

Physiology of "Mutans-Like" *Streptococcus ferus* from Wild Rats

M. L. FREEDMAN,* A. L. COYKENDALL, AND E. M. O'NEILL

Department of Oral Diagnosis, School of Dental Medicine, University of Connecticut Health Center,
Farmington, Connecticut 06032

Received 28 July 1981/Accepted 29 September 1981

Strains of *Streptococcus ferus* isolated from the oral cavities of wild rodents inhabiting sucrose-rich and sucrose-poor environments have many traits in common with the "mutans" streptococci. Thus, *S. ferus* HD3 and 8S1, like cariogenic *S. sobrinus* 6715-13, form adherent, $\alpha(1\rightarrow3)$ glucopyranosyl-glucose linkage-rich, plaque-like deposits in vitro and in vivo through the action of constitutive glucosyltransferase(s) enzymes on sucrose, produce and degrade intracellular polysaccharide, produce short-chain fatty acids from the catabolism of mono- and disaccharides, carry the *c* antigen of *S. mutans*, and penetrate, persist, and proliferate in a sucrose-augmented fashion in the oral cavities of specific-pathogen-free rodent caries models. However, unlike infection with common *S. mutans*, infection with tested *S. ferus* strains does not cause caries. This avirulence appeared to result more from the reduced aciduricity of *S. ferus* than from differences in glucosyltransferase complements. Studies showed that despite generally similar growth rates and extracellular glucan syntheses, the acidogenic metabolism of *S. ferus* was more inhibited by declining environmental pH than was cariogenic *S. sobrinus* 6715-13 and that, in vitro, less hydroxyapatite was solubilized by *S. ferus* metabolic end products. The physiology of these *S. ferus* strains demonstrated that, in addition to plaque formation and acid production, acid tolerance was crucial to the carious process.

Strains of *Streptococcus ferus* isolated from wild rats have many caries-associated traits in common with virulent "mutans-type" streptococci. *S. ferus* strains (i) form adherent, plaque-like deposits in vitro when cultured in the presence of sucrose, (ii) synthesize, sequester, and degrade glycogen-type intracellular polysaccharide, (iii) produce acid by metabolism of common sugars, (iv) ferment polyols, and (v) carry the *c* antigen of *S. mutans* (7, 8, 10, 15). Despite these similarities, the DNAs of *S. ferus* strains are not homologous with common human and animal isolates of cariogenic *S. mutans*, *S. cricetus*, *S. sobrinus*, or *S. rattus* (7, 8); thus, *S. ferus* may be considered to be a "mutans-like" streptococcus.

Because *S. ferus* possesses such physiological traits, it was anticipated that the bacterium would be cariogenic in laboratory rats consuming the nonabrasive, sucrose-rich test diet 2000 (27). However, when compared with uninfected controls and animals infected with the known cariogen *S. sobrinus* 6715-13, two tested *S. ferus* strains were not found to be virulent (9), although they were observed to penetrate, persist, and proliferate in the presence of dietary sucrose in the oral ecology of specific-pathogen-free Sprague Dawley rats.

This study was undertaken to find the basis of the apparent avirulence of *S. ferus* by examining quantitative and qualitative aspects of its (i) extracellular glucan synthesis and associated enzymology, (ii) glucan-induced agglutination in vitro, and (iii) acid production and resulting hydroxyapatite solubilization.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. sobrinus* 6715-13 (19) and *S. ferus* HD3 and 8S1 (8) were grown in (i) broth (26) containing yeast extract and tryptic digest of casein (Difco Laboratories), (ii) Todd-Hewitt broth which contains glucose (Difco), or (iii) the complete defined medium, FMC, which contains assorted inorganic salts and macromolecular precursors (35), supplemented with separately sterilized glucose, sucrose, or equimolar amounts of glucose and fructose. Cultures were grown in flasks in candle jars at 37°C or stirred in vessels in which the surface of the medium was washed with 5% CO₂ plus 95% N₂. In some instances, the culture pH was maintained at 6.8 to 7 during growth by the automatic addition of 10 N NaOH (pH stat). In all cases, inocula from stock cultures (34) were acclimatized to the media by back-culturing.

Glucan analyses. Glucans were isolated from both the media and the cells from cultures grown in the dialyzate of Jordan broth supplemented with 1% su-

crose. These 400-ml cultures were maintained at pH 7 throughout cell growth. Polysaccharide samples were analyzed for glucose content by the glucose oxidase procedure (PGO enzymes, Sigma Chemical Co.) and, after purification, were methylated for glucan linkage analysis (1, 14). Bacterial samples were also taken for the colorimetric determination of DNA (4).

In vitro GTF and dextranase activities. Glucan-synthesizing (glucosyltransferase [GTF]) and -degrading (dextranase) enzymes from the cell-free medium of cultures grown in FMC supplemented with 2% glucose were prepared by the affinity chromatography method of McCabe et al. (30), with the exception that the enzymes were precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 80% saturation. Material absorbing 280-nm light was eluted from the Sephadex G-50 affinity matrix with 6 M guanidine-HCl (GuHCl) after extensive washing with 0.02% NaN_3 and quantified by Coomassie blue dye staining (2); bovine serum albumin was used as the standard.

After polyacrylamide gel electrophoresis (PAGE) (11), gels were incubated at 37°C in 0.1 M KPO_4 buffer, pH 6, containing 0.02% NaN_3 and 2% sucrose which had been purified by dialysis. Glucan synthesis was visualized as zones of swelling or turbidity (5) or as fuscia bands after periodic acid-Schiff staining (37). Other gels contained 0.45% blue dextran (molecular weight, 2×10^6 ; Sigma); in situ dextranase activity was demonstrated by the appearance of zones of clearing in the blue gels after incubation at 37°C in the above buffer minus sucrose. Protein was visualized by staining with 0.5% Coomassie blue in 7% acetic acid followed by decolorization in 7% acetic acid.

In vitro agglutination. Cells were grown in FMC medium supplemented with 0.1% glucose. Before inoculation, the medium was treated overnight at 37°C with 50 U of invertase (Sigma, grade X) per ml to remove any precursors of extracellular glucan. The cells were harvested, washed, and suspended to an optical density at 550 nm (OD_{550}), of 2 (1-cm path length; 1 OD = 200 μg of cells [dry weight] per ml) in either 0.1 M glycylglycine buffer, pH 8.5, or phosphate-buffered saline (12) with and without 0.001 M Ca^{2+} . Portions of the cell suspensions were added to serial 1:10 dilutions of commercial *Leuconostoc mesenteroides* dextran with approximate molecular weights of 2×10^4 , 2×10^5 , and 2×10^6 or to purified water-soluble and water-insoluble glucans which had been isolated from *S. sobrinus* 6715-13 cultured in sucrose-supplemented broth, as described above. Agglutination in individually stirred samples was observed at $\times 20$ after 20 min and 24 h.

Production, identification, and action of acid end products. Cultures were grown in either Jordan or Todd-Hewitt broth supplemented with 1% glucose, 1% sucrose, or 0.5% glucose and 0.5% fructose and were maintained at pH 7 to minimize cell death. Cells were harvested by centrifugation ($10,000 \times g$, 10 min, 4°C), washed with and held in buffer (0.001 M KPO_4 , 0.05 M KCl; pH 7, 4°C). The pH drop effected by cell suspensions, the OD_{550} values of which were known and which were held in a water-jacketed spinner flask at 37°C, was followed with a recording pH meter after addition of glucose, sucrose, or equimolar amounts of glucose and fructose. Cell samples were removed for quantitation of DNA and for assessment of viability by growth on mitis salivarius agar.

Short-chain volatile and nonvolatile acids were prepared for gas-liquid chromatography (GLC) (24). A Capco 700 gas chromatograph (Clinical Analytical Products Co., Sunnyvale, Calif.) was fitted with an SP1000 column, 6 ft by 0.25 in. (182.88 by 0.635 cm), containing 1% H_3PO_4 on Chromosorb 100/120 mesh and operating isothermally at 110°C for nonvolatile or 140°C for volatile acids. The helium carrier gas had a flow rate of 120 ml/min. Metabolic end product standards were treated similarly. Acids in culture media were identified by the similarities of their retention times to those of the standards and were quantitated by measurement of the areas under recorder output peaks.

The ability of metabolic end products to solubilize hydroxyapatite (HA) was measured by exposing 0.2-g portions of material marketed as spheroidal HA (BDH Chemicals, Ltd., Poole, England), slurried in 1 ml of water and sequestered within dialysis bags, to 2 liters of cell-free Todd-Hewitt broth from 24-h cultures which had been supplemented with 5% sucrose. This spent culture medium was sterilized by filtration, and the dialysis bags with their HA contents were autoclaved. Triplicate dialysis bags containing HA were removed from the media after week 1 and thoroughly rinsed with water. Their HA contents were washed by centrifugation ($10,000 \times g$, 15 min, 4°C) and trapped on membrane filters (0.45- μm pore size). The filters with retained HA were dried (30 min, 100°C) and weighed; longer drying times did not affect the weights. Other HA samples (0.039 ± 0.0004 g) were placed in 10-ml solutions of lactic acid plus formic acid and diluted with fresh broth to reflect the concentrations determined by GLC to be present in early-stationary-phase cultures of the three bacterial strains. Quadruplicate samples were taken every day for 3 days and treated as described above.

RESULTS

Glucan analyses. Table 1 presents the types and quantities of glucans formed by strains 6715-13, HD3, and 8S1 cultured in sucrose-supplemented Jordan broth. The glucose contents of hydrolyzed glucan samples were normalized to cellular DNA. The polysaccharides formed were extracellular, water soluble and 1 N NaOH solubilized, cell associated. As shown by the mole percentages of glucopyranosyl-glucose linkages, cell-associated polysaccharide tends to be $\alpha(1 \rightarrow 3)$ rich and less branched.

In vitro GTF and dextranase activities. Figure 1 depicts the profiles of 280-nm-absorbing material eluted by 6 M GuHCl from Sephadex G-50 affinity columns. The ordinate has been normalized to reflect protein recovered per milligram of cell material in the three cultures. Material from the *S. sobrinus* 6715-13 culture eluted as two peaks denoted A and B in Fig. 1. There were clear quantitative and qualitative differences among the three strains. Figure 2 diagrams the combined results of PAGE of the eluate peaks from the three bacterial strains observed visually for clouding, swelling, or periodic acid-Schiff staining (glucan synthesis), for Coomassie blue

TABLE 1. Analysis of glucans from *S. sobrinus* 6715-13 and *S. ferus* HD3 and 8S1

Strain	Glucan class ^a	mg of glucose/mg of DNA ^b	mol% ^c			
			End groups	α (1 \rightarrow 3) linkages	α (1 \rightarrow 6) linkages	Glucan branches
6715-13	WS	0.23	24	27	30	19
	AS	6.46	9	69	10	12
HD3	WS	0.01	25	21	36	18
	AS	3.11	6	56	30	7
8S1	WS	0.03	26	28	23	22
	AS	2.61	6	63	23	8

^a WS, Extracellular, water soluble; AS, cell associated, alkali solubilized.

^b Glucose oxidase-positive material in purified glucans was normalized to bacterial DNA in culture.

^c Determined by GLC analyses of glucopyranosyl-glucose linkages.

dye staining (protein), and for clearing of blue dextran contained in the gel (dextranase activity). Strain 6715-13 had the greatest multiplicity of glucan-synthetic and -degradative activities; dextranase activity in this strain was concentrated in peak A and was absent from both *S. ferus* isolates.

In vitro agglutination. The degree of in vitro agglutination of 6715-13 showed the customary dependence on the molecular weight of the exogenously added α (1 \rightarrow 6)-linked *L. mesenteroides* dextran (unpublished data). Both HD3 and 8S1 agglutinated spontaneously, forming macroscopic aggregates within 24 h in the pH-8.5 glycyglycine buffer. The appearance of these *S. ferus* aggregates was macroscopically indistinguishable from those of *S. sobrinus* 6715-13 exposed to low concentrations of dextran. Agglutination assays conducted in pH-6.8 phosphate-buffered saline showed that 6715-13 agglutinated strongly with exogenous dextran,

whereas HD3 and 8S1 spontaneously formed fine, barely perceptible aggregates, the size of which was not augmented by the presence of dextran.

Acid production. Figure 3 shows the kinetics of acid production by 25-ml buffer suspensions of the three streptococci maintained at different

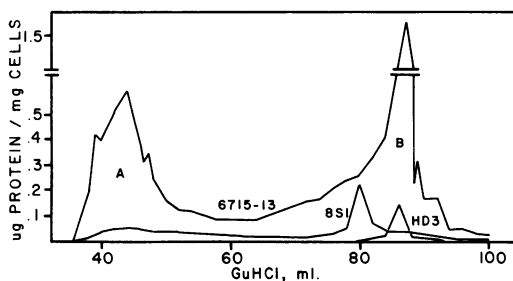


FIG. 1. Profiles of material released from Sephadex G-50. Media from cultures of *S. sobrinus* 6715-13 and *S. ferus* HD3 and 8S1 were pumped onto the affinity matrix, unbound components were washed away with 0.02% NaN₃, and bound material was eluted with 6 M GuHCl (30). Material absorbing 280-nm UV light was converted to protein content with Coomassie dye and corrected for the amount of cells in the cultures. For explanation of peaks, see text.

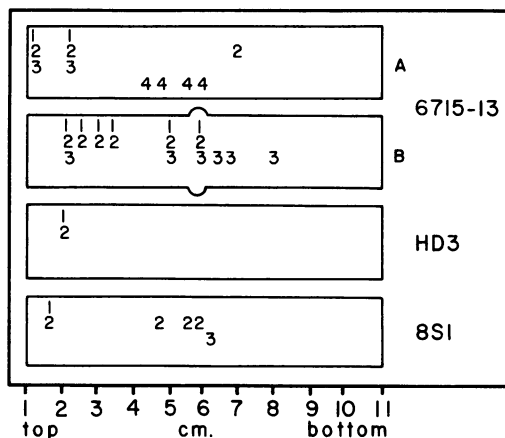


FIG. 2. Diagrams of polyacrylamide gels of material eluted from Sephadex G-50 (profiled in Fig. 1). The numbers 1 through 4 in each gel diagram refer to the positions of bands in four different gel types. Number 1 is a cloudy band signifying insoluble glucan synthesis from sucrose; number 2 is a band of polysaccharide synthesis from sucrose, stained by periodic acid-Schiff; number 3 is a band of protein stained by Coomassie blue dye; number 4 is a clear band in blue dextran-containing gels, denoting dextranase activity. The swelling in 6715-13 gel B denotes soluble glucan synthesis. In some cases, enzyme activity is shown only by product synthesis (numbers 1 and 2 together); in other cases, enzyme activity is shown by both product and protein staining (numbers 1, 2, and 3 together); in yet other cases, nonenzymatically active protein is bound to the affinity matrix (number 3 alone).

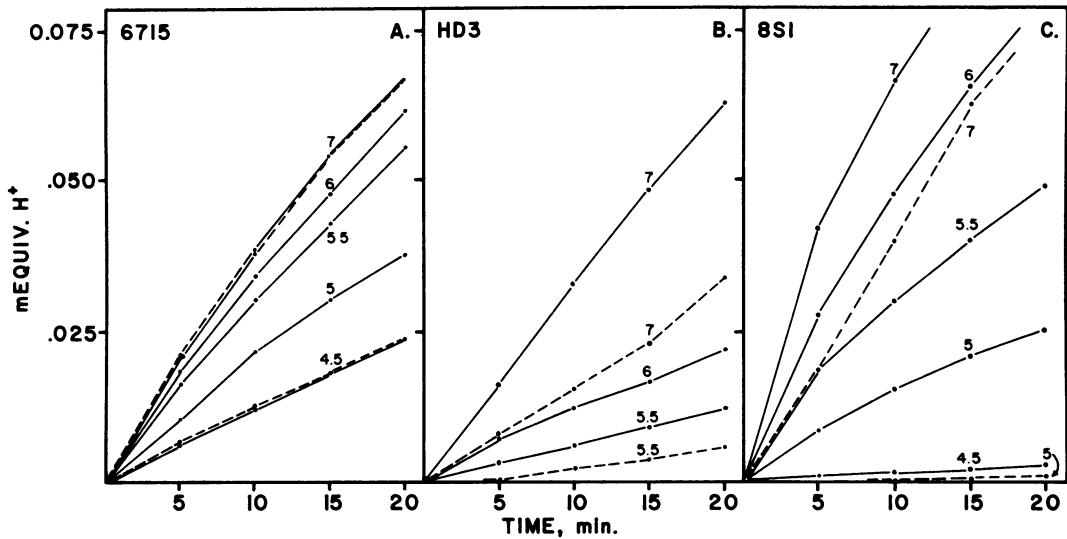


FIG. 3. Acid production kinetics of *S. sobrinus* 6715-13 and *S. ferus* HD3 and 8S1. Standardized, buffer-held cell suspensions were exposed to sucrose (---) or to equimolar amounts of glucose and fructose (—) at the indicated pH values at 37°C. Acid production was followed by the automatic addition of NaOH.

constant pH values. The cells were grown in Jordan broth containing 1% glucose, washed, and held in buffer at pH 7 to minimize loss of viability. There was no endogenous acid production to suggest metabolism of intracellular nutrient pools before the cell suspensions were adjusted to the indicated pH values and sucrose or glucose plus fructose was added to final concentrations of 0.0022 M. Acidogenesis was followed and the particular pH was maintained by the automatic addition of 0.5 N NaOH. Cell viabilities had not diminished by the conclusion of the experiments (unpublished data).

In Fig. 4 are growth curves obtained from the *S. sobrinus* and two *S. ferus* strains growing in Todd-Hewitt broth. Although the culture doubling times during the exponential growth phase are quite comparable, the *S. ferus* strains neither attained culture densities as high as that of *S. sobrinus* nor attained pH values as low as that of *S. sobrinus*. The *S. ferus* culture growth rates diminished at lower optical densities and higher pH values than did the growth rates of *S. sobrinus*. The three cultures whose growth rates are plotted in Fig. 4 were each sampled at the four times and optical density values indicated by the triangles on the abscissae; the samples were prepared for characterization of metabolic acids by GLC. Strain 6715-13 attained lower pH values than did either HD3 or 8S1, and these values were reflected by the greater concentrations of lactic acid (Table 2). Formic acid was the only other metabolic acid product apparent.

The acids produced during growth for 24 h in

Jordan both supplemented with 1% sucrose affected the solubilization and subsequent loss of HA spheroids from within dialysis bags. Exposure for 1 week to the medium from a 6715-13 culture was more effective in solubilizing HA than was exposure to either HD3 or 8S1 medium (unpublished data). The same trends were seen when dilutions of lactic and formic acids were mixed together in amounts reflecting their GLC-determined percent abundances in stationary-phase cultures (Fig. 5). Before the formic acid was added, the dilute lactic acid was heated to 60°C for 4 h to hydrolyze lactic anhydride. There was a background of 4% HA loss in the plain broth control, probably from abrasion.

DISCUSSION

There is abundant evidence that the water-insoluble, $\alpha(1\rightarrow3)$ linkage-rich glucans of mutans-type streptococci are indispensable for adherent proliferation and virulence on smooth tooth surfaces (14, 22, 28, 31, 34). Also, they augment virulence in retentive sulcal sites (34), probably by acting as diffusion barriers to the metabolic acids produced by the plaque flora. A virulent *S. sobrinus* mutants which have defective glucan synthesis do not form such $\alpha(1\rightarrow3)$ -rich polymers (14), nor do they form adherent plaques in vitro or in vivo. However, *S. ferus* strains HD3 and 8S1, although not virulent in the rodent caries model (9), did form adherent plaque in vitro (10; unpublished data) and in vivo (9), albeit less than the renowned cariogenic *S.*

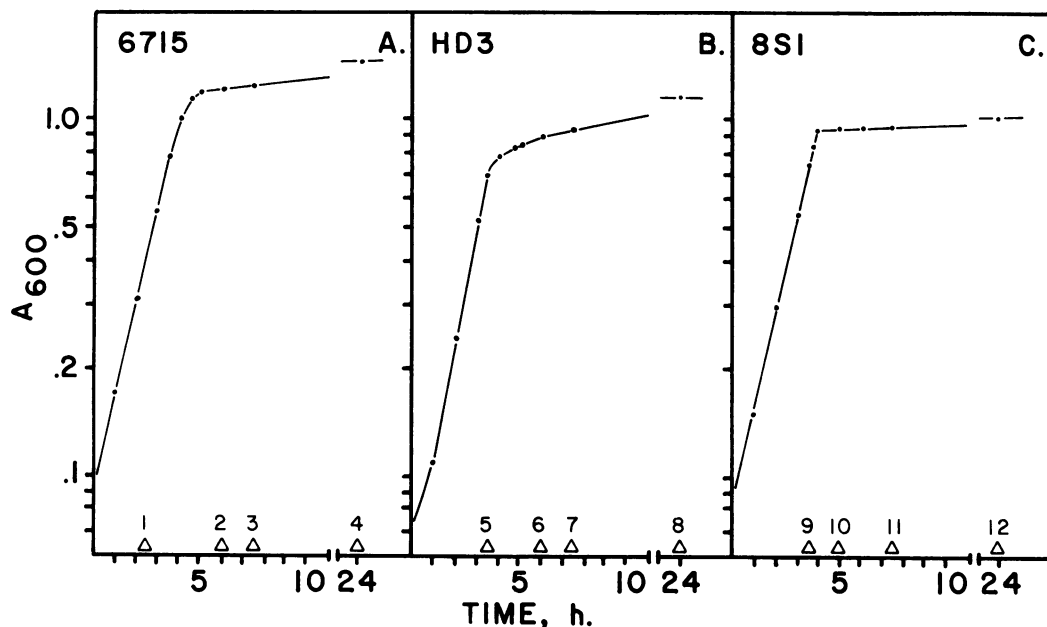


FIG. 4. Growth curves from Todd-Hewitt broth cultures of *S. sobrinus* 6715-13 and *S. ferus* HD3 and 8S1. Culture growth was followed at 600 nm (1-cm light path, 200 μ g [dry weight] of cells per optical density), and samples were removed for analysis of metabolic acids at time points 1 through 12 (Δ).

sobrinus 6715-13. The quantities of insoluble cell-associated glucans formed in vitro by *S. ferus* were two- to threefold less than that of *S. sobrinus*, but they were of generally similar linkage composition. Several *S. sanguis* and *S. mitior* strains, like the adherence-defective mutants of *S. sobrinus* (16) and *S. mutans* (31), form an abundance of $\alpha(1\rightarrow6)$ -rich, water-soluble, dextran-like polysaccharides (13). Further-

more, some *S. sanguis* strains have been reported to be cariogenic (21), adherent (36), or both. However, our findings show that the glucan synthesis of *S. ferus* is not *S. sanguis*-like but rather is mutans-like and appears to mediate the adhesion of strains HD3 and 8S1, despite their avirulence.

Neither *S. ferus* strain demonstrated the diversity of glucan-synthetic and -degradative activities of *S. sobrinus* 6715-13, although both HD3 and 8S1 formed the turbid bands, after PAGE, associated with insoluble glucan synthesis. Neither gels of strain 8S1 nor gels of strain HD3 demonstrated the swelling associated with the synthesis of hydrophilic, $\alpha(1\rightarrow6)$ -rich, soluble glucans, nor did the isolates appear to produce endohydrolytic glucanases. Dextranase activity was not detected by agar plate assay (32) nor in sonically disrupted cells (unpublished data). That 8S1 and HD3 agglutinated (spontaneously) and formed adherent plaquelike deposits suggests that dextranase is neither required for $\alpha(1\rightarrow3)$ -rich, plaque-glucan synthesis nor is it the cell surface glucan receptor in agglutination.

Dextran-induced agglutination in vitro has been suggested to be an analog of in vivo plaque formation and virulence-related phenomena (18). However, agglutination-defective mutants of *S. sobrinus* have proven to be virulent (33; unpublished data), whereas nonvirulent mutants remain agglutination competent (16). Although a dextran-binding lectin has been isolated and

TABLE 2. Acid end products produced by *S. sobrinus* 6715-13 and *S. ferus* HD3 and 8S1 cultures shown in Fig. 4

Strain	Sample ^a		mg of acid per ml ^b	
	No.	pH	Lactic	Formic
6715-13	1	5.85	0.98	0.06
	2	4.52	1.62	0.08
	3	4.55	2.34	1.5
	4	4.15	3.15	1.47
HD3	5	5.5	1.14	0.14
	6	4.95	1.55	0.2
	7	4.85	1.64	0.05
	8	4.65	1.91	0.05
8S1	9	5.6	1.23	0.04
	10	5.05	1.47	trace
	11	4.95	1.66	0.33
	12	4.55	1.78	1.7

^a See Fig. 4.

^b GLC analysis and quantitation of acids produced during 24 h of growth in Todd-Hewitt broth.

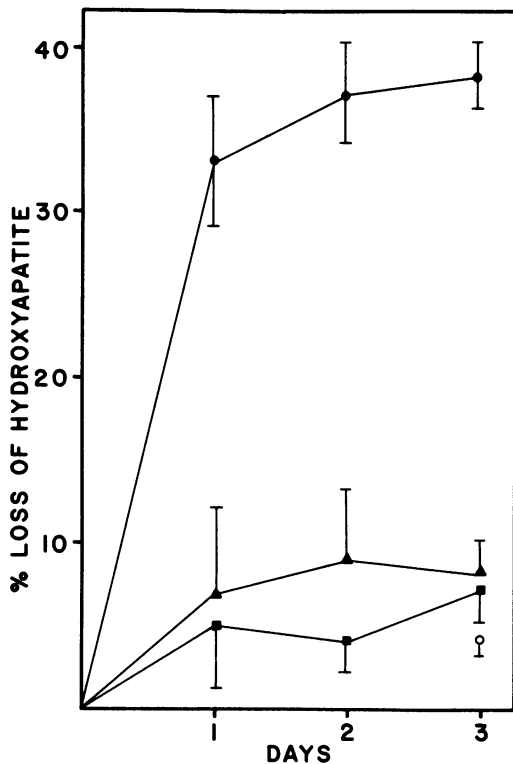


FIG. 5. Solubilization of HA by lactic acid plus formic acid diluted in fresh broth to reflect the metabolic acid concentrations in early-stationary-phase cultures of *S. sobrinus* 6715-13 (●), *S. ferus* HD3 (■), and 8S1 (▲); the control (O) consisted of HA beads in plain broth.

described (29, 30), its absence in mutants has not corresponded well with the loss of ability to bind dextran or to form plaque (M. McCabe, personal communication). Sucrose-induced agglutination which depends on de novo glucan synthesis has also proven to be labile in animal-passaged *S. mutans* (20). Furthermore, in the original descriptions of the phenomenon (18), representative strains of prevalent and virulent *S. mutans* serogenetic group Ic,e (3, 6) agglutinated less strongly than did others, e.g., *S. sobrinus* III,d,g strains. Perhaps of importance is the coincidence that mutans-like *S. ferus* has the *c* antigen (8), and although the cells spontaneously clump, they do not agglutinate more strongly with exogenous dextran.

Measurements of growth and the fermentation characteristics of *S. ferus* give better insight into the reasons for its failure to cause tooth decay despite its sucrose-dependent formation of insoluble glucans and plaque. Although the culture densities of the tested strains doubled at rates comparable to that of 6715-13 in exponential

phase (specific growth rate constants: HD3, 1.00 h^{-1} ; 8S1, 1.1 h^{-1} versus 6715-13, 1.1 h^{-1}), exponential growth of HD3 ceased at 64% of the final culture density of 6715-13, whereas 8S1 stopped at 79%. The culture pH measured close to the points of cessation of rapid growth showed that 6715-13 continued to grow rapidly at one full pH unit lower than the pH at which HD3 and 8S1 grew. Although HD3 did continue to grow slowly for an additional 24 h, 8S1 did not, and neither *S. ferus* strain attained the high final density or low terminal pH of cariogenic 6715-13.

In vitro, washed cells suspended in buffer at pH values from 4.5 to 7 metabolized added carbohydrate to acid. Here the difference in acidogenicity between *S. ferus* strains and *S. sobrinus* was apparent, and greater acidogenicity of *S. sobrinus* was shown at lower pH values. Also, both glucose plus fructose and sucrose were metabolized at similar rates at pH 7 or 4.5 by glucose-cultured *S. sobrinus*, whereas *S. ferus* strains metabolized sucrose more slowly. This perhaps indicates a degree of inducibility in sucrose transport, degradation, or both by *S. ferus* which was not present in *S. sobrinus*. In the *S. ferus* strains, therefore, sucrose had both a lower acidogenic potential and yielded less glucan than in *S. sobrinus*.

At high culture densities, *S. sobrinus* produced more lactic and formic acids than either *S. ferus* isolate. A comparable acidogenic advantage of *S. mutans* PK1 over an *S. sanguis*-type strain has been reported (17) and has been attributed to the greater acidogenicity of the ATP-glucose phosphotransferase system of PK1 (25). The net result of greater acid production by *S. sobrinus* 6715-13 may be more efficient solubilization of the mineral phase of the tooth. This likelihood is illustrated in vitro by the dissolution and loss of HA seen when sequestered, measured quantities were exposed either to spent culture medium or to dilute solutions of the major metabolic acids of cultures of 6715-13, HD3, and 8S1. *S. ferus*, therefore, lies between the *S. sanguis*-*S. mitior* oral bacteria which neither form mutans-like glucans nor are as acidogenic as cariogens such as *S. sobrinus* and virulent mutans streptococci. Because *S. ferus* apparently has evolved the glucans of efficient plaque formation, selection for increased acidogenicity should favor the emergence of the cariogenic potential. Thus, the pathophysiology of the *S. ferus* isolates described above has elements in common with pH mutants of *S. rattus* BHT which are defective in acidogenicity (23; J. Hillman, U.S. patent application 4133875, 1979). These *S. ferus* strains will be tested for their abilities to innocuously occupy the tooth niche of the mutans-type streptococci.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants DE-03758 and DE-04721 from the National Institute of Dental Research.

LITERATURE CITED

- Björndal, H., C. Hellerqvist, B. Lindberg, and S. Svensson. 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. *Angew. Chem.* **9**:610-619.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein type binding. *Anal. Biochem.* **72**:248-254.
- Bratthall, D. 1970. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. *Odontol. Revy* **21**:143-152.
- Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
- Ciardi, J., G. Hageage, and C. Wittenberger. 1976. Multi-component nature of the glucosyltransferase system of *Streptococcus mutans*. *J. Dent. Res.* **55**:C87-C96.
- Coykendall, A. 1974. Four types of *Streptococcus mutans* based on their genetic, antigenic, and biochemical characteristics. *J. Gen. Microbiol.* **83**:327-338.
- Coykendall, A. 1977. Proposal to elevate the subspecies of *Streptococcus mutans* to species status, based on their molecular composition. *Int. J. Syst. Bacteriol.* **27**:26-30.
- Coykendall, A., D. Bratthall, K. O'Connor, and R. Dvarkas. 1976. Serological and genetic examination of some nontypical *Streptococcus mutans* strains. *Infect. Immun.* **14**:667-670.
- Coykendall, A., and M. Freedman. 1981. Colonization and cariogenicity of *Streptococcus ferus* in rats. *Infect. Immun.* **32**:80-85.
- Coykendall, A., P. Specht, and H. Samol. 1974. *Streptococcus mutans* in a wild sucrose-eating rat population. *Infect. Immun.* **10**:216-219.
- Davis, B. 1964. Disc electrophoresis II: methods and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**:404-427.
- Dulbecco, R., and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis virus. *J. Exp. Med.* **99**:167-182.
- Freedman, M., D. Birkhed, A. Coykendall, and D. Rizzo. 1979. Linkage analyses of extracellular glucans from *Streptococcus sanguis* and *Streptococcus mitior*. *Infect. Immun.* **23**:907-909.
- Freedman, M., D. Birkhed, and K. Granath. 1978. Analyses of glucans from cariogenic and mutant *Streptococcus mutans*. *Infect. Immun.* **21**:17-27.
- Freedman, M., and A. Coykendall. 1975. Variation in internal polysaccharide synthesis among *Streptococcus mutans* strains. *Infect. Immun.* **12**:475-479.
- Freedman, M., and J. Tanzer. 1974. Dissociation of plaque formation from glucan-induced agglutination in mutants of *Streptococcus mutans*. *Infect. Immun.* **10**:189-196.
- Futakami, K., S. Sato, and Y. Iwami. 1976. Lactate formation at various pH levels by the wild strain of *Streptococcus mutans* PK1, its variant, and *S. sanguis*. *J. Dent. Res.* **55**:1131.
- Gibbons, R., and R. Fitzgerald. 1969. Dextran-induced agglutination of *Streptococcus mutans* and its potential role in the formation of microbial dental plaque. *J. Bacteriol.* **98**:341-346.
- Gibbons, R., and P. Keyes. 1969. Inhibition of insoluble dextran synthesis and dental caries in hamsters by low molecular weight dextran. *Arch. Oral Biol.* **14**:721-724.
- Gibbons, R., and J. Qureshi. 1980. Virulence-related physiological changes and antigenic variation in populations of *Streptococcus mutans* colonizing gnotobiotic rats. *Infect. Immun.* **29**:1082-1091.
- Guggenheim, B. 1968. Streptococci of dental plaque. *Caries Res.* **2**:147-163.
- Guggenheim, B., and H. Schroeder. 1967. Biochemical and morphological aspects of extracellular polysaccharides produced by cariogenic streptococci. *Helv. Odontol. Acta* **11**:131-152.
- Hillman, J. 1978. Lactate dehydrogenase mutants of *Streptococcus mutans*: isolation and preliminary characterization. *Infect. Immun.* **21**:206-212.
- Holdeman, L., and W. Moore (ed). Anaerobe laboratory manual, 2nd ed., p. 113-115. Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg, Va.
- Iwami, Y., and T. Yamada. 1980. Rate-limiting steps of the glycolytic pathway in the oral bacteria *Streptococcus mutans* and *Streptococcus sanguis* and the influence of acidic pH on the glucose metabolism. *Arch. Oral Biol.* **25**:163-169.
- Jordan, H., R. Fitzgerald, and A. Bowler. 1960. Inhibition of experimental caries by sodium metabisulfite and its effect on the growth and metabolism of selected bacteria. *J. Dent. Res.* **39**:116-123.
- Keyes, P., and H. Jordan. 1964. Periodontal lesions in the Syrian hamster. III: findings related to an infectious and transmissible component. *Arch. Oral Biol.* **9**:337-400.
- Koga, T., and M. Inoue. 1978. Cellular adherence, glucosyl-transferase adsorption, and glucan synthesis of *Streptococcus mutans* AHT mutants. *Infect. Immun.* **19**:402-410.
- McCabe, M., and R. Hamelik. 1978. Multiple forms of dextran-binding proteins from *Streptococcus mutans*. *Adv. Exp. Med. Biol.* **107**:749-759.
- McCabe, M. M., R. Hamelik, and E. E. Smith. 1977. Purification of dextran-binding protein from cariogenic *Streptococcus mutans*. *Biochem. Biophys. Res. Commun.* **78**:273-278.
- Michalek, S., J. Shiota, T. Ikeda, J. Navia, and J. McGhee. 1975. Virulence of *Streptococcus mutans*: biochemical and pathogenic characteristics of mutant isolates. *Proc. Soc. Exp. Biol. Med.* **150**:498-502.
- Staat, R. H., T. H. Gawronski, and C. F. Schachtele. 1973. Detection and preliminary studies on dextranase-producing microorganisms from human dental plaque. *Infect. Immun.* **8**:1009-1016.
- Tanzer, J., and M. Freedman. 1978. Genetic alterations of *Streptococcus mutans* virulence, p. 661-672. In J. McGhee, J. Mestecky, and J. Babb (ed.), *Secretory immunity and infection*. Plenum Publishing Corp., New York.
- Tanzer, J., M. Freedman, R. Fitzgerald, and R. Larson. 1974. Diminished virulence of glucan-synthesis defective mutants of *Streptococcus mutans*. *Infect. Immun.* **10**:197-203.
- Terleckyj, B., N. Willett, and G. Shockman. 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. *Infect. Immun.* **11**:649-655.
- Westergren, G., and M. Freedman. 1979. Comparative studies of two mutants of the mouth *Streptococcus sanguis* with different colonial morphologies and abilities to adhere. *Arch. Oral Biol.* **24**:667-672.
- Zacharius, R. M., T. E. Zell, J. H. Morrison, and J. J. Woodlock. 1969. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **30**:148-152.