading to demineralization of tooth enamel by virtue of its fermentative production of lactic acid (for reviews, see references 9 and 17). Sucrose, glucose, fructose, mannose, lactose, maltose, mannitol, and glucitol (sorbitol) have all been shown to be taken up in *S. mutans* by a high-affinity phosphoenolpyruvate (PEP)-dependent carbohydrate phosphotransferase system (PTS) (for a review, see reference 10a), while non-PTS uptake systems have also been suggested, at least for glucose (5, 10, 11) and sucrose (11, 27, 28). In addition, the general PTS phosphotransfer proteins enzyme I and HPr have been purified from *S. mutans* (19, 29), PTS enzymes III specific for lactose and fructose have also been purified (7, 31), and the gene (*scrA*) encoding the sucrose-specific transport protein of the PTS, enzyme II^{Scr}, has recently been cloned and sequenced (26).

In order to define the types of transport systems for each carbohydrate utilized by *S. mutans* and to investigate their regulation, a general method for the isolation of carbohydrate-negative mutants would be extremely useful. Although sugar-specific *S. mutans* mutants apparently defective in PTS-mediated uptake of glucose (3, 10, 15, 30), sucrose (28), and fructose (8) have been isolated, mutant selection methods have relied primarily on the use of toxic sugar analogs such as 2-deoxyglucose (3, 15, 30) and xylitol (8), and, as yet, have not yielded pleiotropic mutants which might, for example, be defective in one of the general PTS proteins, enzyme I and HPr. For the isolation of sugar-specific mutants, this approach is also limited by the number of toxic sugar analogs available.

In this report, we describe the use of the antibiotic streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranoside] (STZ) for the selection of both pleiotropic and sugar-specific carbohydrate-negative mutants of *S. mutans*. Selection of mutant bacteria with STZ relies on its ability to be taken up into cells, where it is broken down to yield the highly toxic compound diazomethane, which kills cells by excessive DNA alkylation. Therefore, in contrast to penicillin, which kills bacteria from the outside, STZ selects for mutants unable to take up the antibiotic (12–14). Such mutants may be of two general types: auxotrophic mutants, which, during the preinduction period in minimal medium, do not synthesize the antibiotic uptake system and thus are resistant during the subsequent killing phase when STZ is added; or mutants unable to take up the analog because they either lack an intact transport system or are unable to energize transport. The class defective in transport energization normally contains mutants with a defect in the catabolism of the carbohydrate used to energize cells during the killing phase (12, 14).

The mutant selection procedure for *S. mutans* that we have developed in this report is similar to that developed for gram-negative enterobacteria such as *Escherichia coli* (12). However, the different growth requirements of *S. mutans*, as well as an apparently more complex uptake mechanism for STZ in *S. mutans*, which we also describe, has required some significant modifications to the method. A preliminary characterization of some of the mutants obtained is also presented.

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MATERIALS AND METHODS

Chemicals. STZ was purchased from Serva (Heidelberg, Federal Republic of Germany) and was freshly dissolved in distilled water immediately prior to use. (It should be noted that STZ is a strong mutagen and potential carcinogen.) Radiochemicals were from NEN Chemicals GmbH (Dreieich, Federal Republic of Germany) or Dupont, NEN Research Products (Boston, Mass.), except for phospho [^{14}C]enolpyruvate, which was from Amersham (United Kingdom). Other chemicals were of reagent grade or higher purity and were purchased from commercial sources.

Bacteria, culture media, and growth conditions. *S. mutans* GS-5 (serotype c, strain V843) was obtained from F. Macrina. Cells were routinely maintained on TYE-MOPS agar plates (16) containing 0.4% glucose or sucrose, with monthly transfers. Wild-type and mutant cells were routinely checked for purity both by microscopy and by their ability to grow on a selective medium (19). The compositions of TYE and TYE-MOPS broth and plates (pH 7.2) were previously described (16). TYE-triphenyl-tetrazolium chloride (TTC) plates contained TTC (30 mg/liter), which was added as a concentrated solution in absolute ethanol after autoclaving. Tryptone broth (T-broth) contained the following components per liter: 20 g of Select Peptone 140 (Gibco), 4 g of K_2HPO_4, 1 g of KH_2PO_4, and 2 g of NaCl (final pH, 7.2). T-broth at pH 6.2 contained, in addition to these components, 0.1 M bis-Tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)aminomethane], and its pH was adjusted to 6.2 with glacial acetic acid prior to autoclaving. Tryptone plates (T-plates) consisted of T-broth (pH 7.2) plus 15 g of Bacto-Agar (Difco) per liter. Carbohydrate supplements, when present in these media, were included at a concentration of 0.4% unless otherwise indicated. All media were stored at 4°C for no longer than 1 month or at room temperature for no longer than 1 week, because a significant decrease in both growth rate and growth yield of *S. mutans* was noted in older preparations of media.

S. mutans was grown aerobically in liquid culture in loosely stoppered tubes or flasks without shaking at 37°C, unless otherwise indicated. Growth on plates was also aerobic and at 37°C. Growth in liquid culture was monitored at 420 nm (one absorbance unit = 3×10^8 CFU/ml).

Growth phenotypes of *S. mutans* and mutant derivatives on various carbohydrates were determined in TYE-MOPS broth containing 0.4% carbohydrate. Tubes were inoculated with a 1% volume of an overnight culture grown on TYE-MOPS–glucose broth and were incubated at 37°C for 24 h. A positive phenotype was scored as a growth yield at least four times that of the control, which lacked carbohydrate. (Some growth occurs in the absence of added carbohydrate under these conditions because of sugar present in the TYE-MOPS broth [see the Discussion].) A negative phenotype was scored as a yield <1.5-fold that of the control.

For growth rate studies, overnight cultures grown in TYE broth plus appropriate carbohydrates were harvested, washed once with T-broth lacking carbohydrate, and resuspended in carbohydrate-free T-broth. This suspension was then used as an inoculum (4%) for T-broth cultures containing various carbohydrates. Specific conditions for each experiment are given in the Results section and in the figure legends and table footnotes. Cell viability during growth in liquid culture was determined by plating 100 μl of appropriate dilutions at various times onto TYE-MOPS plates containing 0.4% glucose.

Assays. PEP-dependent phosphorylation of sugars by the PTS was assayed as described previously (19), except that 1 mM PEP was used. Enzyme I activity of the PTS was determined by the PEP:pyruvate exchange assay (19, 25), and HPr activity was determined by complementation of a *Staphylococcus aureus* mutant defective in HPr, also as previously described (19). For some of these assays, toluene-treated whole cells (16) were used instead of cell extracts. Uptake of ^{14}C-labeled sugars was measured as described previously (16). Specific conditions for each experiment are given in the figure legends and table footnotes.

RESULTS

Growth inhibition of *S. mutans* by STZ and mechanisms of uptake. V843, the *S. mutans* GS-5 strain used in this study, gave a positive growth phenotype (see Materials and Methods) on TYE-MOPS broth containing the following carbohydrates: glucose (Glc), fructose (Fru), glucosamine, mannose (Man), galactose (Gal), mannitol (Mtl), glucitol (Gut), lactose (Lac), sucrose (Scr), maltose (Mal), melibiose (Mel), and trehalose (Tre). Unexpectedly, this strain failed to grow with *N*-acetylglucosamine (NAG) when it was the sole carbohydrate present (Nag^-), unlike most other serotype c strains (G. Jacobson, unpublished observations). However, derivatives able to utilize NAG were readily isolated on T-plates containing NAG as the sole carbohydrate. One such Nag^+ derivative was named V843-1 and was used along with V843 (Nag^-) for all of the studies reported below.

To determine the effects of STZ on the growth of *S. mutans*, overnight cultures of V843-1 (TYE plus 0.4% NAG) and V843 (TYE plus 0.4% glucosamine and 0.4% NAG) were washed free of medium and each culture was inoculated into several sidearm flasks of T-broth containing 0.4% mannitol. After 2 h at 37°C, when cultures had increased in density about 1.5-fold, STZ (final concentration, 50 μg/ml) plus, in some cases, various sugars were added to all but one flask of each strain. Growth was allowed to continue at 37°C. As shown in Fig. 1, STZ inhibited the growth of both V843 (Fig. 1A) and V843-1 (Fig. 1B), but inhibition was much more immediate and complete for V843-1. Complete protection of V843-1 against STZ inhibition was observed when glucose (0.4%) was added simultaneously with STZ, whereas only partial protection was observed when either NAG or fructose was added with the antibiotic (Fig. 1B). In contrast, both glucose and fructose completely protected V843, while NAG afforded only partial protection (Fig. 1A). Moreover, whereas addition of mannose simultaneously with STZ completely protected V843, only partial protection was observed with V843-1 (data not shown). Thus, STZ apparently is taken up in *S. mutans* by two different systems, one specific for NAG (that also recognizes glucose) and a nonspecific system that recognizes glucose, fructose, and mannose. Both systems are present in V843-1, while only the latter is present in V843.

The identity of the NAG-specific uptake system in V843-1 was investigated further. Uptake measurements obtained by using [^{14}C]NAG showed that V843-1, but not V843, was able to take up NAG at low concentrations (Table 1). Moreover, toluene-treated cells of V843-1 catalyzed the PEP-dependent phosphorylation of NAG, while V843 cells did not (Table 1), suggesting that the NAG-specific transport system is a PTS, which is defective in strain V843.

Both STZ uptake systems identified above appeared to be inducible, as judged by the relative sensitivities of strains V843-1 and V843 to STZ when pregrown on various sugars.

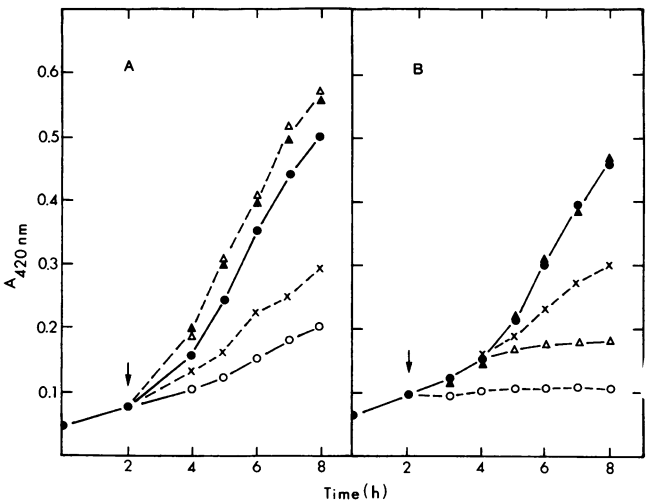

FIG. 1. Inhibition of *S. mutans* growth by STZ and protection by various sugars. Cells were pregrown and inoculated into T-broth containing mannitol as detailed in the text. STZ was added to all but one culture after 2 h, together with various sugars, as indicated (vertical arrow). (A) V843 (Nag^- strain); (B) V843-1 (Nag^+ strain). Symbols: ●, control minus STZ; ○, control plus STZ; X, NAG and STZ; ▲, glucose and STZ; △, fructose and STZ.

Thus, V843-1 was relatively insensitive to STZ when pregrown on mannitol compared with pregrowth on NAG, and V843 showed similar insensitivity to STZ when pregrown on mannitol but was sensitive to the antibiotic when pregrown on glucosamine, glucose, fructose, or mannose (Table 2).

Killing efficiency of STZ. The use of STZ in the selection of carbohydrate-negative or auxotrophic mutants depends upon its ability to selectively kill preinduced and energized cells (12, 13). Therefore, we tested the ability of STZ to kill both V843-1 and V843 cells under conditions in which growth inhibition by STZ was observed (Fig. 1 and Table 2). Strain V843-1 was pregrown on NAG, washed, and inoculated into T-broth (pH 7.2) containing 0.4% mannitol. After 2 h at 37°C, STZ (50 μg/ml) was added, and at various time intervals thereafter samples were removed, serially diluted, and plated to determine viability of cells. As shown in Fig. 2, cell viability progressively dropped over a period of hours, until the viable cell population was reduced by nearly a factor of 10^4 at 6 h after addition of STZ. Under the same conditions, the killing of strain V843 (pregrown on glucosamine) was much less efficient. In this case, the cell viability was reduced only about 10-fold after 6 h of STZ treatment. However, it was possible to increase the killing efficiency of STZ against strain V843 to nearly the same extent as that observed against V843-1 by lowering the pH to 6.2 during the STZ treatment (Fig. 2). Presumably, this is the result of the increased stability of STZ at low pHs (32) or the increased uptake of the antibiotic at this pH or both. In contrast to the efficiency of killing of strain V843, however, lowering the incubation pH of strain V843-1 had little effect on the killing efficiency (data not shown).

TABLE 1. Uptake and PEP-dependent phosphorylation of NAG by strains V843 and V843-1

Strain	Phenotype	NAG uptake[a]	PEP-dependent NAG phosphorylation[b]
V843-1	Nag^+	6.2	6.8
V843	Nag^-	<0.1	<0.1

[a] Measured as described in Materials and Methods, by using 25 μM [^{14}C]NAG (5 μCi/μmol). Units are nanomoles of NAG taken up per minute per milligram (dry weight) of cells.

[b] Measured in toluene-treated whole cells as described in Materials and Methods, by using 100 μM [^{14}C]NAG (5 μCi/μmol). Units are nanomoles of NAG phosphorylated per minute per milligram (dry weight) of cells.

TABLE 2. Sensitivities of strains V843 and V843-1 to STZ when pregrown on various carbohydrates

Strain	Pregrowth carbohydrate[a]	No. of doublings after STZ addition[b]
V843 (Nag^-)	Glucosamine	1.0
	Glc	0.8
	Fru	0.5
	Man	0.4
	Mtl	2.0
V843-1 (Nag^+)	NAG	0.3
	Mtl	2.1

[a] Cells were pregrown overnight in TYE-MOPS broth containing 0.4% of the indicated carbohydrate. They were then inoculated into T-broth containing 0.4% mannitol and incubated at 37°C. After 2 h, STZ (50 μg/ml) was added and growth was monitored spectrophotometrically, as shown in Fig. 1.

[b] The number of doublings achieved by the culture 6 h after STZ addition. At this time, control cultures (no STZ) had reached early stationary phase and had achieved 2.5 to 3 doublings (Fig. 1).

Selection and identification of *S. mutans* mutants by using STZ. The results presented above suggested that STZ could be used to select for cells unable to grow or energize transport under certain conditions (e.g., auxotrophic and carbohydrate-negative mutants) because the killing efficiencies were similar to those found for *E. coli*, for which this antibiotic is useful in such selections (12–14). Indeed, a presumptive auxotrophic mutant of *S. mutans* (mutant 3-7; see below), when incubated with STZ under conditions in which it cannot grow, had a survival rate of over 85% after 5 h of STZ (50 μg/ml) treatment, as shown in Fig. 3. Therefore, among the survivors of STZ treatment under the conditions shown in Fig. 2, there should be both auxotrophic mutants requiring compounds not present in T-broth and mutants unable to transport and/or metabolize mannitol, and we attempted to identify such mutants by using both strains.

For selection of potential mutants of strain V843, we first treated cells pregrown on glucosamine, and then suspended in T-broth containing mannitol, with STZ for 6 h at pH 7.2 under the conditions shown in Fig. 2. The survivors (approx-

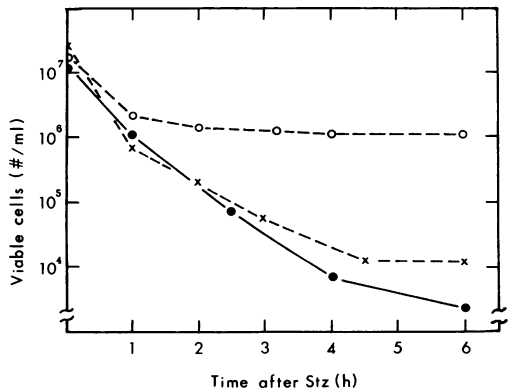

FIG. 2. Cell viability after STZ treatment. Viable cells were counted as described in the text as a function of time after addition of STZ. Symbols: ●, V843-1 (Nag^+ strain), pH 7.2; ○, V843 (Nag^- strain), pH 7.2; X, V843, pH 6.2.

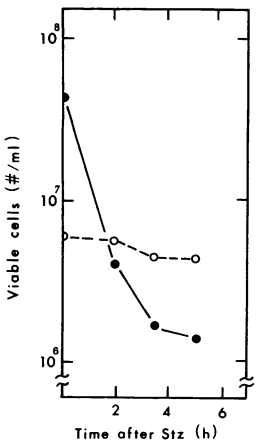

FIG. 3. Effect of STZ on the viability of an auxotrophic mutant of *S. mutans* under conditions in which it cannot grow. Strain V843 and an auxotrophic derivative, mutant 3-7, were pregrown overnight as described in Fig. 2, washed, and then mixed such that the concentration of wild-type cells was roughly 10 times that of the mutant cells. This mixture was diluted into T-broth (pH 7.2) containing mannitol and incubated at 37°C for 2 h, also as described in Fig. 2. STZ (50 μg/ml) was then added, and cell viabilities for strain V843 (●) and the mutant (○) were determined at various times thereafter by plating dilutions onto TYE-TTC–mannitol plates, on which the mutant can readily be distinguished from the wild type (see text). As shown, little killing of the auxotrophic mutant, which cannot grow under these conditions (Table 3), was observed, while the parent strain was killed. This experiment thus directly demonstrates the enrichment of the auxotrophic mutant in a mixed culture.

imately 10% of the original population) were collected by centrifugation and grown out in TYE broth containing glucosamine. In addition to being somewhat enriched in potential mutants, this population was also likely to be heavily mutagenized by this STZ treatment, since STZ is a potent mutagen (13). These cells were then washed, suspended in T-broth (pH 6.2) containing mannitol to a concentration of ca. 5×10^7 cells per ml, and subsequently treated with STZ as shown in Fig. 2 to reduce the cell population by a factor of 10^3 to 10^4. For the selection of potential mutants of strain V843-1, a single STZ selection was performed on cells, pregrown on NAG, in T-broth containing mannitol at pH 7.2 (Fig. 2). In either case, survivors after the final 6-h STZ selection were serially diluted into T-broth, and 100-μl portions were plated onto TYE-TTC plates containing 0.4% mannitol and incubated aerobically at 37°C for 48 to 72 h. Between 100 and 200 colonies per plate was the density found to be the most useful for the identification of mutants.

In Fig. 4 we show the various types of colony morphologies observed on the TYE-TTC plates after STZ selection. Wild-type cells of either strain gave a typical "bull's-eye" colony, with an orange-red to maroon center surrounded by a lighter halo (Fig. 4A). Over 90% of the cells surviving either STZ selection procedure had this appearance. From 1 to 5% of the colonies, however, had a distinctly different colony morphology. Typical examples are shown in Fig. 4B to E. Mutant colonies were consistently smaller than those of the wild type on this medium and showed pigmentation ranging from a "bloodshot-eye" appearance (Fig. 4B) to "mottled" (Fig. 4D and E) to nearly colorless (Fig. 4C). Identification of mutant colonies was greatly facilitated by examination of these plates at low magnification (×10 to ×40) under a light microscope.

Characterization of *S. mutans* mutants isolated by using the STZ selection procedure. In Table 3 are listed the growth phenotypes of a number of representative mutant strains identified as shown in Fig. 4. In each case, the growth phenotype, initially determined with patch tests on T-plates containing various sugars, was confirmed by growth experiments in liquid culture. Presumptive auxotrophs were identified on the basis of their ability to grow on TYE-sugar plates but not on T-plates plus sugar (e.g., mutant 3-7), suggesting that they require a compound for growth that is present in yeast extract but not in tryptone. Mutant 3-7 was used to show that STZ selectively kills growing cells, because this mutant was resistant to the antibiotic when treated with it in T-broth media (Fig. 3). Of over 15 pleiotropic, carbohydrate-negative mutants isolated, all were negative for at least mannitol, glucitol, and melibiose (e.g., mutants 3-8, 4-5, and 4-23 [Table 3]). Most were also negative for, or grew only slowly on, lactose (e.g., mutants 4-5 and 4-23). A final class of mutants, represented by mutant 9-7 in Table 3, grew exceedingly slowly on all media tested but grew best on sucrose-containing media.

Of possible defects that could give rise to pleiotropic, carbohydrate-negative mutants, the most likely would seem to be mutations affecting the PTS, since many sugars are transported in *S. mutans* by this mechanism. Therefore, we assayed several of the carbohydrate-negative mutants listed in Table 3 for PEP-dependent phosphorylation of glucose and mannitol and for activities of the general PTS proteins enzyme I and HPr. The results, presented in Table 4, showed that mutants 4-5, 4-8, and 4-23 all had somewhat depressed levels of PEP-dependent glucose phosphorylation activity, as well as markedly lower PEP-dependent mannitol phosphorylation activity, compared with the wild type. Surprisingly, mutant 9-7 had a significantly elevated level of glucose phosphorylation activity but a very low mannitol phosphorylation activity. Assays of enzyme I and HPr activities suggest that mutant 4-5 could be a leaky HPr mutant because it possessed only 25% of the activity of the wild type, while mutant 9-7 could be a very leaky enzyme I mutant (Table 4). However, we have not yet been able to culture enough of the latter mutant to measure HPr levels reliably in vitro, because it grows exceedingly slowly, especially in liquid culture, with a high reversion frequency. Mutant 4-8 most likely has a defective mannitol-specific enzyme II of the PTS because it is specifically mannitol negative (Table 3) and possesses less than 10% of the activity of the wild type for mannitol:mannitol 1-phosphate phosphoexchange (data not shown), an activity specific for this protein (24).

DISCUSSION

In this report, we have investigated the mechanisms of uptake of and inhibition by the antibiotic STZ for the oral pathogen *S. mutans*. STZ very effectively inhibited growth of this organism and appears to be taken up by a NAG-specific PTS, as well as by a less specific system that also recognizes glucose, fructose, and mannose. Furthermore, we have shown that under appropriate conditions, STZ kills growing or energized *S. mutans* cells by a factor of 10^3 to 10^4 and thus can be used to select for mutants against a large background of wild-type cells, as has been reported for other bacteria (12, 13). In this regard, STZ appears to be superior to penicillin for this type of selection, because in our hands penicillin selection typically enriches for nongrowing mutants of *S. mutans* by a factor of only 10^2 to 10^3 (unpublished observations). Undoubtedly, this is at least partially the result of the fact that, unlike STZ, penicillin lyses sensitive

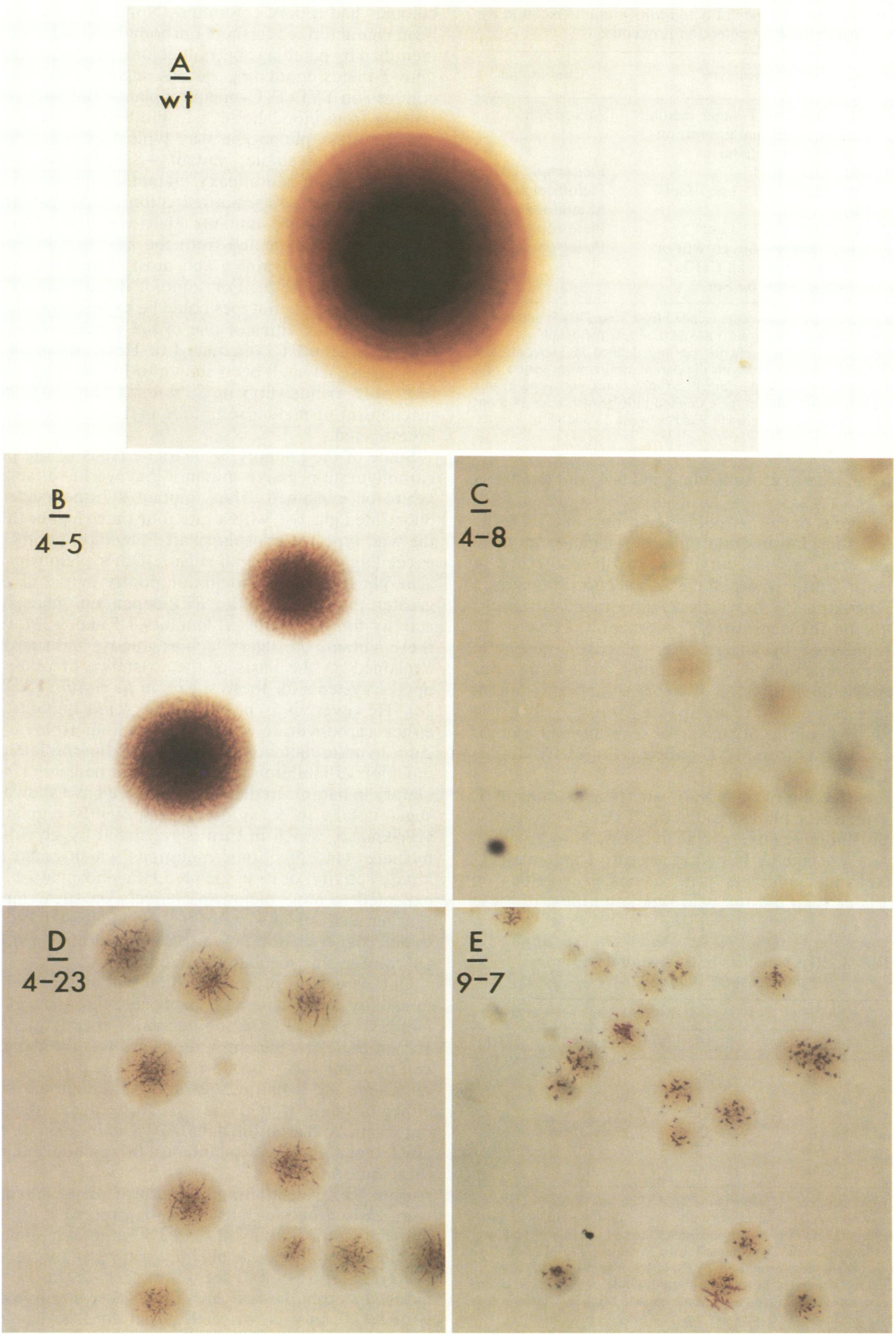

FIG. 4. Colony morphologies of wild-type and carbohydrate-negative mutants of *S. mutans* on TYE-TTC–mannitol plates incubated aerobically. (A) Wild-type; (B) mutant 4-5; (C) mutant 4-8; (D) mutant 4-23; (E) mutant 9-7. Magnification, ×140. See the text and Tables 3 and 4 for further characterization of these mutants.

TABLE 3. Growth phenotypes of *S. mutans* mutants isolated by using the STZ selection procedure

Mutant designation[a]	CHO phenotype[b]	Classification
3-7	No growth on T-sugar media, slow to normal growth on TYE-sugar media	Auxotroph
3-8	Mtl^- Gut^- Mel^-	Pleiotropic CHO
4-5	Mtl^- Gut^- Mel^- Lac^- Gal^-	Pleiotropic CHO
4-8	Mtl^-	Mannitol specific
4-23	Mtl^- Gut^- Mel^- Lac^{slow}	Pleiotropic CHO
9-7	Exceedingly slow growth or no growth on all CHOs; best growth on Scr	Pleiotropic CHO

[a] Mutants of the 3- and 4- series were isolated from strain V843; mutants of the 9- series were isolated from strain V843-1. (See text for details.)

[b] Carbohydrate (CHO) growth phenotypes are defined in Materials and Methods. All carbohydrates tested with the parent strains were tested with these mutants, which were positive for all carbohydrates not listed in this table. Mutants with "slow" phenotype exhibited little growth after 24 h but eventually reached the wild-type growth yield between 24 and 48 h.

cells, which can lead to cross-feeding and lysis of mutants as well.

Although there is one other published report of STZ being used to select for carbohydrate (glucose)-negative mutants of *S. mutans* (15), this selection also involved the use of 2-deoxyglucose. Furthermore, the STZ selection was carried out in TYE medium (15), in which we have found *S. mutans* to be relatively resistant to STZ, probably because of protection conferred by sugars (e.g., glucose) present in yeast extract (unpublished observations). Therefore, our studies on STZ inhibition of *S. mutans* and on mutant selection were carried out in sugar-free T-broth, which will support the growth of this organism for several generations if the inoculum used is from TYE-grown cells (see Materials and Methods).

Using STZ selection of both Nag^- and Nag^+ strains of *S. mutans* and indicator plates containing TTC, we isolated a number of presumptive auxotrophic mutants, as well as both pleiotropic and specific-carbohydrate-negative mutants. Typically, auxotrophic mutants yielded smaller colonies on TYE-TTC plates than did the wild type, and most of these colonies had a pink coloration. None of these mutants has been characterized further. Carbohydrate-negative mutants, identified by patching aberrant colonies from indicator plates onto T-plates containing various sugars, also yielded small colonies on TYE-TTC–mannitol plates, but with colorations ranging from bloodshot-eye to mottled to nearly colorless. The colorless phenotype was typical of the tightest Mtl^- mutants, for example, mutant 4-8, which is a mannitol-specific mutant. More leaky mutants, such as 4-5 and 4-23, typically appeared as mottled colonies. All pleiotropic carbohydrate-negative mutants that we have characterized were Mtl^- (as expected from the selection procedure), as well as Gut^- and Mel^-, and most were Lac^- or Lac^{slow} (Table 3, footnote *b*). For some of these mutants (e.g., 4-5 and 9-7), these phenotypes could be explained on the basis of the lowered activities of one of the general phosphocarrier proteins of the PTS (enzyme I or HPr). Mannitol, glucitol, and lactose have all been shown to be taken up by the PTS (probably exclusively) in *S. mutans* (1, 2, 18), while the mechanism of melibiose uptake in *S. mutans* has not been investigated.

TABLE 4. PTS activities in carbohydrate-negative mutants of *S. mutans*

Mutant	Activity of:			
	Glc PTS[a]	Mtl PTS[a]	Enzyme I[b]	HPr[c]
Wild type	100	100	100	100
4-5	61	21	90	25
4-8	60	11	ND[d]	90
4-23	60	23	75	90
9-7	400	10	45	ND

[a] Percent PEP-dependent phosphorylation of ^{14}C-carbohydrate (0.1 mM) in toluene-treated whole cells relative to wild type. For Glc and Mtl, 100% equals 1.1 nmol and 23 nmol of sugar phosphate formed per min per mg (dry weight) of cells under conditions referred to in Materials and Methods. All cells listed in this table were grown on TYE-MOPS–mannitol broth.

[b] Percent enzyme I activity in toluene-treated whole cells relative to the wild type under conditions referred to in Materials and Methods. 100% equals 40 nmol of pyruvate formed per min per mg (dry weight) of cells.

[c] Percent HPr activity in *S. mutans* cytoplasm relative to wild-type cells under conditions referred to in Materials and Methods. 100% equals 0.27 nmol of sugar-phosphate formed per min per mg of *S. mutans* cytoplasmic protein.

[d] ND, Not determined.

Some of the phenotypic characteristics of the pleiotropic carbohydrate-negative mutants that we have isolated have yet to be explained. Thus, mutant 9-7 unexpectedly phosphorylated glucose with a rate four times higher than that of the wild type yet phosphorylated mannitol poorly and grew exceedingly slowly on all sugars. Other pleiotropic mutants also phosphorylated mannitol poorly but still possessed greater than 50% of the PEP-dependent phosphorylation activity for glucose (e.g., mutants 4-5 and 4-23). Growth of these mutants on sugars such as glucose and sucrose can be explained on the basis of the existence of other non-PTS uptake systems for these sugars in *S. mutans* (5, 10, 11, 27, 28). However, it is possible that some mutants could be either carbohydrate sensitive, resulting from a defect in carbohydrate metabolism that allows the intracellular accumulation of inhibitory metabolic intermediates (14), or regulatory in nature. In the latter case, we have shown that PTS sugar uptake in *S. mutans* is apparently regulated by an HPr-kinase, which in turn is regulated by glycolytic intermediates (16, 20). Some mutations which could affect the kinase activity, or its associated HPr-phosphatase, would be expected to have pleiotropic effects on sugar metabolism. Further analysis of some of these mutants will be necessary to determine whether any are carbohydrate sensitive or are aberrant in this regulatory system.

Our screening of several hundred mutant colonies obtained by using the STZ procedure has as yet failed to identify any tight mutations in genes encoding enzyme I or HPr of the PTS. Such mutations are readily identified in *E. coli* by using this procedure (14). Thus, it may be that such mutations are lethal in *S. mutans*, although a vital role for either of these proteins has yet to be demonstrated in this organism. A search for tighter PTS mutations or conditionally lethal mutations would be useful in finding an answer to these questions.

Both STZ selection and growth of the resultant mutants were carried out aerobically in the present study. It is well known that the activity of the PTS for at least some sugars is regulated by oxygen availability, at least in gram-negative bacteria (for reviews, see references 21 to 23). In these cases, anaerobiosis activates the PTS severalfold compared with aerobically grown cells. Therefore, it would also be informative to attempt to isolate carbohydrate-negative mutants under anaerobic conditions. If tight general PTS mutations are not lethal, it may be easier to select for and identify

them under anaerobic conditions, in which only relatively tight mutations may give a negative phenotype on indicator plates. A TTC indicator plate method that has been developed for *S. mutans* growing anaerobically (6) should be useful in this regard.

Finally, it is worth noting that STZ selection can also be used to cure cells of plasmids carrying genes for antibiotic resistance, as has been shown for both *E. coli* (12) and *Streptococcus faecalis* (4). In this case, cells are first preinduced for NAG uptake in the presence of the antibiotic, the resistance to which is encoded by the plasmid (there will be no induction in cured cells), and then treated with STZ in a medium containing a carbohydrate that does not compete for STZ uptake (e.g., mannitol). In principle, this procedure should also be applicable to *S. mutans* and may prove quite useful, especially because of the recent advances in cloning technology in gram-positive bacteria.

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