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Repressed Respiration of Oral Streptococci Grown in Biofilms

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Abstract. The respiratory activities of oral streptococci grown in biofilms were found to be markedly repressed compared with those of cells from aerobic culture, or for *Streptococcus mutans* GS-5, even for those grown in static culture. Respiration rates generally reflected levels of NADH oxidase activities in cell extracts. Superoxide dismutase levels were somewhat reduced in biofilm cells. However, sensitivities to oxidative damage caused by H_2O_2, *t*-butylhydroperoxide, or 8-hydroxyquinoline were not greatly different for cells from suspension cultures and those from either intact or dispersed biofilms. The capacities of *S. sanguis* and *S. gordonii* to produce H_2O_2 also were markedly repressed by biofilm growth, and presumably this repression would affect the ecology of dental plaque by reducing oxidative stresses under crowded conditions.

Oral streptococci are capable of high levels of respiration involving mainly NADH oxidases and a number of other enzymes in certain species, for example, pyruvate oxidase or glycerophosphate oxidase in organisms such as *Streptococcus sanguis* and *S. mitis* [16]. These enzymes are cytoplasmic and are not connected to electron transport systems capable of oxidative phosphorylation. The need for respiratory activity unconnected to oxidative phosphorylation is somewhat of a mystery. Two functions have been proposed relative to the ecology of dental plaque. One, proposed mainly for the form of NADH oxidase that catalyzes 4-electron reduction of O_2 to yield H_2O, is in reducing O_2 levels in the environment to diminish types of O_2 metabolism producing reactive oxygen species such as hydrogen peroxide or superoxide radical [5]. For organisms such as *S. mutans*, this function could be paramount because the predominant form of NADH oxidase in the organism is the water-yielding form. *Streptococcus mutans* is not a net producer of H_2O_2 during respiration, although it does have AhpCF (alkyl hydroperoxide reductase) proteins and has capacity both to produce and degrade H_2O_2 [11]. The other function for NADH oxidase is an aggressive one. The H_2O_2-yielding form of the enzyme catalyzes production of peroxide, which could then be converted to toxic products to damage other organisms in mixed populations. In the mouth, the toxic products may include hypothiocyanite produced by salivary peroxidases reacting with H_2O_2 and thiocyanate, although there is debate about which is more toxic, H_2O_2 or $OSCN^-$ [15]. Oral streptococci that are net producers of H_2O_2 include *S. gordonii*, *S. sanguis*, and *S. oralis* [12, 14]. Generally, net producers of H_2O_2 are more peroxide resistant than are non-producers.

Most bacteria in nature live in communities; for example, in biofilms. Dental plaque is a good example of this biofilm mode. Living conditions in plaque are crowded in that some 50% of the volume of plaque is made up of bacteria. The rest is mainly hydrated intercellular polysaccharides. Thus, for studying the respiratory capacities of oral streptococci, it is reasonable to use biofilms rather than suspension cultures. In this paper, we consider respiration by oral streptococci in mono-organism biofilms grown in batch cultures with excess carbohydrate and in aerated or static suspension cultures. Clearly, the biofilms used do not mimic the diversity of plaque communities but do mimic the crowded conditions. In addition, information is presented on levels of protective enzymes in biofilm cells compared with cells grown in suspension cultures, and the new information is related to current views of the roles of respiration and oxidative stress in the ecology of dental plaque.

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Table 1. Respiration of intact cells and biofilms and NADH oxidase and superoxide levels in cell extracts of oral streptococci grown under a variety of conditions

Organisms	Growth mode	NADH-oxidase activity (Units/mg protein)	SOD activity (Units/mg protein)	Respiration rate (nmole O_2/min/mg cell dry weight)
S. mutans GS-5	aerobic	1.300 ± 0.060 [0.0114][a]	152.047 ± 27.590 [0.0011]	19.200 ± 3.500 [0.0004]
	static	0.136 ± 0.015 [0.0567]	36.070 ± 1.071 [0.0004]	6.576 ± 1.819 [0.0323]
	biofilm	0.031 ± 0.020	14.713 ± 0.881	2.267 ± 0.262
S. mutans UA159	aerobic	0.185 ± 0.041 [0.0010]	71.983 ± 2.368 [0.0010]	4.233 ± 0.795 [0.0064]
	static	0.109 ± 0.008 [0.0001]	20.333 ± 7.507 [0.3142]	2.300 ± 0.141 [0.0004]
	biofilm	0.013 ± 0.002	13.267 ± 1.522	1.210 ± 0.070
S. sanguis NCTC 10904	aerobic	0.007 ± 0.001 [0.1388]	20.833 ± 6.712 [0.0922]	0.977 ± 0.162 [0.1919]
	static	0.045 ± 0.012 [0.0723]	6.013 ± 0.389 [0.0529]	2.233 ± 0.377 [0.0608]
	biofilm	0.017 ± 0.009	9.447 ± 1.539	1.333 ± 0.287
S. gordonii ATCC 10558	aerobic	0.015 ± 0.002 [0.2203]	76.730 ± 10.522 [0.0017]	1.567 ± 0.368 [0.4301]
	static	0.026 ± 0.002 [0.0169]	11.783 ± 1.065 [0.1969]	3.267 ± 1.302 [0.2094]
	biofilm	0.012 ± 0.002	8.637 ± 2.188	1.933 ± 0.450

[a] Numbers after ± signs are standard deviation, usually with n = 3. The numbers in brackets are *p*-values derived by applying the *t*-test to comparison of the values shown with the corresponding values for cells of the same organism grown in biofilm rather than suspension culture.

Materials and Methods

Bacteria and biofilms. *Streptococcus mutans* GS-5 and UA159, *Streptococcus sanguis* NCTC 10904, and *Streptococcus gordonii* ATCC 10558 are maintained routinely in our laboratory with weekly subculture on tryptic-soy agar plates (Difco, Detroit, MI) and with long-term storage at −70°C in 50% (vol/vol) glycerol solution. For the work with cell suspensions and cell extracts described here, the organisms were grown at 37°C in tryptone-yeast-extract (TY) medium containing 1% (wt/vol) glucose [1]. For killing experiments, suspensions containing ca. 10^9 colony-forming units per ml were used. Samples taken at intervals were diluted in 1% Difco peptone broth. Samples of diluted suspensions were plated on Difco tryptic-soy agar and incubated at 37°C until colony formation was complete.

Mono-organism biofilms were grown on glass slides, as described previously [8], basically in static batch cultures on glass slides in TY-sucrose broth in a 37°C incubator. One day before use, the biofilms were transferred to medium with sucrose replaced by glucose. The slides were covered with a dense film of about 3×10^8 cells/mm^2 on a total area of 18.75 cm^2. Viable counts were determined as described previously [8] by scraping the biofilms from the slides and dispersing the cells with an IKA Labortechnik T25 homogenizer (Janke and Kunkel, Staufen, Germany), followed by brief sonication to obtain suspensions of single cells. The suspensions were then diluted with 1% peptone (Difco, Detroit, MI) broth and spread plated on Difco tryptic-soy agar. Biofilms of the GS-5 strain of *S. mutans* tended to be somewhat less dense in the early phases of growth and matured more slowly, while the UA159 strain formed dense biofilms more rapidly. For killing experiments with intact biofilms, the films were treated with the specified agents. Then, at specified times, the entire biofilm was dispersed by means of scraping, homogenization, and brief sonication with an ice bath. D values (time for killing of 90% of the population) were assessed from the straight-lines parts of plots of logarithms of the fraction of the initial population surviving timed treatments versus exposure time, as described previously [8].

Oxygen uptake measurements. Cell cultures were harvested at stationary phase by centrifugation in a refrigerated centrifuge, washed once with 50 mM KCl plus 1 mM $MgCl_2$ and resuspended in this same salt solution. An aliquot of the cell suspension was transferred to potassium phosphate buffer to yield a 50 mM solution at pH 7.0 containing 0.5% (wt/vol) glucose, to give a cell density of about 1.5 mg cell dry weight per ml. The cell suspension was then vortexed vigorously to be air-saturated and immediately used for assessment of oxygen uptake at room temperature by using a VWR Model 4000 Dissolved Oxygen Meter. Biofilms were treated in the same way but were not centrifuged. Suspensions of biofilm cells were obtained by scraping, homogenizing, and sonicating, as described previously [2]. The dispersed biofilms could then be washed for determinations of dry weight or protein.

Enzyme assays. Cell-free extracts were prepared by homogenization with glass beads. Briefly, washed cells were resuspended in 20 mM Tris-HCl, pH 7.0, containing 50 mM KCl and 1 mM $MgCl_2$. An equal volume of 0.1-mm diameter, acid-washed glass beads was added, and the mixture was then homogenized by using a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK) for six times 30 s until complete cell disruption was observed under the phase microscope. The disrupted cells were then centrifuged at 15,000 *g* for 10 min at 4°C; the clear supernatant was used for assays. NADH oxidase was assayed at 25°C by the procedure of Poole and Claiborne [10]. Briefly, the assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, 0.3 mM EDTA, 0.5 mg/ml bovine liver catalase, and cell extract. NADH was added at a final concentration of 0.16 mM to start the reaction. The decrease in absorbance at 340 nm associated with oxidation of NADH was recorded over 3 min, during which time the reaction was linear. One unit of NADH oxidase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmole NADH/min under the assay conditions. Superoxide dismutase (SOD) was assayed by the cytochrome *c*-xanthine oxidase method used previously [9]

Hydrogen peroxide production. For assessment of H_2O_2 production by cells of *S. sanguis* or *S. gordonii*, cell suspensions or biofilms were prepared as described above for oxygen uptake measurement, except that for the suspensions the cell density was lower, only about 1 mg cell dry weight/ml. The suspensions contained 0.5% glucose. One-milliliter samples were removed at intervals of 20–30 min and centrifuged rapidly in a microcentrifuge to obtain a clear supernatant. The supernatant was diluted with distilled water and assayed for H_2O_2 produced by the reaction with leuco crystal violet in the presence of horseradish peroxidase [7].

Table 2. Killing of oral streptococci by hydrogen peroxide, t-butylhydroperoxide and 8-hydroxyquinoline

		D value (min)			
		H_2O_2	t-BOOH	8-HQ	
Organisms	Growth mode	0.25%	3%	0.002%	0.02%
S. mutans GS-5	aerobic	15	31	11	
	static	11	33	9	
	biofilm	12	34		14
S. mutans UA 159	aerobic	41	49	17	
	static	29	35	10	
	biofilm	13	64		15
S. sanguis NCTC 10904	aerobic	15	53	11	
	static	15	24	13	
	biofilm	27	30		17
S. gordonii ATCC 10558	aerobic	16	16	5	
	static	22	29	7	
	biofilm	27	67		20

Results

Table 1 presents data on the respiratory capacities of the four oral strepococci chosen for this study. The cells were grown in aerated cultures in Erlenmeyer flasks on a shaker-incubator at 250 rpm or in static cultures in milk-dilution bottles with little head space above the cultures, which were essentially anaerobic because of the restricted capacity for O_2 transfer, or in batch-cultivated biofilms. The suspension cultures were harvested at the early stationary phase. The variation among the organisms is striking. For *S. mutans*, aerobic growth greatly enhanced respiratory activity and NADH oxidase activity. The latter is the major base for oxygen metabolism of the organism. Even when using pyruvate or glycerophosphate for respiration, the substrates are first acted on by dehydrogenases, and then the NADH produced is oxidized through NADH oxidase [16]. The GS-5 strain was much more responsive than the UA159 strain and was capable of much higher rates of respiration. For both strains, respiration was markedly reduced for biofilm cells. The low level of respiration in biofilms was not significantly changed when the biofilms were dispersed and then respiratory rates determined with the dispersed cells (see below). Thus, the low respiration of biofilms is not due mainly to the biofilm state, but to the physiological peculiarities of the biofilm cells, indicated by the low levels of NADH oxidase activities in extracts (Table 1).

Streptococcus sanguis NCTC 10904 behaved very differently from the *S. mutans* organisms in that respiration and NADH oxidase levels were actually higher for cells grown in static culture than for those grown in aerated cultures (Table 1). However, this organism grew less well in aerated culture, with final optical densities only about 80% of those of static cultures. Still, it is clear that biofilm cells were repressed in respiration and in NADH oxidase activities compared with cells from static cultures. *Streptococcus gordonii* ATCC 10558 showed a pattern similar to that of *S. sanguis* NCTC10904, with lower respiration and NADH oxidase activities for cells grown with aeration. Aerobic cultures were only some 43% as dense as static cultures. Again, respiratory and NADH oxidase activities were repressed in biofilm cells compared with those from static cultures.

Levels of SOD activity for all of the organisms were increased by growth in aerated cultures (Table 1), independently of whether or not NADH oxidase activities were increased or decreased. Thus, production of enzymes for protection against oxidative damage appeared to be regulated separately from enzymes involved in respiration. Biofilm cells were deficient in SOD activity compared with cells grown aerobically; for example, for *S. mutans* GS-5 they were deficient even compared with cells from static cultures.

Streptococcus sanguis, and *S. gordonii* are net producers of H_2O_2, while *S. mutans*, especially the GS-5 strain, is not. As shown by the data presented in Fig. 1, growth with aeration decreased the capacity of *S. sanguis* for H_2O_2 production, but had only minor effects for *S. gordonii*. For both organisms, biofilms had dramatically reduced capacities to produce H_2O_2. Again, this reduced capacity was evident even when biofilms were initially dispersed before measurements of H_2O_2 production. The reduced production can be related to the low NADH oxidase capacities of biofilm cells.

The cells grown in different modes showed some differences in sensitivities to the oxidative damage agents H_2O_2, *t*-butyl hydroperoxide (t-BOOH), and 8-hydroxyquinoline (8-HQ), but the differences were not

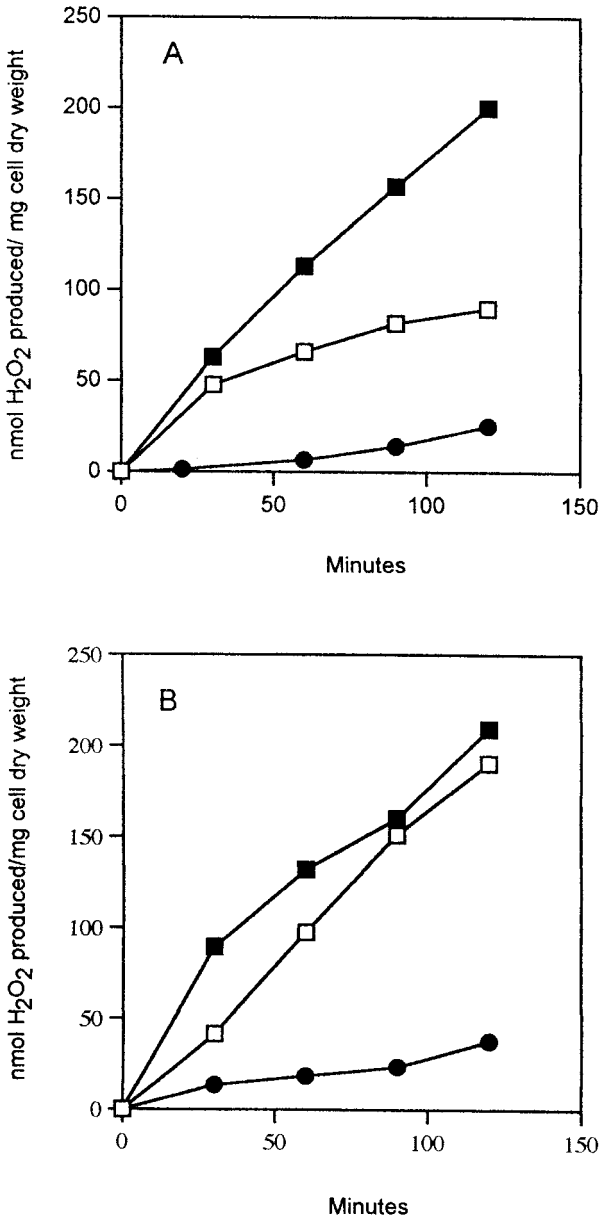

Fig. 1. H_2O_2 production by *S. sanguis* NCTC 10904 (A) or *S. gordonii* ATCC 10588 (B) grown aerobically in suspension cultures (□), statically in suspension cultures (■), or in biofilms (●).

dramatic (Table 2). Biofilm cells were more resistant to oxidative damage caused by 8-HQ than were cells from suspension cultures, and high levels of the agent were required for killing of biofilms. Differences were much less marked for killing by H_2O_2 or t-BOOH. Oral streptococci do not have catalase, but do have peroxidase activities associated with AhpCF proteins. However, peroxidase activity is limited by availability of NADH and so is not as great a factor in resistance as is catalase, which does not require a reducing substrate.

The biofilm state appeared to have little to do with the resistance of biofilm cells to peroxide killing or to their low rates of respiration. For example, biofilms of *S. mutans* GS-5 from a set of films were found to have respiration rates of 0.83 nmol O_2 per min per mg cell dry weight. When the biofilms were dispersed by scraping, homogenization, and brief sonication, the respiration rate was found to be 0.87 nmol O_2 per min per mg. Comparable values for biofilms and dispersed biofilm cells of *S. mutans* UA159, *S. sanguis* NCTC 10904, and *S. gordonii* ATCC 10588 were, respectively, 0.30 and 0.43, 1.60 and 1.3, 2.5 and 1.8. D values (time for killing of 90% of the population) for intact biofilms of *S. mutans* GS and dispersed biofilm cells exposed to 0.1% H_2O_2 at 25°C were, respectively, 25 and 35 min, while those for biofilms or dispersed biofilm cells of *S. sanguis* exposed to 0.3% peroxide solution were 47 and 54 min. Overall, it seems there was little or no difference in resistance between cells in intact or dispersed biofilms, at least for these organisms. Our findings with enzyme assays can be related to those of the mutation studies of Yamamoto et al. [17]. They found that deletion of *ahp*F and *ahp*C did not have major effects on sensitivity of *S. mutans* to hydroperoxide, because there appeared to be a compensatory system involving the iron-binding protein Dpr, which probably has multiple functions.

Discussion

Overall, the results presented here are somewhat difficult to reconcile with commonly held views of the ecology of oral streptococci in dental plaque with H_2O_2 as an antimicrobial and a substrate for production of HOSCN by salivary peroxidases. Oxidative stress appears to be minimized in streptococcal biofilms, and presumably also in plaque, simply by the low capacities of the streptococci to carry out respiration. Of course, plaque has a very diverse population, and many plaque organisms may not be as repressed for respiration as are the streptococci. However, the streptococci tend to be numerically dominant organisms in plaque. The residual O_2 in plaque has been estimated to be some 10% the saturation value for water in contact with air [6, 13]. This residual O_2 is difficult to explain considering the dense population of plaque, including organisms capable of high respiratory activities when grown in suspension cultures. Even extreme anaerobes, such as *Treponema denticola*, have been found [3] to have high respiratory capacities. However, the reduced capacities found in this study for streptococci grown in biofilms provide a plausible explanation.

H_2O_2 also is considered to play important roles in innate immunity in the mouth. Plaque does have catalase

activity that would act to diminish the effects of H_2O_2. However, plaque usually also has high levels of fluoride, usually about 0.1–0.5 mM [4], which is highly inhibitory for catalase in acidified environments [9]. Still, the data presented here indicate that major producers of H_2O_2 have greatly reduced peroxide-generating capacities when they are grown in biofilms. Again, plaque has a diverse population, and more work needs to be carried out with a variety of plaque bacteria in biofilms. Still, the findings for the streptococci are indicative of a state of reduced oxidative stress in plaque. There seems not to be as great a reduction in levels of protective enzymes against oxidative damage, especially superoxide dismutase, in biofilms, and biofilm cells were not highly sensitive to peroxide damage of exogenous origin. Apparently, the levels of protective enzymes were sufficient. However, the data presented here indicate major changes in the oxidative physiology of oral streptococci associated with growth in biofilms.

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Literature Cited

1. Belli WA, Marquis RE (1991) Adaptation of *Streptococcus mutans* and *Enterococcus hirae* to acid stress in continuous culture. Appl Environ Microbiol 57:1134–1138
2. Burne RA, Marquis RE (2001) Biofilm acid/base physiology and gene expression in oral bacteria. Methods Enzymol 337:403–415
3. Caldwell CE, Marquis RE (1999) Oxygen metabolism by *Treponema denticola*. Oral Microbiol Immunol 14:66–72
4. Duckworth RM, Morgan SN, Murray AM (1987) Fluoride in saliva and plaque following use of fluoride-containing mouthwashes. J Dent Res 59:1187–1191
5. Highuchi M, Yamamoto Y, Poole LB, Shimada M, Sato Y, Takahashi N, Kamio Y (1999) Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. J Bacteriol 18:5940–5947
6. Marquis RE (1995) Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. J Indust Microbiol 15:198–207
7. Motolla HA, Simpson BE, Gorin G (1970) Absorptiometric determination of hydrogen peroxide in submicrogram amounts with leuco crystal violet and peroxidase as catalyst. Anal Chem 42:410–411
8. Phan T-N, Reidmiller JS, Marquis RE (2000) Sensitization of *Actinomyces naeslundii* and *Streptococcus sanguis* in biofilms and suspensions to acid damage by fluoride and other weak acids. Arch Microbiol 174:248–255
9. Phan T-N, Kirsch AM, Marquis RE (2001) Selective sensitization of bacteria to peroxide damage associated with fluoride inhibition of catalase and pseudocatalase. Oral Microbiol Immunol 16:28–33
10. Poole LB, Claiborne A (1986) Interactions of pyridine nucleotides with redox forms of the flavin-containing NADH peroxidase from *Streptococcus faecalis*. J Biol Chem 261:14525–14533
11. Poole LB, Higuchi M, Shimada M, Li Calzi M, Kamio Y (2000) *Streptococcus mutans* H_2O_2-forming NADH oxidase is an alkyl hydroperoxide reductase protein. Free Radic Biol Med 28:108–120
12. Ryan CS, Kleinberg I (1995) Bacteria in human mouths involved in the production and utilization of hydrogen peroxide. Arch Oral Biol 40:753–763
13. Tanaka M, Hanoi T, Takaya K, Shizukuishi S (1998) Association of oxygen tension in human periodontal pockets with subgingival inflammation. J Periodontol 69:1127–1130
14. Thomas EL, Pera KA (1983) Oxygen metabolism of *Streptococcus mutans:* uptake of oxygen and release of superoxide and hydrogen peroxide. J Bacteriol 154:1236–1244
15. Thomas EL, Milligan TW, Joyner R, Jefferson M (1994) Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral streptococci. Infect Immun 62:529–535
16. Yamada T (1987) Regulation of glycolysis in streptococci. In: Reizer J, Peterkofsky A. (ed.) Sugar transport and metabolism in gram-positive bacteria. Chichester: Ellis Horwood Ltd, pp. 69–93
17. Yamamoto Y, Higuchi M, Poole LB, Kamio Y (2000) Role of the *dpr* product in oxygen tolerance in *Streptococcus mutans*. J Bacteriol 182:3740–3747