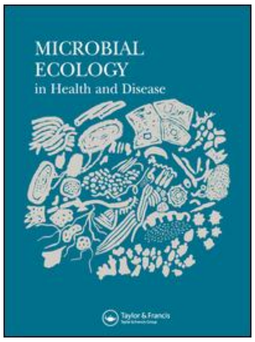

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Post-hydrogen Peroxide Effect in Peroxidogenic Oral Streptococci

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The effects of inhibitory concentrations of hydrogen peroxide on the growth of 11 strains of four peroxidogenic species of oral streptococci (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sanguis* and *Streptococcus sobrinus*) were studied. The effect of H_2O_2 was measured as the post-hydrogen peroxide effect (PHPE), defined as the difference in the time necessary for the bacterial population in batch culture to increase by one decimal logarithmic unit of the number of colony forming units per millilitre, between cultures exposed to a concentration equal to the corresponding minimum inhibitory concentration of H_2O_2, and non-exposed cultures. No PHPE was shown by *S. oralis* NCTC 11427; other strains tested gave times ranging from 20 min (*S. sanguis* JENA 2697) to 9 h 15 min (*S. mitis* OGS 232). The PHPE appears to be strain- and species-dependent.

KEY WORDS—Hydrogen peroxide; Oral streptococci; Post-hydrogen peroxide effect.

INTRODUCTION

Several species of oral streptococci produce hydrogen peroxide,[9,11,16,17,23] and the ability of saliva to inhibit bacterial growth has been traced to hydrogen peroxide produced by these microorganisms.[10,13,14,21,24] The toxic action of H_2O_2 on mammalian cells has been shown with cell culture techniques,[1,12] and salivary lactoperoxidase may play a role in the detoxication of the oral cavity.[4,20] Other studies have investigated the relation between the peroxidogenic capacity of oral streptococci and the available substrate, such as glucose.[14,19,22]

In streptococci lacking cytochromes and catalase,[7,19] flavoproteins such as NADH-oxidase[7] make oxygen consumption possible, and give rise to superoxide ions, hydrogen peroxide or water. Of these metabolites, the first two are highly toxic to the microorganism, and are rapidly removed from the environment by superoxide dismutase[5,8] and NADH-peroxidase[6] respectively. However, at certain concentrations, H_2O_2 inhibits the growth of oral streptococci, and higher concentrations can be bactericidal. The effect of inhibitory concentrations on the recovery of these microorganisms has not been clearly documented, but is a factor that needs to be taken into account in attempts to explain the selective function of H_2O_2. As a strong but unstable oxidant,[15,18] the '*in vivo*' action of H_2O_2 is likely to be transient. It is therefore of interest to study the ability of oral streptococci exposed briefly to H_2O_2 to recover their optimal growth rate.

The present study investigated the post-hydrogen peroxide effect (PHPE) on the development of peroxidogenic species equipped with NADH-peroxidase and NADH-oxidase,[25] as a factor that may influence the interrelations in the oral cavity.

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MATERIALS AND METHODS

Microorganisms tested

The reference strains were *Streptococcus oralis* NCTC 11427, *S. mitis* NCTC 3161, *S. sanguis* NCTC 7863 and *S. sanguis* JENA 2697. Autochthonous strains from the collection of the Microbiology Laboratory, University of Granada Hospital, were *S. mitis* OGS 218, *S. mitis* OGS 232, *S. mitis* OGS 628, *S. sobrinus* OGS 415, *S. sobrinus* OGS 324, *S. sobrinus* OGS 529 and *S. mitis* OGS 420 (OGS = *Odontología Granada Streptococcus*). These strains were identified according to the criteria described by Hardie[11] and Loesche.[16]

Minimum inhibitory concentration and minimum bactericidal concentration

Trypticase soy broth (TSB) (Scott 4900-5207) was used in all assays. Commercial hydrogen peroxide (Panreac 141076), 30 per cent wt/vol., was titrated according to the technique of Bernt and Bergmeyer,[2] and used to prepare serial double dilutions in tubes containing 5 ml TSB. The final dilutions ranged from 900 to 1·46 μg/ml; some intermediate dilutions were also used with *S. mitis* OGS 420. The tubes were inoculated with 0·1 ml of bacterial suspension (turbidity 0·5 on the Macfarland scale), viable counts ranged from 10^6 to 10^7 c.f.u./ml determined by counting in plate, prepared from logarithmic growth phase cultures in TSB.

Colony forming units from control (TSB without H_2O_2) and test (TSB with H_2O_2) tubes were counted on Mitis Salivarius Agar (MSA, Difco 0298-01-0) plates; after 24 h anaerobic incubation (Heraeus 433 incubator, PA 262) at 36 ± 1°C.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of H_2O_2 in cultures of peroxidogenic oral streptococci

Microorganism	MIC H_2O_2 (μg/ml)	MBC H_2O_2 (μg/ml)
S. oralis NCTC 11427	7·0	14·1
S. mitis NCTC 3161	7·0	14·1
S. sanguis JENA 2697	7·0	14·1
S. sanguis NCTC 7863	7·0	14·1
S. mitis OGS 218	14·1	28·2
S. mitis OGS 232	14·1	28·2
S. mitis OGS 628	14·1	28·2
S. sobrinus OGS 324	14·1	28·2
S. sobrinus OGS 415	14·1	28·2
S. sobrinus OGS 529	7·0	14·1
S. mitis OGS 420	2·3	3·5

All experiments were performed in triplicate.

Evaluation of post-hydrogen peroxide effect

Automatic method. To tubes containing 5 ml TSB and H_2O_2 at the MIC for each strain tested was added 0·5 ml of a bacterial suspension (Turbidity 0·5 on the Macfarland scale), with viable counts ranging from 10^6 to 10^7 c.f.u./ml. Controls without H_2O_2 were also prepared for each strain. All tubes were incubated for 1 h at 36 ± 1°C, after which 2 ml of the contents were transferred to automatic analysis bottles (Organon Teknika 52269) and placed in a BacT/Alert incubator-reader (Organon Tecknika Microbial Detection System 031BT5024). This automated system continuously detects positive samples as they appear, based on the change in the pH of the medium resulting from bacterial acid production.

The PHPE was defined as the difference between time to detection of growth in cultures exposed to H_2O_2 and in non-exposed cultures.

Viable counts method. Tubes containing TSB, H_2O_2 and bacterial suspension were prepared as described above for automatic detection, and incubated for 1 h at 36 ± 1°C. The contents of each tube were added to 100 ml Erlenmeyer flasks containing 75 ml TSB, and incubated at 36 ± 1°C. Periodically, 2 ml aliquots were taken to prepare serial dilutions ranging from 10^{-1} to 10^{-10}, depending on the duration of incubation. The dilutions were used to inoculate Mitis Salivarius Agar plates, which were then incubated for 48 h at 36 ± 1°C anaerobically. After counting the numbers of colonies, the concentration of c.f.u. per millilitre of original culture was calculated conventionally.

The PHPE was defined as the difference in time needed for the number of c.f.u. per millilitre to increase by one decimal logarithmic unit between unexposed cultures and cultures incubated in the presence of H_2O_2 at the level of the MIC for the particular strain.

RESULTS

Table 1 shows the MIC and MBC of H_2O_2 for the different strains of oral streptococci assayed. Three values for MIC were obtained: 2·3 μg/ml (*S. mitis* OGS 420), 7·0 μg/ml (*S. oralis* NCTC 11427, *S. mitis* NCTC 3161, *S. sanguis* JENA 2697, *S. sanguis* NCTC 7863, *S. sobrinus* OGS 529), and 14·1 μg/ml for the remaining five strains.

The times until growth in unexposed cultures and cultures exposed to H_2O_2, together with the PHPE measured with the automatic system (BacT/Alert), are shown in Table 2. The figures given are the means (± standard deviations) of triplicate determinations. The PHPE ranged from 52 min in *S. mitis* OSG 420 to 6 h 31 min in *S. mitis* strains OSG 232 and OSG 628. Little or no concordance between this method and the results obtained after viable counts was observed (Table 3). The only strain that showed no delay in PHPE after exposure was *S. oralis* NCTC 11427; all other strains showed PHPE

Table 2. Recovery times of cultures of peroxidogenic oral streptococci after transferral to medium with (T_1) or without H_2O_2 (T_2), determined automatically (BacT/Alert). The post-hydrogen peroxide effect (PHPE) was calculated by subtracting T_2 from T_1

Microorganism	T_1 (h)	T_2 (h)	PHPE (h)
S. oralis NCTC 11427	9:41 ± 0:18*	6:38 ± 0:22	3:03 ± 0:11
S. mitis NCTC 3161	7:41 ± 0:33	6:00 ± 0:25	1:41 ± 0:16
S. sanguis JENA 2697	8:01 ± 0:23	6:50 ± 0:18	1:11 ± 0:20
S. sanguis NCTC 7863	11:22 ± 0:35	9:10 ± 0:15	2:12 ± 0:10
S. mitis OGS 218	14:52 ± 0:35	9:51 ± 0:28	5:01 ± 0:18
S. mitis OGS 232	14:51 ± 0:40	8:20 ± 0:31	6:31 ± 0:25
S. mitis OGS 628	18:12 ± 0:38	11:41 ± 0:25	6:31 ± 0:22
S. sobrinus OGS 324	10:22 ± 0:42	7:50 ± 0:21	2:32 ± 0:15
S. sobrinus OGS 415	13:52 ± 0:39	9:30 ± 0:32	4:22 ± 0:26
S. sobrinus OGS 529	10:42 ± 0:45	8:21 ± 0:35	2:21 ± 0:26
S. mitis OGS 420	9:52 ± 0:30	9:00 ± 0:35	0:52 ± 0:13

All experiments were performed in triplicate.
*Standard deviations.

Table 3. Recovery times of cultures of peroxidogenic oral streptococci after transferral to medium with (T_1) or without H_2O_2 (T_2), determined by counting viable cells method. The post-hydrogen peroxide effect (PHPE) was calculated by subtracting T_2 from T_1

Microorganism	T_1 (h)	T_2 (h)	PHPE (h)
S. oralis NCTC 11427	4:30 ± 0:21*	4:35 ± 0:20	−0:05 ± 0:10
S. mitis NCTC 3161	5:40 ± 0:35	4:50 ± 0:25	0:50 ± 0:15
S. sanguis JENA 2697	5:50 ± 0:38	5:30 ± 0:31	0:20 ± 0:10
S. sanguis NCTC 7863	9:20 ± 0:42	4:50 ± 0:22	4:30 ± 0:20
S. mitis OGS 218	6:10 ± 0:26	2:40 ± 0:11	3:30 ± 0:16
S. mitis OGS 232	15:30 ± 0:50	6:15 ± 0:34	9:15 ± 0:29
S. mitis OGS 628	10:30 ± 0:45	6:35 ± 0:35	3:55 ± 0:22
S. sobrinus OGS 324	8:30 ± 0:43	3:45 ± 0:12	4:45 ± 0:22
S. sobrinus OGS 415	6:10 ± 0:32	1:50 ± 0:09	4:20 ± 0:25
S. sobrinus OGS 529	7:20 ± 0:35	4:30 ± 0:16	2:50 ± 0:13
S. mitis OGS 420	4:00 ± 0:18	3:00 ± 0:10	1:00 ± 0:08

All experiments were performed in triplicate.
*Standard deviations.

ranging from 20 min (*S. sanguis* JENA 2697) to 9 h 15 min (*S. mitis* OGS 232).

The growth curves of the four different strains, based on counts of viable cells in control cultures and cultures exposed to H_2O_2, are shown in Figure 1a–d.

DISCUSSION

The colonisation of different habitats in the oral cavity by streptococci is influenced by many factors,[3] including the effects of growth-inhibiting substances such as hydrogen peroxide.[13,14,22,24] This

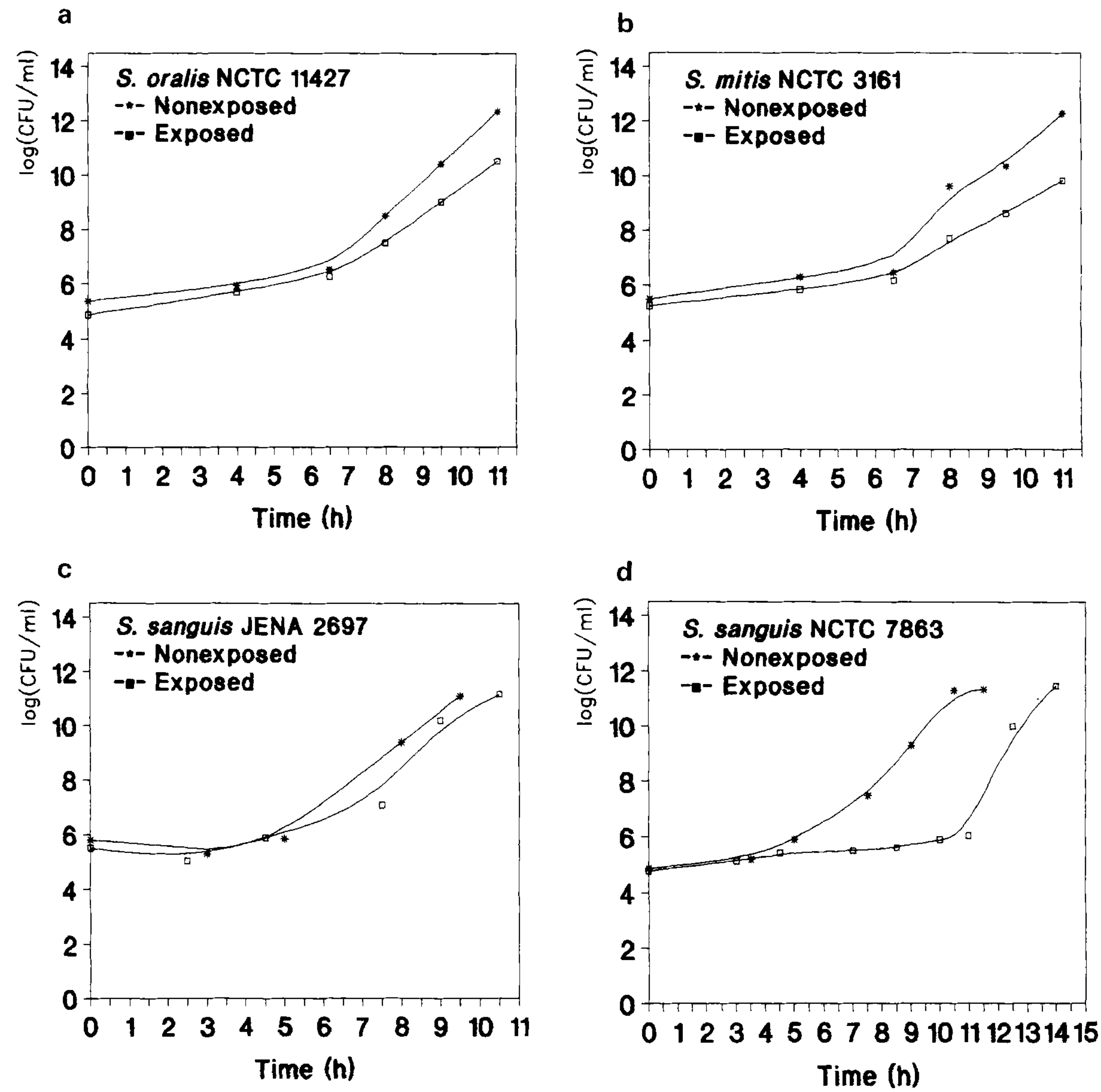

Figure 1a–d. Post-hydrogen peroxide effect in four strains exposed and non-exposed to the minimum inhibitory concentration of H_2O_2

metabolite modifies the development of some peroxidogenic strains themselves, and of strains equipped with NADH-peroxidase. The effect of H_2O_2 is influenced by the amount excreted, and by the microorganism's resistance. The range of concentrations of H_2O_2 able to inhibit growth of peroxidogenic streptococci '*in vitro*' is narrow, except in the case of *S. mitis* OGS 420. Bactericidal concentrations of H_2O_2 were usually double the inhibitory concentrations. It is difficult to relate these findings with the situation in the open ecosystem of the oral cavity in living organisms, where there are countless interrelations between microbes, saliva and cells.[3] Moreover, in quantitative terms at least, the peroxidogenic capacity of oral streptococci appears to be strain or species dependent. For example, *S. sanguis* NCTC 7863 produces relatively little H_2O_2 (0·84 μg/ml) in comparison with *S. oralis* NCTC 11427 (6·7 μg/ml) (unpublished data). Because H_2O_2 is labile and readily inactivated in the environment, '*in vivo*' antibacterial activity is likely to be transient, possibly causing a lag in the growth of bacteria located near the H_2O_2 producer. Hydrogen peroxide may thus be an important ecological factor in the oral cavity: strains that are more resistant to the effects of H_2O_2 may well have an advantage in the competition for a specific habitat.

The findings obtained with the automated system of growth detection differed markedly from those obtained by periodic counting of viable cells method. The automated method detects the decrease in pH caused by bacterial acid production, whereas the viable counts depends on the appearance of c.f.u. on culture plates. Although the latter method is considerably more labour intensive, the data generated are more informative, allowing the evaluation of growth curves, a feature not available with the automated method.

When determined on the basis of viable cell counts, the effect of H_2O_2 on growth was apparent in all strains except the highly peroxidogenic (unpublished data) *S. oralis* NCTC 11427. It seems reasonable to assume that this strain is able to withstand concentrations of H_2O_2 approaching that of the H_2O_2 it excretes. The effects of exposure to H_2O_2 varied widely in the other strains, suggesting that peroxidogenic oral streptococci respond differently to H_2O_2 depending on the species and strain.

In conclusion, growth of 10 of 11 peroxidogenic oral streptococci studied was affected by exposure to the MIC (for a given strain) of H_2O_2. This effect may well influence microbial colonisation of oral habitats, and have some bearing on the interrelationships between bacteria in the mouth.

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