

Growth of Oral *Streptococcus* Species and *Actinomyces viscosus* in Human Saliva

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Microorganisms in dental plaque live in constant association with saliva. The role of saliva in the adherence of bacteria to the teeth and the antibacterial properties of saliva have been well investigated; less interest has been shown in the possible role of saliva as a substrate for oral microorganisms. In this study it was shown that saliva can serve as a growth medium for oral *Streptococcus* spp. and *Actinomyces viscosus*. The cell production of these organisms on saliva was carbohydrate limited. The doubling times for growth on glucose-supplemented saliva (4 to 5 mmol/liter) ranged from 1.6 to 4.0 h. The availability of carbohydrate sources for the oral microflora is discussed in relation to microbial growth in the oral cavity.

Dental plaque is a complex microbial ecosystem in which facultative anaerobic bacteria such as *Streptococcus* and *Actinomyces* species are predominant in addition to anaerobic species such as *Veillonella* and various gram-negative rods. Saliva is known to have a number of functions in the mouth, most of which are protective of the teeth and the soft tissues. The microorganisms in dental plaque live in constant association with saliva, and many studies have dealt with the role of saliva in the adherence of bacteria to teeth or the antibacterial properties of saliva (12, 19).

Less interest has been shown in the growth of the oral microflora and the possible role of saliva as a substrate, yet the rapid accumulation of bacteria on tooth surfaces (21), even in the absence of diet (1), indicates that a continuous supply of nutrients supports the growth of the oral microflora. Possible sources of nutrients for bacteria in the mouth include the host diet, saliva, crevicular fluid, and desquamated epithelial cells. Due to its chemical composition and its continuous production of about 800 ml/day (19), saliva is likely to be one of the main substrates. It has been observed that in vitro various oral bacteria can grow to a certain extent in saliva (20, 24) or on agar medium prepared from heated saliva (17). Cowman et al. (4, 7) reported that, although saliva could not serve as a sole source of nutrients, various oral streptococci utilized specific proteins from saliva as a nitrogen source. Data from Brecher and van Houte (2) showed some increase in the number of *Actinomyces viscosus* cells during overnight incubation in rat saliva. From these in vitro data it would seem that the growth of oral streptococci and *A. viscosus* in saliva is limited by some essential nutrient. From the high protein to carbohydrate ratio in saliva (15) it seems logical to assume that the growth-limiting substrate is carbohydrate.

To test this assumption we studied the growth of some oral streptococci and *A. viscosus* in human whole saliva with and without the addition of glucose.

MATERIALS AND METHODS

Microorganisms. The strains used in this study were *Streptococcus mutans* Ingbritt (Krasse, Malmö, Sweden), *Streptococcus sanguis* P₄A₇ (Weerkamp, Nijmegen, The Netherlands), *Streptococcus milleri* 675B₁ (Huis in 't Veld, Utrecht, The Netherlands), and *A. viscosus* Ut2 (Van Palen-

stein Helderman, Utrecht, The Netherlands). All strains were originally isolated from the human oral cavity. The strains were kept in skim milk (Difco Laboratories, Detroit, Mich.) at -80°C.

Media and culture conditions. The following liquid media were used: TPY broth supplemented with 0.2% glucose (Trypticase peptone; BBL Microbiology Systems, Cockeysville, Md.), 20 g/liter, yeast extract (Difco), 10 g/liter, and D(+)-glucose · H₂O (analyzed reagent; J. T. Baker Chemical Co., Phillipsburg, N.J.), 2 g/liter; and sterile mixed human saliva supplemented with glucose. Saliva used for the pH-stat experiments was supplemented with 4 to 5 mmol of glucose per liter; saliva used for the batch culture experiments was supplemented with 0.0, 0.5, 1.0, 2.5, 5.0, or 10.0 mmol of glucose per liter. The free glucose in saliva with no added glucose was determined by a fluorometric method (13) and was found not to exceed 8 μmol/liter. All cultures were incubated at 37°C under anaerobic conditions in an atmosphere of N₂ (90%), CO₂ (6%), and H₂ (4%).

Preparation of sterile saliva. Mildly stimulated saliva was collected from the members of the laboratory staff by chewing on paraffin wax. The saliva samples were pooled and diluted 1:1 with demineralized (sterile) water; subsequently, 2.5 mmol of dithiothreitol (BDH Biochemicals, Poole, England) was added. The saliva was then centrifuged at 48,000 × g at 4°C for 10 min. The supernatant was sterilized by ultrafiltration, first through a 0.45-μm prefilter (Nuclepore Corp., Pleasanton, Calif.) and subsequently in small portions through sterile disposable 0.2-μm filters (Schleicher & Schüll, Dassel, West Germany). Without the addition of dithiothreitol, ultrafiltration was strongly hampered by gel formation of the salivary mucins. Glucose was added from a 10 times concentrated stock solution before filtration. For all experiments freshly collected saliva was used. The sterility of the saliva was verified by incubating a small portion anaerobically for 48 h at 37°C.

Precultures. The microorganisms were grown anaerobically at 37°C on TPY with 0.2% glucose until their mid-logarithmic growth phase (8 h). The cells were then harvested by centrifugation, washed thrice, and finally suspended in an equal volume of 0.85% (wt/vol) NaCl. Tubes containing 5 ml of saliva plus glucose were inoculated with 0.5 ml of the washed cell suspension. These saliva precultures were incubated anaerobically for 16 h at 37°C and used as an inoculum for the pH-stat and batch culture experiments.

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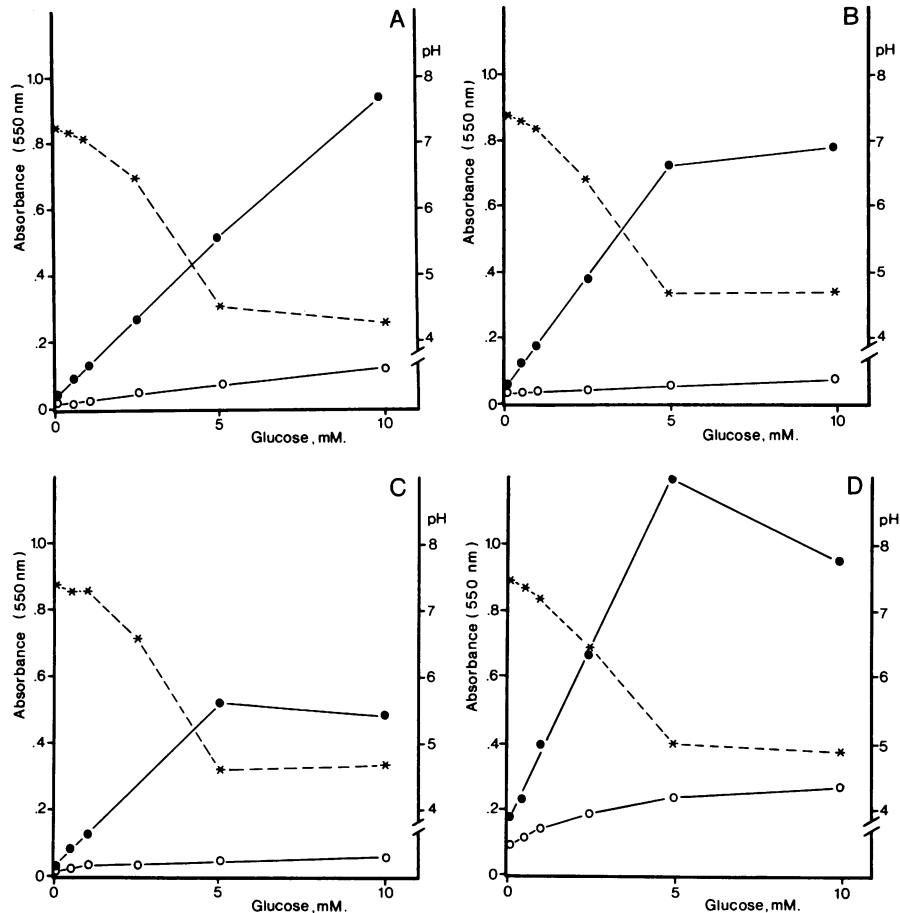


FIG. 1. Relationship between maximum cell production (A_{550}) and glucose concentration in saliva. (A) *S. milleri* 675 B1; (B) *S. sanguis* P4A7; (C) *S. mutans* Ingbritt; (D) *A. viscosus* Ut2. Symbols: \circ , A_{550} , 0h; \bullet , A_{550} , 24 h; *, pH, 24 h.

pH-stat experiments. pH-stat experiments were done at 37°C in 150-ml vessel under a constant flow of sterile anaerobic gas (10 ml/min). The pH was kept at 7.0 by automatic titration with 0.1 N KOH; the alkali consumption was recorded automatically. The pH-stat was inoculated with 5 to 10 ml from a preculture grown on saliva. At regular intervals 3-ml samples were removed via a sample port using a sterile syringe. A 0.5-ml portion of the sample was centrifuged for 3 min at $18,000 \times g$. The supernatant was used to determine the remaining glucose and the fermentation products; the pellet was used to determine bacterial DNA in the culture. The cells in the remaining part of the sample were dispersed ultrasonically for 30 s at 0°C using a Kontes K-881440 cell disruptor provided with a microtip. Immediately after sonication the absorbance at 550 nm (A_{550}) of the culture was measured.

Batch cultures. Five-milliliter portions of sterile saliva with increasing glucose concentrations were poured into sterile 20-ml screw-cap tubes. The tubes were inoculated with 0.5 ml of a preculture on saliva with an identical glucose concentration and incubated anaerobically at 37°C. Tubes were taken for sampling at 0, 24, and 48 h after inoculation. In these samples the pH and the A_{550} were determined after sonication. In none of the samples could cell lysis be detected by phase-contrast microscopy.

Analysis. The residual glucose concentration in the cul-

tures was determined by a fluorometric method with NADP⁺ (13). DNA was assayed fluorimetrically with ethidium bromide (10). DNA from calf thymus (Boehringer, Mannheim, West Germany), was used as a standard. Acidic bacterial fermentation products were analyzed using isotachoforesis (11, 23). The leading electrolyte was 5 mmol of HCl-4-aminobutyric acid (pH 4.1) per liter, and the terminating electrolyte was 4 mmol of octanoic acid-NaOH (pH 5.0) per liter. Separation was performed at a constant current of 50 μ A. Ethanol was determined by gas chromatography.

RESULTS

To obtain reproducible growth of these organisms on saliva it was found to be critical to inoculate the precultures in saliva with cells from the exponential growth phase. The A_{550} measurements of cultures in saliva, despite ultrasonic dispersion, were somewhat more erratic than those of common broth media, due to the presence of aggregates of cells. The optical densities of the test organisms attained in saliva with increasing amounts of added glucose are given in Fig. 1. There was a linear increase of the absorbance up to a glucose concentration of 5 mmol/liter for three of the test strains, whereas *S. milleri* 675B1 showed an increase up to at least 10 mmol of glucose per liter. When *S. milleri* 675B1, *S. sanguis* P4A7, *S. mutans* Ingbritt, and *A. viscosus* Ut2 were grown in saliva supplemented with 4 to 5 mmol of glucose per liter at a

constant pH of 7.0, the optical density and the DNA content of the saliva cultures increased in reasonable agreement with exponential growth. The glucose concentration decreased corresponding with the growth of the microorganisms to a final concentration below 0.05 mmol/liter. Doubling times calculated from logarithmic plots of the A_{550} were 1.6, 2.5, and 4.0 h for *S. sanguis* P4A7, *S. milleri* 675B1, and *A. viscosus* Ut2, respectively. The doubling time of *S. mutans* Ingbritt was calculated from the increase of DNA in the culture and was found to be 3.0 h. The acidic fermentation products of the cultures grown at a constant pH of 7.0 are listed in Table 1. *S. mutans* Ingbritt, *S. sanguis* P4A7, and *S. milleri* 675B1 produced essentially lactic acid during growth in glucose-enriched saliva. *A. viscosus* Ut2 initially produced mainly lactic acid, but at later stages of the growth a shift toward formic, succinic, and acetic acids was observed.

DISCUSSION

The data in Fig. 1 show that the cell production of oral streptococci and *A. viscosus* in saliva was linearly related to the glucose concentration. This result extends the observation of Cowman et al. (4) that sterile saliva can serve as a nitrogen source but not as an energy source for the growth of oral streptococci. Except for *S. milleri*, growth in saliva was likely pH stopped at glucose concentrations above 5 mmol/liter (Fig. 1). The cell production of the streptococcal strains in various batches of saliva was found to be very reproducible. However, *A. viscosus* Ut2 showed more variable yields. At the moment this variability is not understood. The low free glucose concentration ($8 \pm 2 \mu\text{mol/liter}$) in unsupplemented sterilized saliva accounts for the low cell production of the cultures without glucose (Fig. 1).

In parotid saliva glucose concentrations ranging from 5 to 40 $\mu\text{mol/liter}$ (14) up to 100 $\mu\text{mol/liter}$ (9) have been reported. This amounts, at most, to a daily secretion of 14 mg of free glucose in 800 ml of saliva. However, starting from 10^9 bacteria per ml of saliva, the estimated production of microbial biomass in the oral cavity is 100 to 250 mg/day. Obviously the amount of free glucose in saliva is insufficient to account for this biomass production. Therefore, it must be assumed that other carbon and energy sources are used in addition. Potential carbohydrate sources *in vivo* are (i) free glucose in fresh glandular saliva, (ii) the host diet, and (iii) carbohydrates released from salivary glycoproteins.

The contribution of carbohydrates from the host diet to microbial growth in the oral cavity is not well understood, but there is evidence that carbohydrate is a growth-limiting substrate for oral streptococci, except for short periods after carbohydrate intake by the host (22). It has been shown that the carbohydrates from salivary glycoproteins are rapidly elaborated by microorganisms from dental plaque (5, 6, 16, 18). Their contribution to biomass production in the oral cavity is as yet unknown. It has recently been shown that some oral microorganisms can also use amino acids as a carbon and energy source (8; J. S. van der Hoeven, M. H. de Jong, A. H. Rogers, and P. J. M. Camp, *J. Dent. Res.*, in press).

In the pH-stat experiments in saliva the oral streptococci and *A. viscosus* Ut2 were grown under an excess of glucose (4 to 5 mmol/liter). Lactic acid was the only fermentation product of the streptococci, whereas *A. viscosus* produced formic, acetic, and succinic acids in addition to lactic acid (Table 1). This corresponds with fermentation patterns of these microorganisms under conditions of glucose excess (3; van der Hoeven et al., in A. C. R. Dean and D. C. Ellwood, ed., *Continuous Cultivation of Micro-organisms*, in press).

TABLE 1. Fermentation products after growth in saliva with added glucose at a constant pH of 7.0

Microorganism	Time (h)	Acid production (mmol/liter)				C recovery ^a (%)
		Formic	Acetic	Lactic	Succinic	
<i>S. mutans</i> Ingbritt	24	— ^b	—	4.8	—	59
<i>S. sanguis</i> P4A7	24	—	—	4.2	—	53
<i>S. milleri</i> 675 B1	24	—	—	7.5	—	94
<i>A. viscosus</i> Ut2	6	0.3	0.2	0.8	0.0	
	24	0.6	0.6	2.1	0.3	
	54	1.9	1.4	3.4	1.4	64

^a Carbon recovery, calculated on the basis of fermentation products formed from the glucose added to the culture (streptococci, 4 mmol/liter; *A. viscosus*, 5 mmol/liter).

^b Not produced; ethanol was not found in any of the samples.

Carbon recoveries for *S. mutans* and *S. sanguis* calculated from the fermentation products in saliva (Table 1) were slightly lower than those observed in glucose-limited continuous cultures (van der Hoeven et al., in Dean and Ellwood, in press). This is likely due to the production of intracellular polysaccharide by these organisms in the pH-stat cultures. The doubling times observed in saliva supplemented with 4 or 5 mmol of glucose per liter were similar to those observed during the initial colonization of the dentition of gnotobiotic rats (1). These doubling times are independent on whether the rats were starved or fed a sucrose-containing diet. Thus, it would seem that fresh glandular saliva contains sufficient nutrients, including carbohydrates (9, 14), to account for the high growth rates in the initial phase of the colonization of the dentition, when the total number of microorganisms on the teeth is still small. At later stages of colonization the doubling times increase as carbon and energy sources become growth limiting.

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