Accumulation of Streptococci on Teeth in a Laboratory Microcosm (Artificial Mouth)

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The accumulation on teeth of three strains of oral bacteria, 'Streptococcus mitior' LPA–1, Streptococcus mutans C67–1, and Streptococcus rattus BHT, was studied from 8 to 90 hours after inoculation. Direct and viable counts demonstrated that strains BHT and C67–1 accumulated more rapidly than strain LPA–1, with fastest growth during the first 16 hours ($t_{d8-16 h}$ 3·6–4·8 h). In older cultures, S. mutans and S. rattus continued to accumulate and a high proportion of the cells were viable. In contrast, 'S. mitior' cultures contained many non-viable organisms, possibly due to hydrogen peroxide-induced death. When S. rattus was co-cultured with 'S. mitior' it was strongly inhibited during the first 24 hours, but increased in proportion after 66 and 90 hours. It was concluded that in the interaction between 'S. mitior' and S. rattus during initial plaque development, a potentially rapid initial growth rate was of less importance than the ability to antagonise the other organism.

KEY WORDS — *Streptococcus mutans*; *Streptococcus rattus*; *'Streptococcus mitior'*; Oral streptococci; Growth on teeth; Model mouth; Microbial interactions.

INTRODUCTION

Dental plaque is a surface-associated microbial film in an open ecosystem. It is subjected to a fluctuating nutrient supply from the saliva, crevicular fluid, and the host diet. Under these conditions the behaviour of organisms is likely to differ from that of bacteria in batch or continuous cultures,[18] and an artificial mouth apparatus has been developed as an appropriate model of the microbial environment on teeth.[11]

'Streptococcus mitior' is probably the most commonly isolated streptococcal species from dental plaque.[12] It has been shown in previous studies that 'S. mitior' can inhibit the mutans streptococcus S. rattus BHT by production of hydrogen peroxide when co-cultured for 90 hours in the model mouth.[8] In addition, pure cultures of 'S. mitior' are highly acidogenic in this model, and apparently produce larger quantities of acid than equivalent cultures of S. rattus BHT.[7]

Mutans streptococci, i.e. Streptococcus mutans and those streptococci with a similar phenotype, are of particular interest because of their acidogenicity

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and association with dental caries, yet it is not understood how these organisms can become established in a potentially inhibitory plaque. Events during the initial period of colonisation of a tooth surface may be crucial in determining whether any one particular organism is more successful than another. In particular, growth rate may be an important ecological factor in microbial accumulation.[1]

Pure culture studies enable growth rate to be determined in the absence of microbial interactions. The aim of the present study, therefore, was to determine the growth rate and viability of 'S. mitior' LPA–1 and S. rattus BHT, when grown in pure and mixed culture on teeth, in order to assess the possible role of these factors in the ecology of developing plaque. S. mutans C67–1 was included for purposes of comparison, as it is a more typical example of the mutans streptococci found in human dental plaque.[4]

MATERIALS AND METHODS

Microorganisms

'S. mitior' LPA–1, Carlsson's strain 44,[5] produces extracellular polysaccharide. The taxonomy of this group is still the subject of debate, but due to its

familiarity in the dental literature, the name '*S. mitior*' is used in this study. Both strain LPA–1 and *S. rattus* BHT[19] have been described previously.[8] Strain BHT was formerly known as a serotype b strain of *S. mutans*. *S. mutans* C67–1 is a strain which closely resembles the type strain, NCTC 10449.[6] All three strains were originally human oral isolates but have been maintained for several years in the laboratory.

Cultural Conditions

The model mouth described by Hudson *et al.*[11] was used, but without pH electrodes. Methods were similar to those described previously.[8,11] Bacteria were grown on sound, human upper premolar crowns, sliced into halves and mounted back to back to provide a stagnation site. Six replicate culture flasks were generally used per experiment. The assembled apparatus, consisting of reservoirs, tubing, flasks and teeth, was steam-sterilised (134°C for 6 min). Fluids were filtered into the reservoirs using Duropore membranes (Millipore [UK] Ltd, Harrow, Middlesex).

A synthetic saliva based on bovine submaxillary glycoprotein[14] was supplied continuously at 0·5 ml/hour. This was delivered to the teeth as separate drops which were retained there until displaced by the next drop. Preliminary work showed that drops fell on the teeth at intervals of 5·08 min (SE 0·31) when saliva only was being supplied. An additional nutrient supplement, SBM broth, was supplied intermittently, for one hour every six hours, at 0·5 ml/hour, and was first available to the organisms eight hours after inoculation. SBM broth contained (g/l): Bacto-casitone (Difco) 20·0; yeast extract (Oxoid) 5·0; NaCl 2·0; K_2HPO_4 4·0; KH_2PO_4 1·0; pH7·0. After sterilisation (15 min at 121°C) glucose was added from a separately sterilised (10 min at 110°C) 50 per cent (w/v) stock solution to give a final concentration of 1 per cent (w/v). Culture flasks were maintained at 35°C via their water-jackets, and the apparatus was swept with 5 per cent (v/v) CO_2 in air or nitrogen, at 7–14 kPa (1–2 psi). Conditions were not strictly anaerobic with the N_2/CO_2 gas mixture,[8] as fluids in reservoirs were not deoxygenated and the tubing is permeable to gases.

Inoculation

Bacterial strains were maintained by weekly sub-culture on brain heart infusion (Oxoid) agar plates.

For inocula, organisms were grown in brain heart infusion broth for 24 hours at 35°C. From these, rapidly-growing (4 hour) broth cultures were prepared and diluted with fresh, warm broth to give similar numbers of organisms. Inocula were always counted and contained 10^8–10^9 CFU/ml. Before inoculation, the gas and pump were switched on and the synthetic saliva allowed to drop over the teeth for approximately 30 min. A single inoculum (1·5 ml) of a pure culture or freshly-prepared mixture of equal volumes of two strains, was delivered rapidly (30 secs) to each tooth via the vaccine cap, using a hypodermic syringe.

Sampling and Counting Procedure

Teeth were removed at 8, 16, 24, 48, 66 or 90 hours after inoculation. Organisms were released into bottles of 10 ml 1/4 strength Ringer's solution (Oxoid) by placing containers in an ultrasonic cleaning bath for 2 min. Direct cell and CFU counts were performed on the bacterial suspensions using a counting chamber. The Miles and Misra method was used for viable counts, which were performed in triplicate on SBM agar containing 0·5 per cent (w/v) glucose. Differential *S. rattus* counts were obtained using agar with 250 µg/ml streptomycin. Plates were incubated for 48 hours in an atmosphere of 10 per cent (v/v) carbon dioxide and 90 per cent (v/v) hydrogen.

Analysis of Data

Before any calculations were performed, bacterial counts were converted to $\log_{10}$ values to ensure normal distribution of the data. The mean and SEM were determined and Student's t was used as a measure of statistical significance ($P < 0·05$ was deemed to be significant). Bacterial doubling time (t_d) was determined using the formula:

$$t_d = (t_2 - t_1) \log_{10} 2 / \log_{10} N_2 - \log_{10} N_1 \quad (1)$$

The standard error of the mean difference ($S_{\bar{D}}$) was calculated,[15] also the median t_d from all possible combinations of pairs of teeth at t_1 and t_2.

RESULTS

Total Counts

Direct counts demonstrated that there was little clumping of organisms and the mean size of colony-forming units was always less than two bacteria. In
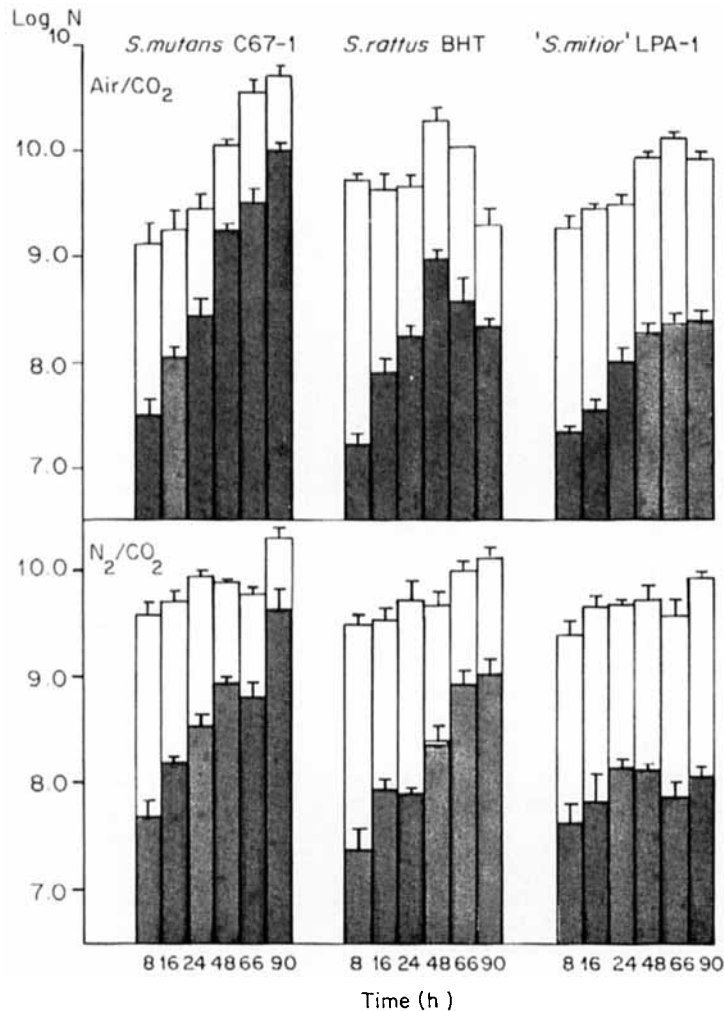
Figure 1. Log_{10} direct and viable count (+SEM) of *Streptococcus mutans* C67–1, *Streptococcus rattus* BHT and '*Streptococcus mitior*' LPA–1 grown in pure culture on teeth in a model mouth

☐ direct count
■ viable count

all cultures there was evidence of an increase in the total number of organisms on teeth during the experimental period (Fig. 1). This was particularly pronounced with *S. mutans* C67–1, and the *S. rattus* BHT cultures with the N_2/CO_2 gas mixture, where accumulation continued up to 90 hours after inoculation. The total numbers of '*S. mitor*' LPA–1 on teeth showed a less pronounced increase with time and had stabilised by 24–48 hours after inoculation. *S. rattus* aerobic cultures showed a drop in direct count between 48–90 hours ($P < 0.001$).

Viable Counts of Pure Bacteria

Similar numbers of viable organisms were recovered from all pure cultures 8 hours after inoculation (Fig. 1). However, populations subsequently increased at differing rates. *S. mutans* and *S. rattus* increased most rapidly between 8 and 16 hours after inoculation (Table 1). *S. rattus* had a faster t_d with the air/CO_2 gas mixture ($P < 0.02$), and accumulated more rapidly between 8–16 hours than *S. mutans* ($P < 0.02$). '*S. mitor*' increased at a slower

Table 1. Minimum initial bacterial doubling time (hours) of *Streptococcus mutans* C67–1, *Streptococcus rattus* BHT and '*Streptococcus mitior*' LPA–1, growing in pure or mixed culture on teeth in the model mouth

Gas Mixture		Bacterial Strain			
		S. mutans C67–1 (8–16)	*S. rattus* BHT (8–16)	'*S. mitior*' LPA–1 (16–24)	'*S. mitior*' + *S. rattus* (8–16)
Air/CO_2	Mean	4·38	3·56	5·33	5·67
	($S_{\bar{D}}$)	(1·50)	(1·48)	(1·47)	(1·21)
	Median	4·64	3·55	5·43	6·40
	(SE median)	(1·29)	(1·20)	(1·22)	(1·11)
	n	4	4	3	4
N_2/CO_2	Mean	4·76	4·25	7·68	11·81
	($S_{\bar{D}}$)	(1·42)	(1·66)	(1·72)	(1·36)
	Median	4·63	5·69	7·91	21·14
	(SE median)	(1·26)	(1·28)	(1·56)	(1·47)
	n	4	4	4	4

n = Number of replicate teeth.
S_D = Standard error of the mean difference.

rate than either of the mutans streptococci ($P < 0.001$), and its fastest t_d occurred 16–24 hours after inoculation.

The '*S. mitior*' viable population continued to accumulate slowly under aerobic conditions but remained stable from 24–90 hours when flushed with N_2/CO_2. Aerobically-cultured *S. rattus* attained its maximum population after 48 hours and then declined. However, under partially anaerobic conditions, *S. rattus* continued to accumulate for 66 hours and then stabilised. Viable *S. mutans* increased in numbers throughout the whole 90 hour experimental period, with either gas mixture. After 90 hours *S. mutans* had attained a larger population than '*S. mitior*' ($P < 0.001$) or *S. rattus* ($P < 0.005$).

Viability of Pure Cultures

The ratio of viable to the direct bacterial count was determined (Fig. 2) to give an indication of the viability of organisms recovered from teeth. With all cultures there was a general trend for the proportion of viable organisms to increase with incubation time. The ratio of viable *S. mutans* was generally higher than in any of the other cultures. The proportions of viable *S. mutans* and *S. rattus* increased substantially during the experiment, up to 21 per

cent and 11 per cent respectively (Fig. 2). The proportion of viable organisms was low (1–3 per cent) in cultures of '*S. mitior*'.

Mixed Cultures

When '*S. mitior*' and *S. rattus* were co-cultured on teeth (Fig. 3), doubling times were slow, especially with the N_2/CO_2 gas mixture (Table 1). Under aerobic conditions, numbers of viable *S. rattus* decreased during the initial 24 hours and its proportion of the total viable count (Table 2) fell from 3 per cent at 8 hours to < 0.1 per cent at 24 hours ($P < 0.005$). It subsequently slowly increased in proportion to 0·3 per cent by 66–90 hours. When the apparatus was flushed with N_2/CO_2, proportions of *S. rattus* were 2–40 times greater ($P < 0.005$). A similar pattern was seen, however, of initial decline followed by an increase, in this case, up to 12 per cent by 90 hours. This difference between aerobic and partially anaerobic cultures at 90 hours was statistically highly significant ($P < 0.001$).

The viability in mixed cultures was only 1–2 per cent (Fig. 2). It was clear, however, that from 8 to 24 hours there was a higher proportion of viable organisms in mixed culture under partially anaerobic conditions, when compared with aerobic cultures
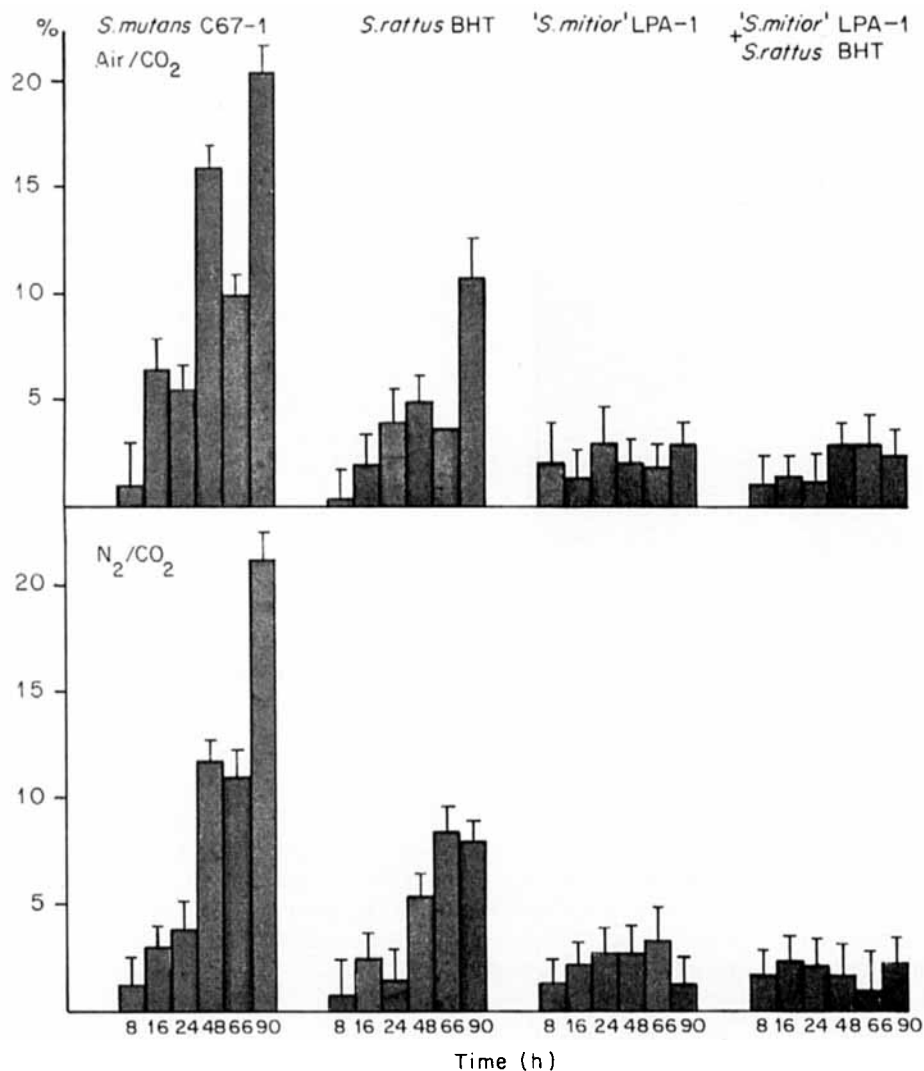
Figure 2. Proportion (%) (+SEM) of viable count compared with the direct count of streptococci grown in pure or mixed culture on teeth in a model mouth

($P<0\cdot01-0\cdot05$). In a pilot experiment with lactoperoxidase included in the saliva,[8] the proportion of viable organisms in aerobic culture at 24 hours was approximately doubled ($P<0\cdot01$).

DISCUSSION

As both direct and viable counts increased with time, it was assumed that bacterial growth occurred on the tooth surfaces. The results with pure cultures enable the potential colonisation ability of each organism to be determined in the absence of heterogeneous microbial interactions. It is of interest,

therefore, that both strains of mutans streptococci examined had a faster initial growth rate than '*S. mitior*' LPA–1, and that *S. mutans* C67–1 continued to accumulate throughout the whole experimental period.

Initial doubling times (t_d) were comparable with those obtained using animal models. For example, *S. mutans* T2, in starved gnotobiotic rats, had a $T_{d12-24h}$ of 4·4 hours,[1] and *S. mutans* C67–1 inoculated into rats with a conventional flora[2] had a t_{d6-24h} of 4·5 hours. Naturally-occurring strains of *S. mutans* had a t_{d6-18h} of 3·2 hours when re-colonising the developmental grooves of the
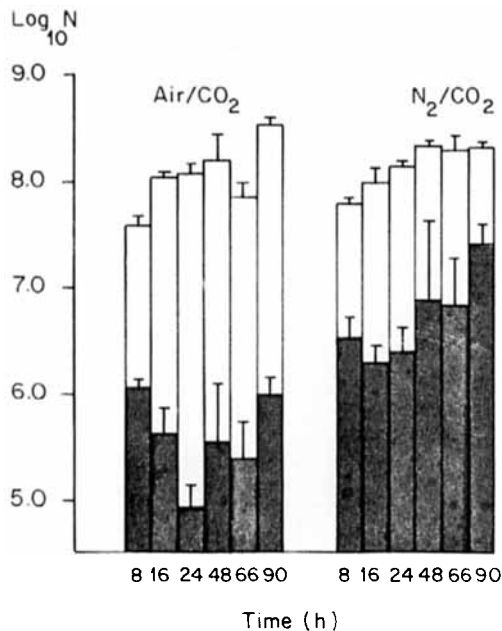
Figure 3. Log_{10} viable count ($+$ SEM) of 'Streptococcus mitior' LPA–1 and Streptococcus rattus BHT grown in mixed culture on teeth in a model mouth

☐ 'S. mitior' + S. rattus viable count
■ differential count of S. rattus

first deciduous molars of conventional macaque monkeys.[3] These results suggest, therefore, that the microbial environment on tooth segments in the model mouth is indeed analogous to that found in vivo, and that the artificial mouth is an appropriate model for the study of dental plaque ecology.

Examination of direct and viable counts enables the proportion to be established. This gives an indication of the viability of the culture, although it is recognised that the use of microscopic counts in this way can give rise to unexpectedly low estimates.[13] The data have been presented, however, as they enable useful comparisons to be drawn. There is also a possibility of non-viable bacteria being washed off the surface, or of being autolysed. This may have led to the values of microbial viability being over-estimated in the case of the aerobic 90 hour S. rattus culture. Otherwise, this does not appear to have been a problem, as the highest proportions of viable organisms were found on teeth with the largest direct counts.

The provision of a glucose-containing nutrient pulse every 6 hours enables all three strains to produce acid, with Stephan-like curves of pH-

fall.[7,10,16] Comparison of viable counts leads to the conclusion that the more rapid and extensive pH-fall noted previously in young cultures of 'S. mitior'[7,10,11] was due to the greater acidogenicity of this strain rather than to any difference in population size. After about 60 hours, however, all three organisms, in pure culture, are exposed to acid for prolonged periods, as the pH does not return to neutrality before the next glucose pulse.[10] The high proportions of viable mutans streptococci recovered after 66–90 hours may thus reflect their greater acid tolerance.

Another factor to be considered is hydrogen peroxide (H_2O_2) production. 'S. mitior' LPA–1 produces H_2O_2 in the model mouth with either gas mixture[8] and the low proportion of viable organisms may be due to self-inhibition. The decline in older aerobic S. rattus cultures may also be due to self-inhibition by H_2O_2, as this strain produces detectable quantities, at least in cell suspensions.[17] The low proportion of S. rattus when co-cultured with 'S. mitior' is assumed to be due primarily to the H_2O_2 which is produced by strain LPA–1 on teeth.[8] This is supported by the observation that the proportion of S. rattus is lower under aerobic conditions, with a minimum value at 24 hours, just after the 'S. mitior' main phase of growth. The increased viability at 24 hours in the presence of lactoperoxidase (LPO) provides indirect evidence for H_2O_2 toxicity, as inclusion of LPO in the saliva removes H_2O_2 by the production of bacteriostatic hypothiocyanite ($OSCN^-$). The high proportion of S. rattus in 90 hour mixed cultures under partially anaerobic conditions appears to reflect the greater importance of acid tolerance in conditions with limiting H_2O_2 levels.

In conclusion, it is not known whether other strains of the three organisms examined here would exhibit similar differences in accumulation and viability, although it seems likely, as the extent of H_2O_2 production and aciduricity are characteristics common to each species. Therefore, considering the initial stages of the establishment of organisms on teeth, the present study suggests that the potential for rapid initial growth by the mutans streptococcus BHT was not sufficient to ensure substantial accumulation when the organisms were co-cultured with a slower-growing H_2O_2-producing strain of 'S. mitior'. However, after prolonged incubation, with regular glucose supplements, it appears that the combined effects of 'S. mitior' self-inhibition, plus the greater aciduricity of the mutans streptococcus, led to an increase in the proportion of S. rattus on

Table 2. Proportion (per cent) (SEM) of *Streptococcus rattus* BHT differential viable count in the model mouth, when co-cultured with '*Streptococcus mitior*' LPA-1

Gas Mixture		Time since inoculation (h)					
		8	16	24	48	66	90
Air/CO_2		2·9	0·4	0·1	0·2	0·3	0·3
		(1·3)	(1·7)	(1·6)	(2·0)	(2·8)	(1·4)
	n	4	4	4	3	6	11
N_2/CO_2		5·2	1·9	1·7	3·3	3·7	11·5
		(1·4)	(1·1)	(1·9)	(4·9)	(2·1)	(1·4)
	n	4	4	4	2	11	11

n = number of replicate teeth.

teeth. This is currently being examined in the model mouth, using *S. mutans* C67–1 and a mixture of typical plaque bacteria.[9]

ACKNOWLEDGEMENT

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