JOURNAL OF BACTERIOLOGY, June 1983, p. 1236–1244
0021-9193/83/061236-09$02.00/0
Copyright © 1983, American Society for Microbiology
Vol. 154, No. 3

Oxygen Metabolism of *Streptococcus mutans*: Uptake of Oxygen and Release of Superoxide and Hydrogen Peroxide

EDWIN L. THOMAS* AND KATE A. PERA

Departments of Biochemistry, St. Jude Children's Research Hospital and The University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38101

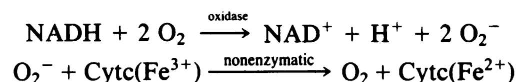
Received 29 November 1982/Accepted 21 March 1983

Oxygen (O_2) uptake and release of O_2 metabolites to the extracellular medium were studied with representatives of serotypes *a* through *g* of *Streptococcus mutans*. When incubated with glucose, washed cells of all strains took up O_2 at rates proportional to the O_2 concentration. When O_2 was held constant at 0.2 mM, 0.2 to 0.5 mol of O_2 was taken up per mol of glucose metabolized. Despite the similar rates of O_2 uptake, the strains fell into three classes according to the amount of hydrogen peroxide (H_2O_2) released. Strains BHT, FA-1, and OMZ-176 released up to 90% of O_2 taken up as H_2O_2, which accumulated in the medium to concentrations as high as 2 mM. The high levels of H_2O_2 accumulation were correlated with low ability to reduce exogenous H_2O_2 to water. Strains Ingbritt and B-13 released about half as much H_2O_2, but H_2O_2 in the medium did not exceed 0.05 to 0.1 mM. Strains HS-6, AHT, GS-5, OMZ-175, LM-7, and 6515-15 released <10% of O_2 taken up as H_2O_2, and H_2O_2 did not accumulate. Within this class, strains HS-6 and AHT released about 6% of O_2 taken up as superoxide (O_2^-). Release of O_2 metabolites was correlated with enzyme activities in cell-free extracts. Extracts from all strains catalyzed NADH-dependent O_2 uptake. Extracts from H_2O_2-accumulating strains produced H_2O_2 when incubated with NADH and O_2 and had low ability to catalyze NADH-dependent reduction of H_2O_2. Extracts from HS-6 and AHT had low superoxide dismutase activity, which may account for O_2^- release and the O_2-sensitive growth of these strains.

Bacteria classified as *Streptococcus mutans* are a heterogeneous group of oral pathogens with some shared characteristics (21, 25). As facultative (aerotolerant) anaerobes, they grow in the presence or absence of oxygen (O_2), but their energy metabolism is of an anaerobic type regardless of growth conditions. They depend primarily on glycolysis for ATP synthesis and excrete lactic acid as an end product (11). Many bacteria share these characteristics. The "lactic acid bacteria" do not synthesize hemes and therefore lack some major enzymes of O_2 metabolism, including cytochrome oxidase, other respiratory cytochromes, and catalase (16, 33).

Nevertheless, many of these bacteria take up O_2 at rates comparable to those of aerobic organisms, due to flavoproteins that act as NADH oxidases (16). Oxidase enzymes catalyze the one-, two-, or four-electron reduction of O_2 to superoxide (O_2^-), hydrogen peroxide (H_2O_2), or water (9, 29, 38).

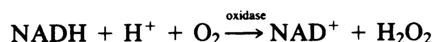
Cell-free extracts from some streptococci carry out NADH-dependent reduction of exogenous cytochrome *c* (Cytc) (14), a reaction that may indicate O_2^- production (31):



$$NADH + 2\,O_2 \xrightarrow{\text{oxidase}} NAD^+ + H^+ + 2\,O_2^-$$
$$O_2^- + Cytc(Fe^{3+}) \xrightarrow{\text{nonenzymatic}} O_2 + Cytc(Fe^{2+})$$

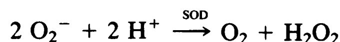
Production of O_2^- accounted for 17% of NADH-dependent O_2 uptake by extracts from *S. faecalis* (10).

Some lactic acid bacteria release H_2O_2 to the extracellular medium, and cell-free extracts produce H_2O_2 (2), which may be due to reduction of O_2 to H_2O_2:



$$NADH + H^+ + O_2 \xrightarrow{\text{oxidase}} NAD^+ + H_2O_2$$

Alternatively, H_2O_2 may arise from spontaneous or enzyme-catalyzed dismutation of O_2^-. Aerotolerant bacteria, including *S. mutans* (43), contain superoxide dismutase (SOD) activities (3, 13, 20):



$$2\,O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

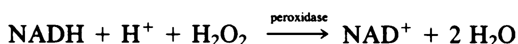
Lactic acid bacteria have one or more enzymes

TABLE 1. Release of O_2 metabolites

Class[a]	Strain	Serotype	Biotype	Species
I	FA-1	*b*	II	*S. rattus*
	BHT	*b*	II	*S. rattus*
	OMZ-176	*d*	IV	*S. sobrinus*
II	B-13	*d*	IV	*S. sobrinus*
	Ingbritt	*c*	I	*S. mutans*
III				
Measurable O_2-release				
	HS-6	*a*	III	*S. cricetus*
	AHT	*a*	III	*S. cricetus*
O_2-release not measurable				
	6715-15	*g*	IV	*S. sobrinus*
	GS-5	*c*	I	*S. mutans*
	LM-7	*e*	I	*S. mutans*
	OMZ-175	*f*	I	*S. mutans*

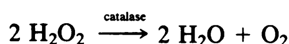
[a] Class I, High levels of H_2O_2 release and accumulation; class II, intermediate H_2O_2 release and low H_2O_2 accumulation; class III, low H_2O_2 release and no H_2O_2 accumulation.

that eliminate H_2O_2 by reduction. The only characterized activity is NADH peroxidase (14, 32, 44):



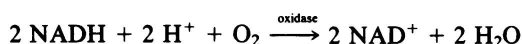
$$NADH + H^+ + H_2O_2 \xrightarrow{\text{peroxidase}} NAD^+ + 2\ H_2O$$

Elimination of H_2O_2 is dependent on metabolism because a continuing supply of reducing equivalents (NADH) is required. In contrast, catalase eliminates H_2O_2 in a dismutation reaction with no net change in oxidation state (36):



$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$

The combination of oxidase and peroxidase activities can reduce O_2 to water at the expense of 2 NADH (16). However, certain bacteria contain a flavoprotein NADH oxidase that reduces O_2 directly to water without producing H_2O_2 as an intermediate (15):



$$2\ NADH + 2\ H^+ + O_2 \xrightarrow{\text{oxidase}} 2\ NAD^+ + 2\ H_2O$$

Some *S. mutans* strains release H_2O_2 (21), although according to one report they do not contain NADH oxidase activity (35). The aim of this study was to characterize O_2 uptake and release of O_2 metabolites by representative *S. mutans* strains and to determine the enzymatic basis for their O_2 metabolism.

MATERIALS AND METHODS

Materials. SOD, xanthine oxidase, horseradish peroxidase (HRP), Cytc type VI, and scopoletin were from Sigma Chemical Co. Catalase and NADH were from Boehringer Mannheim. Catalase crystals were washed by centrifugation in water and then dissolved (2.4 mg/ml) in 20 mM KCl–4 mM phosphate, pH 8. Scopoletin (2 mg/ml) was dissolved in 0.05 M Na_2CO_3 and immediately diluted to 0.4 mM in 0.12 M NaCl–1 mM $MgSO_4$–0.05 M potassium phosphate, pH 7. H_2O_2 (30%) from Fisher Chemical Co. and ethyl hydrogen peroxide (12%) from Accurate Chemical Co. were diluted into water, and concentrations were determined by HRP-catalyzed oxidation of leuco-crystal violet (34).

Growth medium. Todd-Hewitt broth (Difco Laboratories) at 30 g/liter was filtered through an acid-washed 4- to 5.5-μm sintered-glass filter, and then 100-ml portions were sterilized by filtration through 0.22-μm filters (Nalge Co.).

Bacteria. *S. mutans* strains (Table. 1) were grown aerobically to stationary phase, and portions of the cultures were frozen in liquid N_2 and stored at −80°C. Portions were thawed at 37°C, and 1 ml was used to inoculate 100 ml of medium in 250-ml flasks, with a foam plug for aerobic growth ($+O_2$), or interconnected to a flask with 100 ml of 0.1 M phosphate (pH 7) to which 1 g of sodium hydrosulfite (dithionite) was added immediately before closing the system, for anaerobic growth ($-O_2$). Flasks were shaken at 200 rpm at 37°C. Cells were harvested in early (16 to 24 h) or late (40 to 48 h) stationary phase. To obtain cells in logarithmic growth phase, 10 ml of a 16-h culture was transferred to 100 ml of medium, and the cells were harvested about 3 h later when absorbance at 600 nm (Uvikon 810 spectrophotometer) was 60 to 70% of that obtained at stationary phase. Cultures were centrifuged at 5,000 × *g*, resuspended in 2 volumes of cold NaCl-$MgSO_4$-phosphate, centrifuged again, and resuspended with a Teflon glass homogenizer to an absorbance of 4.5 at 600 nm, corresponding to 2.3 mg (dry weight)/ml or two to four times the cell density obtained at stationary phase.

Incubation conditions. Unless otherwise indicated, incubations were at the cell density indicated above in 2 ml of NaCl-$MgSO_4$-phosphate with 10 mM glucose (Glc) at 37°C, either in 15-ml closed tubes with an N_2 atmosphere (anaerobic) or in 50-ml flasks on a reciprocal shaker at 100 cycles/min (continuous aeration). After cooling at 4°C, portions were centrifuged. All centrifugations were at 20,000 × *g* for 15 min at 4°C. Glc in the supernatant fractions was determined by the phenol-sulfuric acid method (4), and rates of Glc metabolism were calculated from the rate of disappearance of Glc from the medium.

O_2 uptake. O_2 uptake was measured in a 3-ml total volume in a stirred chamber at 37°C with a Clarke-type O_2 electrode.

H_2O_2 release. Suspensions were incubated under continuous aeration with 2 μg of HRP per ml–0.1 mM scopoletin as a trap for H_2O_2 (8). Suspensions were cooled and centrifuged, portions of supernatants were diluted 10-fold with cold NaCl-$MgSO_4$-phosphate, and relative fluorescence was measured with excitation at 365 nm and emission at 460 nm. The amount of H_2O_2 released during the incubation was calculated from the loss of scopoletin fluorescence.

H_2O_2 accumulation. Suspensions were incubated under continuous aeration, cooled, and centrifuged, and H_2O_2 concentration in the supernatant was determined with HRP and leuco-crystal violet.

H_2O_2 reduction. Suspensions in the O_2 electrode chamber were deaerated with N_2, H_2O_2 (0.3 mM) and Glc (10 mM) were added, and after 15 min at 37°C, catalase (0.1 mg) was added and the amount of H_2O_2 remaining was calculated as two times the amount of O_2 liberated. Catalase activity was removed from the chamber and electrode surface with 0.1 M HCl (41). Alternatively, suspensions were incubated anaerobically with H_2O_2 and Glc and then cooled and centrifuged, and H_2O_2 remaining in the supernatant was determined with HRP and leuco-crystal violet.

Reduction of Cytc. Suspensions containing Cytc were centrifuged, and triplicate portions of supernatants were diluted into cold, deaerated buffer containing 25 μg of catalase and 25 U of SOD per ml. Concentration of reduced Cytc was calculated from the difference (21.1 mM^{-1} cm^{-1}) in the extinction coefficients of reduced and oxidized Cytc at 550 nm (28, 30).

Cell-free extracts. Ten cultures were pooled and centrifuged. The cells were resuspended and washed by centrifugation in 0.8 liter of cold 20 mM Tris-chloride, pH 8, with 1 mM EDTA and then resuspended to 250 ml in the buffer with 100 ml of 0.1-mm glass beads. The suspension was deaerated with N_2 and subjected to five 2-min periods of agitation in a homogenizer with Teflon blades (Biospec Products), with cooling in between. The suspension was decanted and clarified by centrifugation, and the supernatant was dialyzed against 0.1 M phosphate, pH 7, and stored at −80°C. Protein was determined by the method of Lowry et al. (26), with bovine serum albumin as the standard.

Activities in extracts. NADH oxidase activity was measured by incubating the extract (10 to 200 μg of protein per ml) with 2 mM NADH and 100 U of SOD per ml in 0.3 M phosphate, pH 7, with 0.1 M mannitol at 37°C. Uptake of O_2 was measured with the O_2 electrode, and oxidation of NADH was measured by incubating under continuous aeration, diluting eightfold with cold 0.1 M Tris-chloride, pH 8, and calculating NADH concentration from absorbance at 340 nm. NADH peroxidase activity was measured in the same medium with 0.3 mM H_2O_2 under anaerobic conditions, using the O_2 electrode and catalase to measure H_2O_2 as described above. SOD activity was measured by the method based on inhibition of O_2-dependent reduction of Cytc by xanthine and xanthine oxidase (30).

RESULTS

Table 1 lists *S. mutans* strains used in this study. As described below, similar rates of Glc metabolism and O_2 uptake were observed with all strains. However, the strains fell into three classes in decreasing order with respect to the amount of H_2O_2 released. Classes I to III were not exactly correlated with serotype (21, 25), "biotype" (37), or the four species (12). However, two of three class I strains were of serotype *b*, biotype II, *S. rattus*, and three of six class III strains were of biotype I, *S. mutans*. Within class III, two strains released O_2^- and both were of serotype *a*, biotype III, *S. cricetus*.

O_2 uptake. O_2 uptake required Glc and was not inhibited by cyanide, but was blocked by KF or EDTA or at 4°C. Plots of O_2 concentration, $[O_2]$, versus time were not linear; the rate of O_2 uptake became slower as time progressed and $[O_2]$ decreased. This result was not due to declining ability of the bacteria to take up O_2. If the suspension was reaerated, O_2 uptake resumed at the original rate. Also, if $[O_2]$ was lowered by passing N_2 through the suspension, O_2 was taken up at the same low rate as when O_2 uptake by the bacteria had lowered $[O_2]$ to that level.

Figure 1 (left) shows that plots of the natural logarithm of O_2 concentration ($\ln[O_2]$) versus time were linear at high $[O_2]$. Therefore, O_2 uptake could be described by the equation $\ln[O_2] = \ln a - kt$, where a is $[O_2]$ at the start of incubation (0.2 mM for an air-saturated suspension at 37°C), k is the rate constant, and t is time. This observation indicated that the rate of O_2 uptake was proportional to $[O_2]$. Therefore, the enzymatic process was not saturated with respect to $[O_2]$; i.e., the K_m for O_2 was much higher than 0.2 mM.

At $[O_2]$ below 70 μM, plots deviated from linearity, indicating faster rates than predicted from the equation above. Figure 1 (right) shows the data replotted to evaluate O_2 uptake at low $[O_2]$. The change in $[O_2]$ in successive 1-min intervals is plotted versus one-half the sum of $[O_2]$ at the beginning and end of each 1-min interval. This method provides an estimate of the rate of O_2 uptake as a function of $[O_2]$. At $[O_2]$ below 10 μM, O_2 uptake appeared saturable. At higher $[O_2]$, O_2 uptake was proportional to $[O_2]$. After subtracting the linear portion of O_2 uptake, a K_m for O_2 of 2 to 4 μM and V_{max} of 3 to 4 nmol min^{-1} ml^{-1} were calculated for the remaining, saturable portion of O_2 uptake.

Rates of O_2 uptake (V) that would be obtained under continuous aeration were calculated from the equation $V = k \cdot a$, where $a = 0.2$ mM, with the assumption that the contribution of the saturable process was negligible. Typical rates of O_2 uptake for cells from 24-h cultures were 30 to 70 nmol min^{-1} ml^{-1}, and rates of Glc metabolism under continuous aeration were 100 to 170 nmol min^{-1} ml^{-1}. The molar ratio of O_2 uptake to Glc metabolized was in the range of 0.2 to 0.5 for all strains.

H_2O_2 release. Figure 1 shows O_2 uptake measured in the presence and absence of catalase. With catalase, the rate appeared slower. If reduction of O_2 yields H_2O_2 and H_2O_2 is eliminated by catalase, then the observed rate of O_2 uptake is one-half the rate of O_2 reduction (9, 41).

Linear plots of $\ln[O_2]$ versus time were obtained with or without catalase, so that the rate

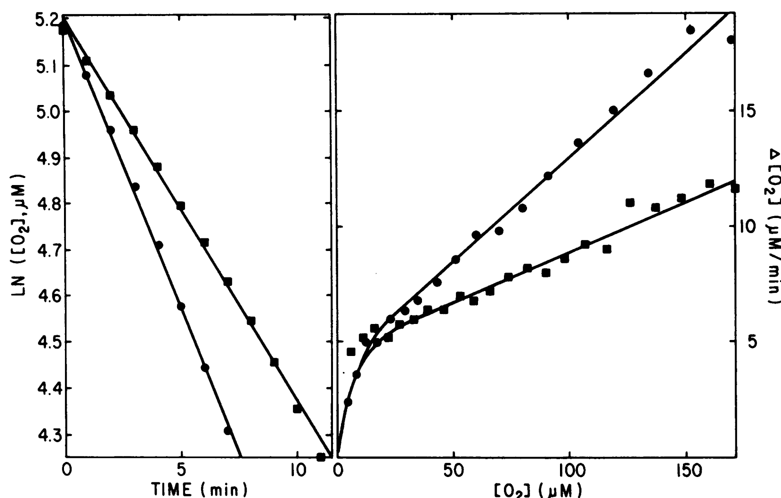


FIG. 1. $[O_2]$ dependence for O_2 uptake. O_2 uptake by OMZ-176 cells (40 h, $+O_2$) was measured in the absence (●) and presence (■) of 80 µg of catalase per ml. The choice of zero time is arbitrary; usually 1 to 2 min were allowed for the electrode response to stabilize.

of H_2O_2 release was proportional to $[O_2]$. The rate constant for H_2O_2 release (k_3) could be calculated as two times the difference between rate constants for O_2 uptake measured in the absence (k_1) or presence (k_2) of catalase. In Fig. 1, $k_1 = 0.126$, $k_2 = 0.084$, and $k_3 = 0.084$, so that about 67% of O_2 taken up was released to the medium as H_2O_2 ($k_3/k_1 = 0.67$). Release of H_2O_2 approached 90% of O_2 uptake with class I strains from late stationary phase.

H_2O_2 reduction. When cells were incubated anaerobically with added (exogenous) H_2O_2, the H_2O_2 was reduced as indicated by disappearance of H_2O_2 from the medium. Reduction of H_2O_2 was dependent on metabolism as indicated by the requirement for Glc and inhibition by KF or EDTA. Cyanide did not inhibit, rates of reduction of H_2O_2 and ethyl hydrogen peroxide were similar, and no O_2 was evolved with or without Glc, confirming that the cells contained no catalase (36).

The H_2O_2-reducing activities of cells of classes I to III differed in their apparent affinity for H_2O_2 (Fig. 2). Cells were incubated anaerobically with Glc and varying $[H_2O_2]$, and the initial rate of H_2O_2 reduction (V_0) was measured and plotted versus the initial H_2O_2 concentration, $[H_2O_2]_0$. With GS-5 cells (class III), V_0 was nearly independent of $[H_2O_2]_0$ over the range tested. Therefore, the K_m for H_2O_2 was <0.04 mM. With OMZ-176 cells (class I), H_2O_2 reduction also appeared saturable, although the K_m for H_2O_2 was higher. The maximum rate was obtained at 0.2 to 0.3 mM H_2O_2. In contrast, with Ingbritt cells (class II), the rate of reduction was proportional to $[H_2O_2]_0$, and no evidence of saturation was obtained at $[H_2O_2]$ up to 0.5 mM.

To compare H_2O_2 reduction by cells of different strains, initial rates of reduction of 0.3 mM H_2O_2 were measured, although this concentration was not saturating for the class II strains. However, this concentration of exogenous H_2O_2 had little or no effect on rates of Glc metabolism or O_2 uptake, whereas 2 to 3 mM H_2O_2 inhibited by about 50%.

H_2O_2 accumulation. Table 2 summarizes results obtained with four representative strains. When cells of class I strains were incubated with Glc under continuous aeration, H_2O_2 accumulated in the medium to concentrations as high as 2 mM. H_2O_2 release increased with cells from stationary phase, and the ability to reduce H_2O_2 decreased. With class II strains, H_2O_2 accumulation stopped at a low level (0.05 to 0.1 mM). Rates of H_2O_2 release were about half those of class I strains, and rates of H_2O_2 reduction were slightly higher. With class III strains, no H_2O_2 accumulated but a small amount was released. Therefore, these cells produced H_2O_2, but H_2O_2 in the medium was eliminated by reduction. These cells had high ability to reduce exogenous H_2O_2. Table 2 also shows that the major differences between classes I to III were in rates of H_2O_2 release and reduction, rather than O_2 uptake.

Within class III, the HS-6 and AHT strains grew faster and to higher cell yields under anaerobic conditions. Table 2 shows that rates of O_2 uptake and H_2O_2 reduction were much higher with HS-6 cells harvested from anaerobic cultures. In other experiments, rates of Glc metabolism were similarly increased. With strains

other than HS-6 or AHT, growth under aerobic versus anaerobic conditions had no consistent effect on the measured rates or on H_2O_2 accumulation.

O_2^- release. Measurable release of O_2^- was obtained only with strains HS-6 and AHT. High [Cytc] was required to detect O_2^-; reduction increased with [Cytc] up to at least 10 mg/ml. Therefore, it is likely that only a portion of the O_2^- was detected (19).

Table 3 shows that O_2^- release appeared greater in the presence of catalase, and H_2O_2 release appeared greater in the presence of SOD. Rates measured in this way were equal (about 1 nmol min^{-1} ml^{-1}). If the H_2O_2 detected in this experiment was due to release of O_2^-, followed by dismutation of O_2^- in the medium, then the rate of O_2^- release should be twice the rate of H_2O_2 release. However, if it is assumed that Cytc trapped only about half the O_2^-, then O_2^- release could account for all of the apparent H_2O_2 release.

At high [Cytc], high levels of added SOD were required to block reduction. Table 3 shows that SOD at 100 U/ml inhibited reduction by about 50%. No reduction occurred without Glc.

Reduction of Cytc was also measured at the end of a period in which O_2 uptake was measured, to obtain a ratio of O_2^- release to O_2 uptake. Uptake of 120 nmol of O_2 (from 0.2 to 0.08 mM) by HS-6 cells (24 h, $-O_2$) in the presence of catalase (80 μg/ml) and Cytc (6 mg/ml) resulted in a 4% increase in absorbance at 550 nm, corresponding to trapping of 8 nmol of O_2^-. This increase was blocked by SOD (100 U/ml). Therefore, as a minimum estimate, 6% of O_2 taken up was released to the medium as O_2^- [8/(120 + 8) = 0.06]. With OMZ-176, Ingbritt, or GS-5, the change in absorbance was ±1%, so that O_2^- release was less than could be detected.

Activities in extracts. Table 4 shows that cell-free extracts had NADH oxidase activity, measured as O_2-dependent oxidation of NADH or NADH-dependent uptake of O_2. Rates of O_2 uptake were proportional to O_2 concentration and were calculated as k_1 times 0.2 mM O_2 as described above. A K_m for NADH of 0.1 to 0.2 mM was calculated from the effect of NADH on the rate of O_2 uptake.

Table 4 also shows that with the extract from GS-5 (class III) cells the rate of NADH oxidation was two times the rate of O_2 uptake, consistent with oxidation of 2 mol of NADH and reduction of 1 mol of O_2 to water. The NADH/O_2 ratio was <2 with extracts from other strains, indicating that a portion of the O_2 taken up was reduced to O_2^- or H_2O_2 rather than to water. With extracts from OMZ-176 (class I) cells, the NADH/O_2 ratio decreased with increasing culture age, consistent with the in-

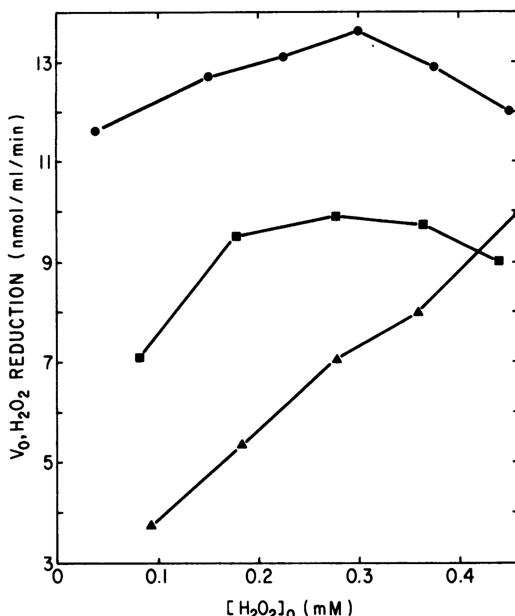


FIG. 2. [H_2O_2] dependence for H_2O_2 reduction. GS-5 cells (24 h, $+O_2$) at one-fourth the usual cell density (●), OMZ-176 cells (24 h, $+O_2$) (■), and Ingbritt cells (24 h, $-O_2$) (▲) were incubated anaerobically with the indicated [H_2O_2]$_0$, and the initial rate of H_2O_2 reduction (V_0) was calculated from the decrease of [H_2O_2] in the medium.

crease in H_2O_2 release by intact cells (Table 2). Accumulation of H_2O_2 was observed during aerobic incubation of these extracts with NADH, and catalase caused a decrease in the observed rate of O_2 uptake similar to that in Fig. 1.

Extracts also had NADH peroxidase activity, measured as NADH-dependent reduction of H_2O_2 under anaerobic conditions. A K_m of 0.3 to 0.4 mM was calculated for NADH. No other H_2O_2-reducing activities were detected, such as with NADPH or glutathione (18) as cofactors. Table 4 shows that extracts from stationary-phase OMZ-176 (class I) cells had the lowest peroxidase activity, consistent with the low H_2O_2-reducing activity of the cells (Table 2).

NADH oxidase activity was at least 10 times greater than NADH peroxidase activity in extracts from all strains. Therefore, the 2/1 stoichiometry of NADH oxidation and O_2 uptake in extracts from GS-5 (class III) could not be due to the sum of NADH-dependent H_2O_2 production and NADH-dependent H_2O_2 reduction. If reduction of O_2 to H_2O_2 was 10 times faster than reduction of H_2O_2 to water, then H_2O_2 would accumulate and the NADH/O_2 ratio would be about 1/1. Instead, the results suggested that most of the O_2 uptake was due to an NADH

TABLE 2. O_2 metabolism[a]

Strain (class)	Culture conditions	H_2O_2 accumulation (mM)	H_2O_2 release (nmol min^{-1} ml^{-1})	H_2O_2 reduction (nmol min^{-1} ml^{-1})	O_2 uptake (nmol min^{-1} ml^{-1})
OMZ-176 (I)	3 h, $+O_2$	0.04 ± 0.03	3	6	57 ± 25
	24 h, $+O_2$	0.51 ± 0.19	7	5	51 ± 28
	24 h, $-O_2$	0.44 ± 0.14	7	4	33
	40 h, $+O_2$	0.30 ± 0.08	10	3	21
Ingbritt (II)	24 h, $+O_2$	0.02 ± 0.01	2	9	48 ± 18
	24 h, $-O_2$	0.05 ± 0.01	3	6	47 ± 18
	40 h, $+O_2$	0.06 ± 0.04	2	2	19
HS-6 (III)	24 h, $+O_2$	0	1	1	3
	24 h, $-O_2$	0	1	15	22 ± 3
GS-5 (III)	3 h, $+O_2$	0	0.5	33	58 ± 29
	24 h, $+O_2$	0	0.5	40	55 ± 10
	40 h, $+O_2$	0	0.5	12	23

[a] Cells harvested at the indicated times from anaerobic ($-O_2$) or aerated ($+O_2$) cultures were incubated aerobically for 1 h with 10 mM Glc to measure H_2O_2 accumulation. Cells were incubated aerobically with HRP-scopoletin–10 mM Glc–100 U of SOD per ml to measure H_2O_2 release. H_2O_2 reduction was measured by incubating anaerobically with 10 mM Glc–0.3 mM H_2O_2. Rates of O_2 uptake were calculated as described in the text. Standard deviation is shown where values were available from three or more experiments with separate cultures.

oxidase that reduced O_2 directly to water without forming H_2O_2 as an intermediate. Consistent with this interpretation, H_2O_2 did not accumulate and catalase had no effect on the observed rate of O_2 uptake by extracts from GS-5.

Table 4 also shows SOD activity of the dialyzed extracts. No dialyzable activity (3) was detected. Extracts from HS-6 cells had the lowest SOD activity, suggesting that low SOD activity was responsible for O_2^- release (Table 3).

DISCUSSION

Significance of O_2 metabolism. O_2 is not essential to growth of lactic acid bacteria, and the reduction of O_2 and H_2O_2 by these organisms consumes NADH in reactions that are not coupled to ATP synthesis (16). Nevertheless, O_2 or O_2 metabolites can have a useful role in carbohydrate metabolism under some conditions (16, 45) and may have a role in microbial ecology. O_2 uptake enables these bacteria to create an anaerobic environment when they are present at high cell densities or in areas of limited O_2 diffusion. Although this anaerobic environment is not necessarily of direct benefit, it could exclude O_2-requiring microorganisms or slow the growth of microorganisms that grow faster when O_2 is available. Also, the release of toxic O_2 metabolites such as O_2^- or H_2O_2 may inhibit metabolism and growth of other microorganisms (17, 23).

On the other hand, O_2^- or H_2O_2 may damage the bacteria that produce them by reacting with cell components or by giving rise to other potentially toxic agents (7). The absence of catalase or the production of toxic agents derived from O_2 may cause these bacteria to be more susceptible to the O_2-dependent antimicrobial activities of

TABLE 3. H_2O_2 and O_2^- release by HS-6 cells[a]

Time (min)	H_2O_2 release (nmol/ml)		O_2^- release (nmol/ml)				
	+SOD	−SOD	+Catalase	−Catalase	+Catalase, +SOD	+SOD	+Catalase, −Glc
15			17	16	12		
30	27	12	31	23	15	9	0
45			36	24	20	6	−1
60	55	48	46	23	20	4	1
90	80	53					

[a] HS-6 cells (24 h, $-O_2$) were incubated with 10 mM Glc under continuous aeration with HRP-scopoletin or 5.3 mg of Cytc per ml. Where present, SOD was 100 U/ml and catalase was 80 μg/ml. Variation between triplicate samples was ±4 nmol/ml.

TABLE 4. Activities in cell-free extracts[a]

Strain (class)	Culture conditions	NADH oxidase ($nmol\ min^{-1}\ mg^{-1}$)		$NADH/O_2$	NADH peroxidase ($nmol\ min^{-1}\ mg^{-1}$)	SOD ($U\ mg^{-1}$)
		NADH	O_2			
OMZ-176 (I)	3 h, $+O_2$	860	470	1.8	35	5
	24 h, $+O_2$	470	310	1.5	9	10
	40 h, $+O_2$	230	180	1.3	9	8
Ingbritt (II)	24 h, $-O_2$	810	440	1.8	16	5
HS-6 (III)	24 h, $-O_2$	570	310	1.8	25	2
GS-5 (III)	24 h, $+O_2$	510	250	2.0	20	6

[a] Cells were harvested at the indicated times from anaerobic ($-O_2$) or aerated ($+O_2$) cultures, extracts were prepared, and oxidase, peroxidase, and SOD activities per milligram of protein were measured.

phagocytic leukocytes (5, 6, 24, 27). Release of H_2O_2 is especially relevant to oral microbial ecology, because the oral bacteria coexist with a H_2O_2-activated, peroxidase-mediated antimicrobial system (22, 40). Classification of *S. mutans* strains according to the amount of H_2O_2 released is correlated with inhibition of their metabolism by the salivary lactoperoxidase system (42).

Relation of O_2 uptake and H_2O_2 release. Differences in H_2O_2 release among lactic acid bacteria were attributed to differences in the ratio of NADH oxidase to NADH peroxidase activities (2, 16). Results presented here are in part consistent with this interpretation. Cells of all strains took up O_2 at similar rates, whereas cells that released large amounts of H_2O_2 had low ability to reduce H_2O_2. Similarly, NADH oxidase activity was high in extracts from all cells, and NADH peroxidase activity was lowest in extracts from cells that released the most H_2O_2.

Nevertheless, rates of O_2 uptake by cells or extracts were much higher than rates of H_2O_2 reduction. This observation suggests that a substantial portion of O_2 uptake occurred by way of enzymatic mechanisms in which H_2O_2 was not an intermediate. Therefore, cells that release relatively little H_2O_2 may have low levels of H_2O_2-producing activity as well as high levels of H_2O_2-reducing activity. *Clostridium perfringens* was reported to contain an NADH oxidase that reduces O_2 directly to water without producing H_2O_2 (15), but to our knowledge such an activity has not been reported in streptococci.

In one study, differences in H_2O_2 release were attributed to qualitative differences in NADH oxidase activity (2). It was reported that the oxidase in extracts from streptococci that release H_2O_2 can be distinguished from the oxidase of other strains on the basis of plots of [O_2] versus time. Linear plots are obtained with the first class (e.g., *S. lactis*), whereas the slope decreases with decreasing [O_2] with the second class (e.g., *S. cremoris*). In results presented here, the second type of plot was obtained with all *S. mutans* strains, regardless of the amount of H_2O_2 released. Although these strains may have differing oxidase activities, it was not possible to distinguish between these activities on the basis of the kinetics of O_2 uptake.

O_2^- release. Release of O_2^- from *S. faecalis* was reported based on SOD-inhibited chemiluminescense (1). However, the quantitative relation between chemiluminescense and release of O_2 metabolites is unclear. In this report, measurable O_2^- release was obtained only with *S. mutans* serotype *a* strains, which had low SOD activity and which are the least aerotolerant (12). The absence of SOD accounts for the O_2 sensitivity of obligate anaerobes (20).

O_2^- may not be highly toxic (7) in that O_2^- is short-lived, nonreactive with most biological materials, and perhaps more likely to act as a reducing agent than as an oxidizing agent. However, O_2^- is responsible for O_2-dependent toxicity when production of O_2^- by *S. sanguis* is stimulated by plumbagin (13). In addition, the toxicity of O_2^- is amplified by H_2O_2 due to reactions that yield other activated species. The reaction of O_2^- with H_2O_2 does not occur under physiological conditions, unless catalyzed by transition metals such as iron (7). Lactic acid bacteria have no hemoproteins, and perhaps no other iron-containing proteins (16), although ferrous iron is required for growth of *S. mutans* in a defined medium (39). Low levels of iron and other metals in these bacteria and in the incubation medium may account for their tolerance to the O_2^- and H_2O_2 that they produced and to added H_2O_2.

ACKNOWLEDGMENTS

We thank M. Morrison and G. Schonbaum for helpful discussions, T. Kral (Temple University, Philadelphia, Pa.) and J. Pollack (State University of New York, Stony Brook) for providing bacterial strains, and Patricia Nicholas and Vicki Gray for manuscript preparation.

This work was supported by Public Health Service grants DE 04235, AI 16795, and CA 21765 from the National Institutes of Health and by ALSAC.

LITERATURE CITED

1. **Allen, R. C.** 1980. Bacterial chemiluminescence: oxygen dependence and inhibition by superoxide dismutase and catalase, p. 116–127. *In* W. H. Bannister and J. V. Bannister (ed.), Developments in biology, vol. 2A. Elsevier/North-Holland, New York.
2. **Anders, R. F., D. M. Hogg, and G. R. Jago.** 1970. Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. Appl. Microbiol. **19:**608–612.
3. **Archibald, F. S., and I. Fridovich.** 1982. The scavenging of superoxide radical by manganous complexes. Arch. Biochem. Biophys. **214:**452–463.
4. **Ashwell, G.** 1966. New colorimetric methods of sugar analysis. Methods Enzymol. **8:**85–95.
5. **Babior, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298:**659–668, 721–725.
6. **Badwey, J. A., and M. L. Karnovsky.** 1980. Active oxygen species and the functions of phagocytic leukocytes. Annu. Rev. Biochem. **49:**695–726.
7. **Bors, W., M. Saran, and G. Czapski.** 1980. The nature of intermediates during biological oxygen activation, p. 1–31. *In* W. H. Bannister and J. V. Bannister (ed.), Developments in biology, vol. 2B. Elsevier/North-Holland, New York.
8. **Boveris, A., E. Martino, and A. O. M. Stoppani.** 1977. Evaluation of the horseradish peroxidase-scopoletin method for the measurement of hydrogen peroxide formation in biological systems. Anal. Biochem. **80:**145–158.
9. **Bright, H. J., and D. J. T. Porter.** 1975. Flavoprotein oxidases, p. 421–505. *In* P. D. Boyer (ed.), The enzymes, vol. 12. Academic Press, Inc., New York.
10. **Britton, L., D. P. Malinowski, and I. Fridovich.** 1978. Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. J. Bacteriol. **134:**229–236.
11. **Cole, J. A.** 1977. A biochemical approach to the control of dental caries. Biochem. Soc. Trans. **5:**1232–1239.
12. **Coykendall, A. L.** 1974. Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. J. Gen. Microbiol. **83:**327–338.
13. **DiGuiseppi, J., and I. Fridovich.** 1982. Oxygen toxicity in *Streptococcus sanguis*. The relative importance of superoxide and hydroxyl radicals. J. Biol. Chem. **257:**4046–4051.
14. **Dolin, M. I.** 1955. The DPNH-oxidizing enzymes of *Streptococcus faecalis*. II. The enzymes utilizing oxygen, cytochrome c, peroxide and 2,6-dichlorophenol-indophenol or ferricyanide as oxidants. Arch. Biochem. Biophys. **55:**415–435.
15. **Dolin, M. I.** 1959. Oxidation of reduced diphosphopyridine nucleotide by *Clostridium perfringens*. J. Bacteriol. **77:**383–392.
16. **Dolin, M. I.** 1961. Cytochrome-independent electron transport enzymes of bacteria, p. 425–460. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 2. Academic Press, Inc., New York.
17. **Donoghue, H. D., and J. E. Tyler.** 1975. Antagonisms against streptococci isolated from the human oral cavity. Arch. Oral Biol. **20:**381–387.
18. **Flohe, L., and W. A. Gunzler.** 1976. Glutathione-dependent enzymic oxidoreduction reactions, p. 17–34. *In* I. M. Arias and W. B. Jakoby (ed.), Glutathione: metabolism and function. Raven Press, New York.
19. **Fridovich, I.** 1970. Quantitative aspects of the production of superoxide anion by milk xanthine oxidase. J. Biol. Chem. **245:**4053–4057.
20. **Fridovich, I.** 1975. Superoxide dismutases. Annu. Rev. Biochem. **44:**147–159.
21. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44:**331–384.
22. **Hamon, C. B., and S. J. Klebanoff.** 1973. A peroxidase-mediated, *Streptococcus mitis*-dependent antimicrobial system in saliva. J. Exp. Med. **137:**438–450.
23. **Holmberg, K., and H. O. Hallander.** 1973. Production of bactericidal concentrations of hydrogen peroxide by *Streptococcus sanguis*. Arch. Oral Biol. **18:**423–434.
24. **Kaplan, E. L., T. Laxdal, and P. G. Quie.** 1968. Studies of polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood: bactericidal capacity for streptococci. Pediatrics **41:**591–599.
25. **Kral, T. A., and L. Daneo-Moore.** 1981. Biochemical differentiation of certain oral streptococci. J. Dent. Res. **60:**1713–1718.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
27. **Mandel, G. L., and E. W. Hook.** 1969. Leukocyte bactericidal activity in chronic granulomatous disease: correlation of bacterial hydrogen peroxide production and susceptibility to intracellular killing. J. Bacteriol. **100:**531–532.
28. **Massey, V.** 1959. The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim. Biophys. Acta **34:**255–256.
29. **Massey, V., G. Palmer, and D. Ballou.** 1971. On the reaction of reduced flavins and flavoproteins with molecular oxygen, p. 349–361. *In* H. Kamin (ed.), Flavins and flavoproteins. University Park Press, Baltimore.
30. **McCord, J. M., C. O. Beauchamp, S. Goscin, H. P. Misra, and I. Fridovich.** 1973. Superoxide and superoxide dismutase, p. 51–76. *In* T. E. King, H. S. Mason, and M. Morrison (ed.), Oxidases and related redox systems, vol. 1. University Park Press, Baltimore.
31. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244:**6049–6055.
32. **Mizushima, S., and K. Kitahara.** 1962. Purification and properties of reduced diphosphopyridine nucleotide (DPNH) peroxidase in *Lactobacillus casei*. J. Gen. Appl. Microbiol. **8:**56–62.
33. **Molland, J.** 1947. Bacterial catalase. Acta Pathol. Microbiol. Scand. Suppl. **66:**1–165.
34. **Mottola, H. A., B. E. Simpson, and G. Gorin.** 1970. Absorptiometric determination of hydrogen peroxide in submicrogram amounts with leuco crystal violet and peroxidase as catalyst. Anal. Chem. **42:**410–411.
35. **Ritchey, T. W., and H. W. Seeley, Jr.** 1976. Distribution of cytochrome-like respiration in streptococci. J. Gen. Microbiol. **93:**195–203.
36. **Schonbaum, G. R., and B. Chance.** 1976. Catalase, p. 363–408. *In* P. D. Boyer (ed.), The enzymes, vol. 13. Academic Press, Inc., New York.
37. **Shklair, I. L., and H. J. Keene.** 1976. Biochemical characterization and distribution of *Streptococcus mutans* in three diverse populations, p. 201–210. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Microbial aspects of dental caries. Sp. Suppl. Microbiol. Abstr., vol. 1.
38. **Singer, T. P., and D. E. Edmondson.** 1974. Biological reduction of O_2 to H_2O_2, p. 315–337. *In* O. Hayaishi (ed.), Molecular oxygen in biology: topics in molecular oxygen research. North-Holland, Amsterdam.
39. **Terleckyj, B., N. P. Willett, and G. D. Shockman.** 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11:**649–655.
40. **Thomas, E. L., K. P. Bates, and M. M. Jefferson.** 1981. Peroxidase antimicrobial system of human saliva: requirements for accumulation of hypothiocyanite. J. Dent. Res. **60:**785–796.
41. **Thomas, E. L., and M. Fishman.** 1982. Hydrogen peroxide release by rat peritoneal macrophages in the presence and absence of tumor cells. Arch. Biochem. Biophys.

215:355–366.

42. **Thomas, E. L., K. A. Pera, K. W. Smith, and A. K. Chwang.** 1983. Inhibition of *Streptococcus mutans* by the lactoperoxidase antimicrobial system. Infect. Immun. **39**:767–768.
43. **Vance, P. G., B. B. Keele, Jr., and K. V. Rajagopolan.** 1972. Superoxide dismutase from *Streptococcus mutans*. Isolation and characterization of two forms of the enzyme. J. Biol. Chem. **247**:4782–4786.
44. **Walker, G. A., and G. L. Kilgour.** 1965. Pyridine nucleotide oxidizing enzymes of *Lactobacillus casei*. II. Oxidase and peroxidase. Arch. Biochem. Biophys. **11**:534–539.
45. **Whittenbury, R.** 1978. Biochemical characteristics of streptococcus species, p. 51–69. *In* F. A. Skinner and L. B. Quesnel (ed.), Streptococci. Academic Press, Inc., New York.