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Growth Rates of *Actinomyces viscosus* and *Streptococcus mutans* During Early Colonization of Tooth Surfaces in Gnotobiotic Rats

H. J. A. BECKERS* AND J. S. VAN DER HOEVEN

Institute of Preventive and Community Dentistry, University of Nijmegen, Nijmegen, The Netherlands

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Germfree Osborne-Mendel rats were monoassociated with *Actinomyces viscosus* or *Streptococcus mutans*. The adherence and subsequent growth of these organisms on the tooth surface was studied by means of total viable cell counts. Both *A. viscosus* and *S. mutans* showed a lag phase and an exponential growth phase, similar to logarithmic growth in batch cultures. The exponential growth rates of *S. mutans* and *A. viscosus* were 0.63 h^{-1} (doubling time [t_d] = 1.1 h) and 0.24 h^{-1} (t_d = 2.9 h), respectively. After a period of rapid growth, the rate declined and the populations approached a steady state. The presence of a sucrose-containing diet did not significantly influence the exponential growth rates of *A. viscosus* and *S. mutans*, but had a slight negative effect on the initial adherence of *S. mutans* at the tooth surface.

The survival of a bacterial species in a natural habitat depends on its ability to grow at a rate sufficient to balance cell death and loss of cells from the population. The growth of bacteria is governed by the availability of nutrients, as is expressed by Monod's (20) empirical equation for specific growth rate, which has served as a basis for ecological studies. Bacteria respond to fluctuations in the environment by changing their metabolism. They have developed different strategies with respect to the utilization of various nutrients and the concentrations thereof (18). The overall result of the physiological adaptation of the cells is reflected by changes of the growth rate. The growth rate is an important factor in the competetiveness of a bacterium (1).

Chemostat studies of phenotypic responses of bacteria to the environment may enable the investigator to predict the outcome of the competition of two or more species under a given set of conditions (13, 33). However, such information is generally insufficient to explain the behavior of these bacteria under natural conditions. In particular, it is extremely difficult to explain the population dynamics in complex heterogeneous ecosystems, such as dental plaque, from in vitro observations only. Complementary results can be obtained from direct observations on natural ecosystems or simplified in vivo model systems. Little data are available on growth rates of bacteria in dental plaque. Generation times of 8 to 12 h for mature plaque have been suggested by Gibbons (10), based on data of Krasse (14). In artificial fissures, Mikx and Svanberg (19) suggested a mean generation time of 6 to 14 h, whereas Socransky et al. (26) reported bacterial generation times of 3 to 4 h in the early phase of plaque formation.

In terms of population dynamics, dental plaque can be considered a dividing transit population (Fig. 1e). The three processes, settling, dividing, and decaying, regulate the extent of the dental plaque in space and time. Depending on the relative rates of the processes of settling and decaying, the dividing transit population merges into the other three types of growing populations (Fig. 1f, g, and h).

A steady state may be reached in mature dental plaque, which means that the processes of settling, decaying, and cell division are in equilibrium. When the processes of settling and decaying are negligible compared with the dividing process, the population can be considered a closed dividing (exponential) one. In nature, an exponential dividing population will only occur transiently. Socransky et al. (26) showed a phase of rapid growth of bacteria in early dental plaque on a cleaned tooth surface. These observations suggested that early dental plaque behaves like a closed dividing population.

We have estimated growth rates of bacteria during early colonization of the tooth surfaces in gnotobiotic rats. To this end, germfree rats were inoculated with *Streptococcus mutans* or *Actinomyces viscosus*, and the increase of the populations of these organisms on the teeth was measured by total viable cell count. The effect of the host's diet on the population increase was evaluated by comparison of bacterial growth in starved and fed rats.

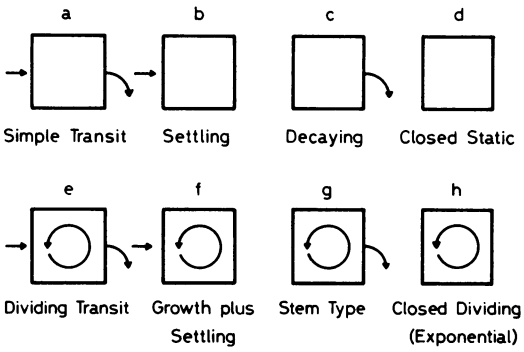

FIG. 1. Diagrammatic representation of possible cell populations. Arrows outside the boxes indicate cell migration; arrows inside the boxes refer to cell divisions taking place within the system. (Reproduced with permission from Brock [3]).

MATERIALS AND METHODS

Microorganisms. *A. viscosus* strain Ny1 (29) and *S. mutans* strain T2 (23) were used in this study. The strains were kept lyophilized in stock.

The inocula were prepared from 16-h cultures of strain Ny1 in actinomyces broth (BBL Microbiology Systems) or strain T2 in brain heart infusion broth (Difco Laboratories) supplemented with 2% (wt/vol) glucose. The bacterial cultures were ultrasonically dispersed with a Branson Sonifier, type B-12, twice for 15 s each at 15-s intervals and with continuous cooling in ice (microwave tip, 75-W ouput). The dispersed cultures were used to inoculate the rats. Viable counts of the inocula were estimated on Trypticase soy agar (BBL) plates supplemented with 1% (wt/vol) yeast extract and on blood agar plates. The inocula contained about 10^{10} viable cells per ml.

Animals and Treatments. Germfree Osborne-Mendel rats, 36 to 45 days old, from six litters were randomly distributed among four groups. Groups 1 and 2, consisting of 12 and 15 rats, respectively, were inoculated with *A. viscosus* Ny1. Groups 3 and 4 consisted of 15 and 21 rats, respectively, and were inoculated with *S. mutans* T2. The rats were housed in plastic isolators containing Macrolon cages.

The rats were individually inoculated with 100 μl of the bacterial suspension. The inocula were applied with a 1-ml hypodermic syringe. The diet was administered at the time of inoculation. The rats were observed to eat from about 30 min after inoculation.

Before the inoculations all rats were starved for 24 h, with only drinking water available. The rats in groups 1 and 3 continued to starve for an additional period of 24 h, during which bacterial growth was monitored. A sucrose-containing diet was available ad libitum to the rats in groups 2 and 4. In these groups the growth of the inoculated strains was monitored for 3 and 6 subsequent days, repectively. The composition of the diet was as follows: sucrose, 16%; wheat flour, 44%; skim milk powder, 32%; yeast extract, 7%; and vegetable oil, 1%. The diet was sterilized with 2.5 Mrads of gamma irradiation.

Bacteriological procedures. After inoculation, samples were taken at the intervals noted in Fig. 2. Each sample consisted of specimens from three rats.

The method used for determination of the colonization of the teeth by bacteria was a modification of the procedure described by van Houte et al. (31). The rats were killed, and the molars of the lower jaw were extracted with a dental probe. The right and left halves of the lower jaw were treated as separate samples. The molars were ground in a mortar in 0.5 ml of 0.85% saline. After the mortar was rinsed with an additional 0.5 ml of saline, the suspension was transferred into a sterile tube and dispersed for 30 s with a Kontes E/MC Sonifier. The undiluted suspension and suitable dilutions were plated onto blood agar plates. The plates were incubated for 48 h at 37°C in an atmosphere of 91% N_2–5% CO_2–4% H_2.

RESULTS

Initial growth. The number of viable cells on molar teeth at increasing time intervals after inoculation are shown in Fig. 2A and B for *A. viscosus* and *S. mutans*, respectively. These curves demonstrate several distinct growth phases. Immediately after inoculation, the viable counts decreased until a minimum level was reached after about 2 h. The decrease then turned into a fast increase, and rapid growth proceeded for 48 h. Next, the rate of increase leveled off, and the number of viable counts approached a maximum value. In contrast to the results found for *A. viscosus* in both groups and *S. mutans* in sucrose-fed rats, no initial decrease of *S. mutans* was observed in starved rats during the first 2 h (Fig. 2).

Doubling times of bacterial populations. From the increase in total viable counts (Fig. 2), the doubling times of the populations of *A. viscosus* and *S. mutans* adhering to the tooth surface were caculated for several intervals during growth. The calculated doubling times are shown in Table 1. In the period of 6 to 24 h, the growth of *A. viscosus* in the fedgroup proceeded with a doubling time of 2.9 h. In the period from 24 to 48 h, its growth continued with a slightly longer doubling time of 3.9 h. The >1,000-fold increase of *S. mutans* between 2 and 12 h in the fed group corresponds to a doubling time of 1.1 h.

S. mutans showed a doubling time of 7.5 h in the period of 12 to 48 h. After 2 days, the populations of *A. viscosus* or *S. mutans* approached maximum values with concomitant increases of the calculated doubling times. The differences in doubling times found between the fed and starved groups were not statistically significant. Because no 12-h sample was available, it is not known whether the doubling time of *A. viscosus* was shorter during the first 12 h than in the period thereafter, as was the case for *S. mutans*.

Adherence. The number of cells attached to

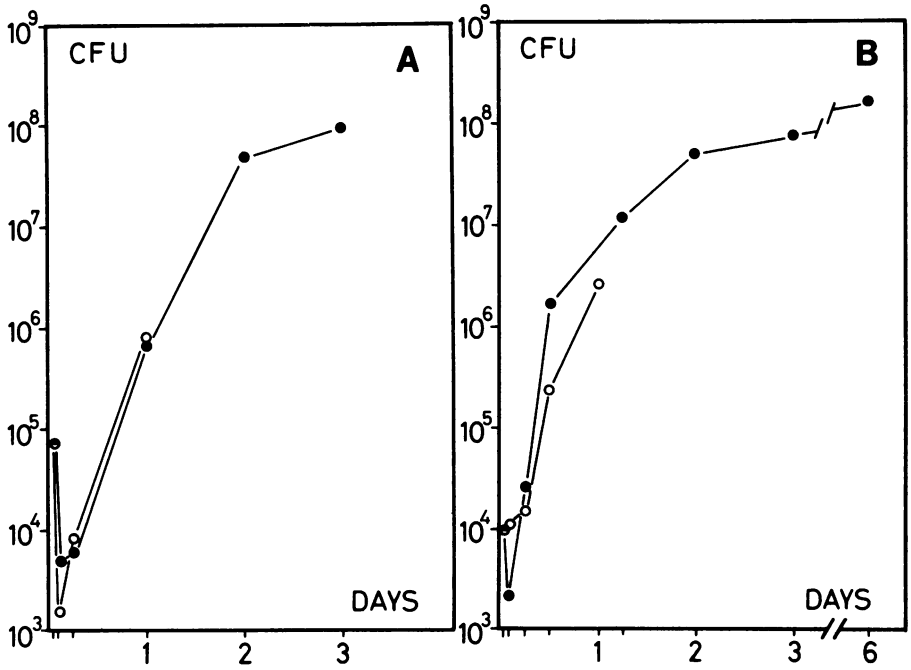

FIG. 2. Viable counts of *A. viscosus* Ny1 (A) and *S. mutans* T2 (B) on molar teeth of gnotobiotic rats. Symbols: (●) sucrose fed; (○) starved. CFU, Colony-forming units.

the molar teeth reached a minimum level of about 2 h after inoculation (Fig. 2; Table 2). At that time growth did not seem to have started, and the loosely bound cells had been lost from the site. The number of adherent cells at 2 h after inoculation likely represented firmly attached cells. No significant differences could be demonstrated in the numbers of *A. viscosus* that were found to adhere in starved or fed rats. The number of adherent *S. mutans* cells found in starved rats was significantly higher than that in sucrose-fed-rats (Table 2).

Establishment after 3 days. The number of bacteria approached a maximum value 3 days after inoculation (Fig. 2). In spite of different growth patterns, the populations of *A. viscosus* and *S. mutans* had reached about the same sizes at 3 days after inoculation (Fig. 2).

DISCUSSION

In this study the initial growth rates of *S. mutans* and *A. viscosus* were estimated in gnotobiotic rats to avoid the effect of intermicrobial interactions upon growth rate. The differences between our data and those reported by Socransky et al. (26) and Mikx and Svanberg (19) may well reflect the influence of microbial interaction on growth rate. The kinetics of the initial growth of bacterial populations on the tooth surface were found to be similar to the logarithmic growth in batch cultures. After the lag phase, there is an exponential increase in cell numbers followed by a decline in growth rate. The decline is likely to reflect the change from a closed dividing transit system (3) (Fig. 1), wherein a steady state is reached. The rate of the exponential increase in cell number is surprisingly high, which reflects an environment that is relatively rich in nutrients. This is supported by the observation that the supply of external nutrients from the host's diet did not significantly

TABLE 1. Doubling times of *A. viscosus* and *S. mutans* in gnotobiotic rats

Bacterial strain	Group	Period of increase ($t_1 - t_2$; h)	Doubling time (h)[a]
A. viscosus Ny1	Sucrose diet	6–24	2.9 ± 0.3
		24–48	3.9 ± 0.3
		48–72	24 ± 1
	Starved	2–24	2.8 ± 0.2
S. mutans T2	Sucrose diet	2–12	1.1 ± 0.2
		12–48	7.5 ± 0.5
		48–72	38 ± 6
		3–6 days	66 ± 7
	Starved	6–12	1.3 ± 0.2
		12–24	4.4 ± 0.8

[a] Doubling time = $[(t_2 - t_1)0.3010]/(\log N_2 - \log N_1)$, where N_2 = mean number of cells at time t_2.

TABLE 2. Initial adherence of *A. viscosus* Ny1 and *S. mutans* T2 to molar teeth in germfree rats 2 h after inoculation

Organism	Group	No. of adherent cells ± SE/10^6 inoculated cells
A. viscosus Ny1	Starved	2 ± 1
	Sucrose diet	5 ± 2
S. mutans T2	Starved	16 ± 4[a]
	Sucrose diet	3 ± 2[a]

[a] $P_t \leq 0.05$.

alter the rate of the exponential growth. It seems that the growth rate has reached a maximum value that is not dependent upon the concentration of limiting substrates. This maximum growth rate can only be reached at the early stages of the formation of a microbial film on the tooth. With increasing thickness of the film, the growth rate decreases due to substrate limitations (22).

The size of the population on the tooth surface is influenced by various processes, including division of cells, decaying due to release of cells or cell death, and settling as a result of the adherence of cells from the saliva.

In the early stage of plaque development, during the period of rapid population increase, the numerical contribution of settling and decaying to the cell numbers is likely to be small compared with the contribution of cell division. As a consequence, the doubling times of the populations calculated for this period may well reflect true rates of cell division. At a later stage, when a steady state is reached, the processes of settling, decaying, and cell division will be in equilibrium.

The substrates for bacterial growth in the mouth arise from saliva, the gingival crevice fluid, and epithelial cells that are shed into the saliva (17). Because of its relative abundance, composition, and continuous production (17), saliva seems to be the main source of nutrients. It is known that *S. mutans* can utilize salivary proteins as a sole source of nitrogen (6) whereas *A. viscosus* may grow slowly in saliva (2). The utilization of various salivary components by these organisms has not been studied yet in sufficient detail to explain their rapid growth on the tooth surfaces. However, in the mouth, macromolecules from saliva become quickly adsorbed to the enamel surfaces (16) and bacteria adhere to the macromolecular layer by various mechanisms (24). According to ZoBell (34) attached bacteria have at their disposal more substrates than do free-floating cells and consequently can grow more rapidly. The mechanisms involved include accumulation of substrate and degradation and utilization of the adsorbed macromolecules by exoenzymes. Esterman and McLaren (8) showed an enhanced protein degradation by extracellular proteinases of strains of *Flavobacterium* and *Pseudomonas* when the proteins were adsorbed by clay minerals. The influence of the presence of solid surfaces on bacterial growth was also demonstrated by Filip (9). He found that the growth of compost bacteria was accelerated by the presence of solid particles such as glass beads; this effect was associated with the surface activity of the particles rather than with an oxidative power or a nutrition factor from the particles.

Intracellular polysacharide is another substrate that could advance the initial growth. Depending on the culture conditions, *S. mutans* (32) and *A. viscosus* (unpublished data) produce a considerabale amount of intracellular polysaccharides. The contribution of intracellular polysaccharides to the increase in cell mass will be low. Assuming a molar growth yield of about 20 to 30 g (dry weight) per mol of glucose (28), it will be clear that to double the cell mass a multiple of the dry weight in the form of glucose is needed.

There is considerable evidence that sucrose promotes the *S. mutans* population on the tooth surface (4, 15, 31). As the initial growth rate of the cells was not influenced by a sucrose-containing diet, other mechanisms are likely more important in the stimulatory effect of sucrose. Sucrose can favor the accumulation of *S. mutans* by various mechanisms, including dextran- or glycosyl transferase-mediated adherence (12, 21). In complex microflora sucrose might sustain the competitive ability of *S. mutans*, because this organism has a high-affinity transport system for sucrose (25, 27), which enables the bacterium to capture low concentrations of sucrose from the environment.

To survive in the oral cavity, bacteria must be able to adhere to the tooth surface or to the soft tissues in the oral cavity. *A. viscosus* and *S. mutans* preferentially adhere to the tooth surface (2, 7, 11, 30). The bacteria in the 2-h samples represent cells that were firmly attached to salava-coated enamel while loosely bound cells were being released into the saliva. No significant difference in initial adherence was found between *A. viscosus* and *S. mutans* in the fed group. For *A. viscosus* no effect of sucrose on the initial adherence was found. Sucrose seemed to have a slight negative effect on the adherence of *S. mutans*. This corroborates the results of Clark and Gibbons (5), who showed that *S. mutans* cells, exposed to sucrose to permit extracellular polysaccharide synthesis before or during adsorption, tended to attach in fewer numbers to saliva-treated hydroxyapatite

surfaces than did cells in the absence of sucrose. Two days after inoculation, growth declined (Fig. 2) and the populations approached a steady state. The increase in population density and the increase in thickness of the bacterial layer will induce substrate limitations in the bacterial layer and consequent reduction in growth rates. Little information is available on generation times in mature dental plaque. Gibbons (10) has argued that steady-state growth proceeds with a mean generation time of 8 to 12 h.

Study of the growth rates of steady-state populations requires special methods, as pointed out by Brock (3). The estimation of steady-state growth rates of microorganisms in dental plaque may help to explain their ecological relationships.

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