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The Microaerophile *Spirillum volutans*: Cultivation on Complex Liquid and Solid Media

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Spirillum volutans grows only under microaerobic conditions in a peptone-succinate-salts broth, but can grow aerobically when the peptone is replaced by vitamin-free acid-hydrolyzed casein broth. The addition of potassium metabisulfite, norepinephrine, catalase or superoxide dismutase (SOD) permitted aerobic growth in peptone-succinate-salts broth. A combination of catalase and SOD had a synergistic effect. *S. volutans* lacked catalase and had only a low level of peroxidase activity, but did possess SOD activity (12 to 14 U/mg of protein). The organism was found to be extraordinarily sensitive to exogenous hydrogen peroxide. Illumination of peptone-succinate-salts broth generated hydrogen peroxide and rendered the medium inhibitory to growth. A combination of catalase and SOD prevented this inhibition. Growth of *S. volutans* on solid media, not previously possible, was accomplished by the use of vitamin-free acid-hydrolyzed casein and peptone-succinate-salts agar media; maximum growth responses were dependent on the following combination of factors: addition of bisulfite, catalase, or SOD, protection of the media from illumination, incubation in a highly humid atmosphere, and incubation under atmospheres of 12% oxygen or less. The results indicate that the microaerophilic nature of *S. volutans* is attributable largely to the high sensitivity of the organism to exogenous hydrogen peroxide and, to a lesser extent, superoxide radicals occurring in the culture medium.

Spirillum volutans is a large, helical bacterium that occurs in stagnant freshwater sources (17, 19, 24). Even during enrichment it is vastly outnumbered by other bacteria; for this reason and also because it fails to form colonies on solid media, *S. volutans* has been isolated only by use of a capillary tube method which allows it to outswim contaminants (21). Purified strains at first could be cultured only in dialysis sacs suspended in cultures of other bacteria (21), but the subsequent recognition that the organism is a microaerophile led to successful cultivation in broth media under atmospheres of 1 to 9% oxygen (24). All efforts to obtain colonies on solid media have so far been unsuccessful despite the use of microaerobic conditions.

Bowdre and Krieg (3) subsequently were able to grow the organism under an air atmosphere (21% oxygen) in casein hydrolysate-succinate-salts (CHSS) broth provided that a particular type of acid hydrolyzed casein was used. Bowdre et al. (4) later devised a defined medium which could also support aerobic growth provided the medium contained low levels of dihydroxyphenyl compounds such as norepinephrine (NE). Similar results were obtained with another microaeroerophile, *Campylobacter fetus*, by using NE or high levels of ferrous or ferric salts (15). Bowdre et al. (4) proposed that NE might increase the availability of ferric iron for *C. fetus* and *S. volutans*, and that the microaerophilic nature of the organism might be related to some iron-dependent physiological process in the cells. More recent studies of *C. fetus*, however, have indicated that this organism is extraordinarily sensitive to the superoxide radicals or hydrogen peroxide which occur spontaneously or by photochemical processes in the culture medium, and that the diverse agents capable of enhancing the aerotolerance of *C. fetus* (NE, superoxide dismutase [SOD], catalase, pyruvate, and a combination of an iron salt and sodium metabisulfite) all have the ability to quench these toxic forms of oxygen (14). Low oxygen tensions would also minimize the formation of superoxide radicals and hydrogen peroxide in the culture medium.

In this investigation, the effect of SOD and catalase on the growth of *S. volutans* is examined. We also report for the first time a method

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to isolate colonies of *S. volutans* on solid medium.

MATERIALS AND METHODS

Media. The composition of modified peptone-succinate-salts (MPSS) broth was as follows (grams per liter): peptone (Difco), 5.0; succinic acid (free acid), 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $FeCl_3 \cdot 6H_2O$, 0.002; and $MnSO_4 \cdot H_2O$, 0.002. pH was adjusted to 7.0 with KOH (5). *S. volutans* grows only under microaerobic conditions (i.e., an atmosphere of 1 to 12% oxygen) in this medium from a small inoculum (i.e., 20 cells per ml, final concentration). However, it grows under an air atmosphere (21% oxygen) in a modification of the medium, CHSS broth (3), in which peptone is replaced by 2.5 g of vitamin-free, salt-free acid hydrolyzed casein (ICN Nutritional Biochemicals, Cleveland, Ohio). A decrease in the level of the peptone component of MPSS broth to 0.25% (to correspond to the level of casein hydrolysate in CHSS broth) failed to support aerobic growth.

For growth experiments under aerobic conditions (static incubation under an air atmosphere), 10-ml portions of liquid media were contained in 20- by 125-mm tubes closed with loosely fitting screw caps. For growth experiments under microaerobic conditions, 5-ml portions of media were contained in 20- by 125-mm tubes stoppered with cotton and incubated under a 6% oxygen atmosphere.

CHSS agar medium was prepared by incorporating 1.5% agar (Difco, bacteriological grade) into CHSS broth, autoclaved, and dispensed at 45°C into plastic petri dishes.

Liquid and solid media were stored at room temperature for at least 24 h before use, to allow equilibration with an air atmosphere. Storage was done in the dark because, as noted by Bowdre and Krieg (3), media stored under ordinary room illumination became unable to support growth.

Organism and maintenance. *S. volutans* ATCC 19554 was used throughout the study and was maintained by daily transfer in 100 ml of CHSS broth (containing 0.01% NaCl) contained in 250-ml cotton-stoppered Erlenmeyer flasks with static incubation under an air atmosphere at 30°C. One milliliter of each previous culture served as the inoculum for the next.

Gaseous conditions. For microaerobic conditions, tubes of broth or plates of solid media were placed into household pressure cookers which had gaskets cemented into place by silicone rubber caulking. The air within the vessels was partially evacuated and replaced by nitrogen gas to give the desired level of oxygen. For 21% oxygen atmospheres, the vessels were not evacuated, and their vents were left open to the air.

Preparation of inocula. The inoculum for liquid media was prepared by centrifuging a 24-h log phase culture at 3,000 × *g*. The cells were washed twice in the defined motility medium of Caraway and Krieg (5) and suspended in 10 ml of defined motility medium. The cells were enumerated with a haemocytometer and diluted in defined motility medium to provide an inoculum of 2×10^3 cells per ml of test medium.

For inoculation of solid media, 0.1 ml from a 24-h culture in CHSS broth was spread evenly over the surface of agar plates. In later experiments involving colony counts, the cell concentration in 24 h cultures grown in CHSS broth was determined with a hemocytometer, and the cultures were diluted with defined motility medium to give ca. 3×10^3 cells per ml. Samples (0.1 ml) of this suspension were then spread on the surface of the test plates.

Supplements for test media. Bovine erythrocyte SOD (Sigma Chemical Co., St. Louis, Mo.) and bovine liver catalase (Sigma) were prepared as sterile stock solutions and added aseptically to test media to give the desired concentrations. For agar media, the enzymes were added to the molten media at 45°C just before dispensing into petri dishes. The lot of SOD used had no detectable catalase activity when assayed by the method of Beers and Sizer (1) and no detectable *o*-dianisidine (ODD) peroxidase activity when assayed by the method of Guidotti et al. (11) with hydrogen peroxide and ODD. The catalase used had no detectable SOD activity when assayed by the method of McCord and Fridovich (20).

Sterile stock solutions of NE were added aseptically to liquid media. Hydrogen peroxide was diluted from a 30% stock solution into sterile liquid media to give the desired concentrations. Potassium metabisulfite was incorporated into liquid or solid media before heat sterilization of the media.

All media containing supplements were stored for at least 24 h in the dark under an air atmosphere before inoculation.

Effect of illumination on culture media. For liquid media, 10-ml portions contained in 20- by 125-mm cotton-stoppered tubes were prepared 24 h in advance and stored in the dark under an air atmosphere. The tubes were then exposed uniformly to 10,760 lx from a battery of white fluorescent lamps for various periods before inoculation. Control tubes were stored continuously in the dark. Bovine catalase or SOD was added to some tubes before or after illumination, but always before inoculation. After inoculation, all tubes were incubated at 30°C in the dark under aerobic or microaerobic conditions.

For solid media, CHSS agar was dispensed in 20-ml portions into petri dishes under dim illumination (less than 54 lx from an orange photographic safelight). After solidification and storage in the dark for 24 h, plates were exposed uniformly to 8,600 lx from a battery of white fluorescent lamps for various periods before inoculation. Control plates were stored continuously in the dark. After inoculation, all plates were incubated in the dark at 30°C under aerobic or microaerobic conditions.

For detecting the photochemical generation of hydrogen peroxide in liquid media, the oxidation of ODD (80 μg/ml) in the presence of horseradish peroxidase (Sigma; 20 μg/ml) was monitored in the presence and absence of catalase (130 U/ml) by using a Klett-Summerson colorimeter at 540 nm with 16-mm cuvettes. For detecting the generation of superoxide radicals (O_2^-), the reduction of Nitro Blue Tetrazolium was monitored in the presence and absence of bovine SOD (85 U/ml) at 540 nm.

Preparation of cell extracts and methods for enzyme assays. Cells were grown statically in MPSS broth under conditions of low oxygenation and also with agitation in CHSS broth under conditions of high oxygenation. For static incubation, the method employed by Cole and Rittenberg (7) was used. Cultures

were then inoculated immediately from a 24-h CHSS broth culture and incubated at 30°C without agitation under an air atmosphere for 48 h. For cultivation with agitation, CHSS broth was inoculated as described above and incubated statically for 4 h to promote initial growth. The flasks were then incubated on a reciprocal shaking machine at 50 oscillations per min under an air atmosphere for 48 h. For both types of cultures, cells were harvested at 10,000 × *g*, washed twice in potassium phosphate buffer (20 mM, pH 7.4), and suspended in 10 ml of buffer. They were disrupted by two passages through a chilled French pressure cell at 20,000 lb/in^2 and centrifuged at 19,500 × *g* for 10 min to remove intact cells and large debris. After additional centrifugation at 100,000 × *g* for 1.5 h, the supernatant fluid was assayed for total SOD activity by the method of McCord and Fridovich (20). Catalase activity was assayed by the method of Beers and Sizer (1), and ODD peroxidase activity was assayed by the method of Guidotti et al. (11) as modified by Hoffman et al. (15) by using a value of 9,450 M^{-1} cm^{-1} for the ϵ of ODD at 450 nm. Cytochrome *c* peroxidase was assayed as described by Ellfolk and Soininen (10). NADH peroxidase was assayed as described by Dolin (9).

Stability of enzymes added to liquid media. Bovine SOD or catalase was added aseptically from sterile stock solutions to 10-ml portions of sterile MPSS broth. Enzyme activities were assayed immediately, after illumination by 96,000 lx for 1 h followed by storage in the dark for 24 h and after storage in the dark for 7 days. Assay methods were as described for cell extracts.

Enzyme-like activities of MPSS and CHSS broth. SOD-, catalase- and ODD peroxidase-like activities were assayed in MPSS and CHSS broth by the same methods used for cell extracts.

RESULTS

Requirement for sodium ions in the growth medium. The particular lot of CHSS (see above) used by Bowdre and Krieg (3) in their investigation was also found to support the aerobic growth of *S. volutans* in this study. However, a new lot purchased from the same manufacturer did not allow growth of *S. volutans* either aerobically or microaerobically. Flame photometry analysis of a 10% (wt/vol) solution of the two lots revealed that the new lot contained 2 mM Na^+, whereas the old lot contained 65 mM Na^+. The addition of 0.01 to 0.02% NaCl to the new lot supported aerobic growth of *S. volutans*. Since an equivalent level of Na_2SO_4 similarly supported growth whereas KCl did not, it appears that *S. volutans* requires a low level of Na^+ for growth. The organism is inhibited by NaCl levels greater than 0.02%. However, the addition of various levels of NaCl to MPSS broth did not allow this medium to support aerobic growth.

Aerobic growth in MPSS broth. Unsupplemented MPSS broth supported growth of *S. volutans* from a small inoculum (final concentration, 20 cells per ml) under a 6% oxygen atmosphere, but not under an air atmosphere. However, when the medium was supplemented with SOD (4 U/ml), catalase (0.8 U/ml), potassium metabisulfite (90 μM, or 0.02%), or NE (10 μM, or 0.002%), visible turbid growth (at least 10 Klett units of turbidity as measured at 660 nm with a Klett colorimeter) occurred aerobically within 48 h. Heat-inactivated SOD or catalase was ineffective. When SOD and catalase were used in combination, a synergistic effect occurred (Table 1); whereas SOD at 0.8 U/ml or catalase at 0.4 U/ml failed to support aerobic growth, they were effective at concentrations as low as 0.08 U/ml when used in combination.

Effect of illumination of MPSS and CHSS broth on growth. Unsupplemented MPSS broth that had been exposed to 10,760 lx for 2 h or more before inoculation failed to support growth of *S. volutans* under a 6% oxygen atmosphere. However, when supplemented with potassium metabisulfite, MPSS broth illuminated for 2 h did support growth even under an air atmosphere (Table 2). Neither SOD nor catalase when used separately permitted aerobic growth to occur in MPSS broth that had been illuminated for 2 h; however, a combination of the two enzymes did permit aerobic growth even in MPSS broth that had been illuminated for 8 h. This result was obtained regardless of whether the enzymes were added to the medium before or after illumination, indicating that the enzymatic activity

TABLE 1. Effect of SOD and catalase singly and in combination on the aerobic growth of *S. volutans* in MPSS broth

Supplements		Growth response at 48 h[a]
SOD (U/ml)	Catalase (U/ml)	
None	None	0
None	4.0	12
4.0	None	11
4.0	4.0	11
None	0.8	11
0.8	None	0
0.8	0.8	12
None	0.4	0
0.4	None	0
0.4	0.4	12
None	0.08	0
0.08	None	0
0.08	0.08	12

[a] The growth response is expressed in Klett units of turbidity at 660 nm. The values represent the mean from triplicate cultures. Initial concentration of cells was 20 cells per ml.

TABLE 2. Effect of SOD and catalase on the aerobic growth of *S. volutans* in MPSS broth exposed to illumination before inoculation[a]

Supplement added to MPSS broth	Growth response at 48h[b]		
	0 h[c]	2 h[c]	8 h[c]
None	−	−	−
Potassium meta-bisulfite, 9×10^{-5} M	+	+	−
Catalase, 130 U/ml	+	−	−
SOD, 58 U/ml	+	−	−
Catalase + SOD	+	+	+
Heat-denatured SOD + catalase	−	−	−

[a] Results are a summary of two similar experiments, each performed with duplicate tubes. Illumination was 10,760 lx from a white, fluorescent lamp.

[b] Symbols: +, visible turbidity; −, no turbidity. Inoculum size was 200 cells; final concentration was 20 cells per ml.

[c] Hours of exposure of broth to illumination before inoculation.

was directed to a toxic product formed during illumination.

In contrast to the inhibitory effects caused by illumination of MPSS broth, illumination of CHSS broth for 8 h failed to abolish the ability of the medium to support aerobic growth of *S. volutans*.

Generation of hydrogen peroxide in MPSS and CHSS broth. Horseradish peroxidase-catalyzed oxidation of ODD occurred during illumination of MPSS and CHSS broth, but was negligible when the media were incubated in the dark (Fig. 1 and 2). The rate of ODD oxidation in illuminated CHSS broth was ca. 15% less than that in MPSS broth. Catalase partially inhibited the oxidation in both media, indicating that some hydrogen peroxide was being photochemically generated. Not all of the ODD oxidation could be attributed to hydrogen peroxide, however, since oxidation occurred even in the absence of horseradish peroxidase; some of this may have been due to the concurrent generation of superoxide radicals (19). Illumination of tubes of MPSS and CHSS broth before the addition of the horseradish peroxidase ODD system also

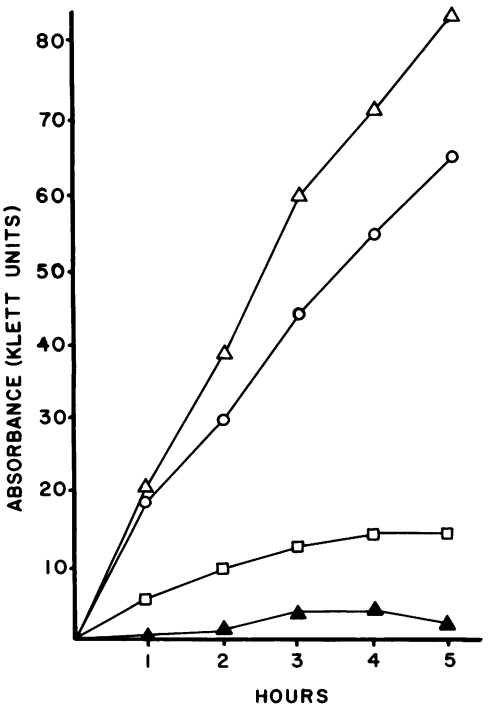

FIG. 1. Photochemical generation of hydrogen peroxide in MPSS broth. The rate of oxidation of ODD (80 μg/ml) was monitored at 540 nm. Symbols: △, MPSS broth + ODD + horseradish peroxidase (HRP; 20 μg/ml), exposed to 10,760 lx of white fluorescent light; ○, MPSS broth + ODD + HRP + catalase (130 U/ml), exposed to light; □, MPSS broth + ODD, exposed to light; ▲, MPSS broth + ODD + HRP, incubated in the dark.

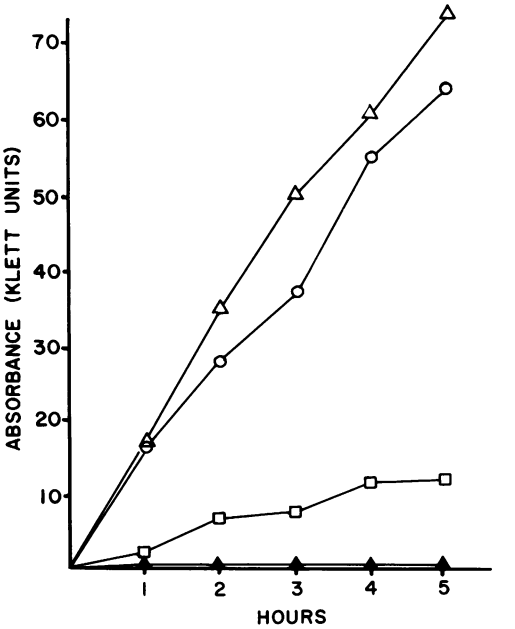

FIG. 2. Photochemical generation of hydrogen peroxide in CHSS broth. The rate of oxidation of ODD (80 μg/ml) was monitored at 540 nm. Symbols: △, CHSS broth + ODD + horseradish peroxidase (HRP; 20 μg/ml), exposed to 10,760 lx of white fluorescent light; ○, CHSS broth + ODD + HRP + catalase (130 U/ml), exposed to light; □, CHSS broth + ODD, exposed to light; ▲, CHSS broth + ODD + HRP, incubated in the dark.

TABLE 3. Effect of illumination of MPSS and CHSS broth before the addition of ODD and horseradish peroxidase

Medium	Additives[a]	OD_{540}[b]	H_2O_2(μM)
New lot casein hydrolysate[b]	CHSS	0	
	CHSS + ODD	0	
	CHSS + ODD + HRP	0.0185	12.56
	CHSS + ODD + HRP + Cat	0.007	
Old lot casein hydrolysate	CHSS	0	
	CHSS + ODD	0	
	CHSS + ODD + HRP	0.026	17.65
	CHSS + ODD + HRP + Cat	0.0075	
	MPSS	0	
	MPSS + ODD	0	
	MPSS + ODD + HRP	0.0315	21.38
	MPSS + ODD + HRP + Cat	0.004	

[a] Final concentrations of additives: ODD, 80 μg/ml; horseradish peroxidase (HRP), 20 μg/ml; catalase (Cat), 130 U/ml. Tubes containing 5 ml of medium were exposed to 10,760 lx of white fluorescent light for 3 h. At the end of 3 h catalase was added to one tube of each set and incubated for 5 min at room temperature. The other additives were then added to all tubes. The tubes were incubated for 10 min and then the absorbance was read at 540 nm. All tubes were run in duplicate.

[b] OD_{540}, Optical density at 540 nm.

resulted in the oxidation of ODD (Table 3). This indicated that hydrogen peroxide was indeed generated in the media and that the observed color change was not due to photochemical oxidation of the ODD. Direct measurement of hydrogen peroxide production by the Beers and Sizer method (1) was not successful since both MPSS and CHSS broth absorb too strongly at 240 nm to be an effective blank. Attempts were also made to use the method of Carlsson et al. (6) for the measurement of hydrogen peroxide production in anaerobic medium. This method, however, proved to be unsuitable to measure the small amounts of H_2O_2 being produced. In MPSS broth illuminated for 5 h (Fig. 1), the amount ODD oxidation that was inhibitable by catalase corresponded to a hydrogen peroxide concentration of 21 μM; this was at least 70 times the level of hydrogen peroxide required to inhibit the growth of *S. volutans* (see below). Although hydrogen peroxide generation could not be definitively demonstrated in either medium in the dark, the previously described effectiveness of catalase in enhancing aerotolerant growth of *S. volutans* implies that low levels of hydrogen peroxide might indeed have been present, but at levels below the limit of detection by ODD. As shown below, even extremely low levels of hydrogen peroxide can inhibit the growth of *S. volutans*.

Tolerance of *S. volutans* to hydrogen peroxide. An extraordinarily high sensitivity of *S. volutans* to hydrogen peroxide was indicated by the addition of decimal concentrations of this agent to a series of duplicate tubes of CHSS broth inoculated with 2×10^3 cells and incubated under an air (21% oxygen) atmosphere. In two separate experiments, growth (measured by observing turbidity) occurred within 72 h in all tubes containing 0.029 μM or less hydrogen peroxide, but not in any tube containing 0.29 μM or more of the agent. This sensitivity was far greater than that reported for *Campylobacter fetus* subsp. *jejuni* (35 μM) by Hoffman et al. (14) or for *Escherichia coli* (14.71 mM) by Yoshpe-Purer and Henis (25).

Generation of superoxide radicals in MPSS and CHSS broth. In contrast to results reported previously for Brucella broth (14), only very low levels of reduction of nitro blue tetrazolium occurred in MPSS and CHSS broth, even when the media were strongly illuminated. Although superoxide radical generation could not be clearly demonstrated in media, the effectiveness of SOD in enhancing aerotolerant growth of *S. volutans* and also the synergistic effect of a combination of SOD and catalase on aerotolerant growth indicate that low levels of superoxide radicals may have been present, but at levels below the limit of detection by the system employed.

Cultivation of *S. volutans* on solid media. Based on the ability of CHSS broth to support aerobic growth of *S. volutans*, a solid CHSS medium was prepared by the addition of 1.5% agar. However, growth failed to occur on this medium at any oxygen level.

Since light inhibited growth in broth, stringent precautions were taken to protect plates of CHSS agar from illumination. Growth now oc-

curred on this medium at oxygen levels of 12% or less. Results were erratic in that growth did not always occur from one experiment to the next. Growth did invariably occur, however, when the medium was supplemented with 0.002% potassium metabisulfite. Lower levels of bisulfite were not effective, and higher levels were inhibitory. Photographs of typical colonies growing on CHSS bisulfite agar are presented in Fig. 3. Catalase (230 U/ml) and bovine SOD (30 U/ml) also allowed consistent growth responses. Results identical to those with supplemented CHSS agar were subsequently obtained with supplemented MPSS agar.

Although >300 colonies could be obtained consistently from an inoculum of 0.1 ml from a 24-h broth culture, provided that the supplemented media were protected from illumination, more precise quantification of the growth response indicated that erratic results were still occurring. For example, when dilutions of a broth culture were spread onto a series of replicate plates, so that each plate was inoculated with 300 cells (as determined by a haemocytometer), the resulting number of colonies per plate might vary from 2 to 140. Colonies from these plates were grown up in broth and replated onto both MPSS and CHSS agar. The resulting number of colonies per plate was still highly erratic. Alterations in the diluent, the spreading technique, the orientation of the plates within the culture vessel, or the concentration of agar used had no effect on this variability; however, the relative humidity during incubation was found to be an important factor. When plates were stored for 24 h in the dark in an atmosphere having a relatively high humidity (achieved by lining the culture vessel with wet filter paper) before inoculation and incubated after inoculation for 5 days under similar highly humid conditions, more reproducible results were obtained. In many experiments with duplicate or triplicate plates of MPSS agar supplemented with bisulfite, SOD, or catalase and incubated under moist conditions, the colony counts per plates differed from the mean colony count for a set of plates by ±0 to 34%, with an average of ±14%. The percent recovery (mean colony count for a set of

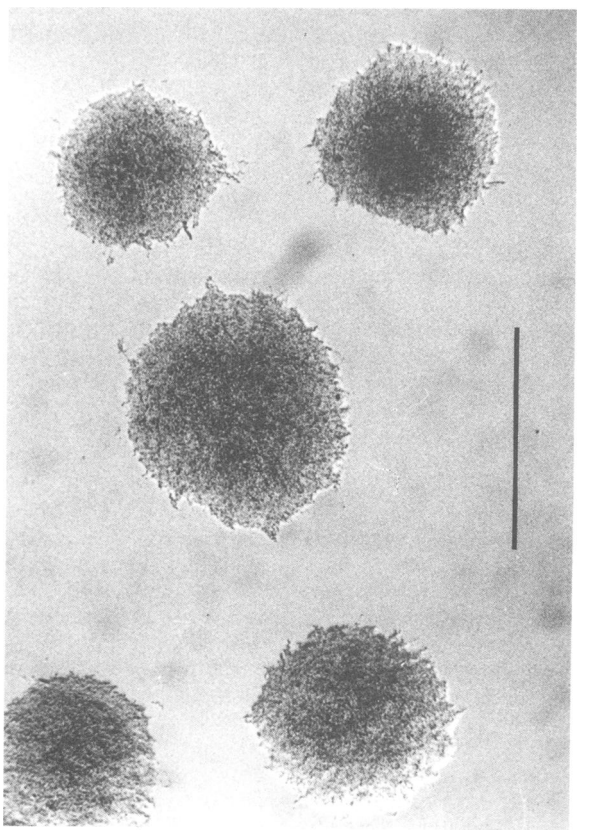

FIG. 3. Colonies of *S. volutans* cultured under a 6% oxygen atmosphere on CHSS agar containing 0.002% potassium bisulfite at 30°C under humid conditions for 5 days. Bar, 0.5 mm.

plates divided by the number of cells inoculated, multiplied by 100) averaged 42% and ranged from 22 to 72%. In contrast, when wet filter paper was not used in the culture vessels the colony counts per plate differed from the mean colony counts per plate differed from the mean colony count for a set of plates by ±14 to 186%, with an average of ±79%. The percent recovery these results, it can be seen that even with the beneficial effect of humid conditions, supplements, and protection from illumination, quantification of growth of *S. volutans* by means of colony counts was still not entirely satisfactory, and that other unknown factors appeared to be involved in the variability that still occurs.

With regard to potassium metabisulfite, its effectiveness in promoting colony formation on MPSS agar was dependent on the simultaneous presence of the $FeCl_3$ component of the medium, even though the peptone component of the medium contains a low level of iron. This was in accordance with previous studies by Hoffman et al. (14) in which sodium metabisulfite was ineffective in stimulating the growth of *C. fetus* subsp. *jejuni* unless used in combination with a high level of an iron salt.

In contrast to experiments with MPSS broth, potassium metabisulfite failed to support colony formation on MPSS agar (or CHSS agar) at oxygen levels greater than 12%, although an occasional colony would appear on plates incubated under a 14% oxygen atmosphere. Catalase, SOD, or a combination of catalase plus SOD similarly failed to support growth at oxygen levels higher than 12%.

Although potassium metabisulfite, catalase, or SOD failed to increase the aerotolerance of *S. volutans* at oxygen levels of >12%, catalase was able to prevent the inhibitory effect caused by strong illumination of MPSS agar. As shown in Table 4, only plates containing catalase permitted growth when the medium had been subjected to 10,760 lx before inoculation. This suggests that the photochemical generation of hydrogen peroxide, rather than superoxide radicals, was the major cause of the inhibitory effect produced by illumination.

Stability of enzymes in media. The stability of SOD and catalase in solid media could not be assayed, since the media could not be liquefied without denaturing the enzymes. However, incubation of SOD or catalase in MPSS broth in the dark for 7 days under a 6% oxygen atmosphere yielded residual activities of 42 and 51%, respectively. Moreover, illumination of MPSS broth by 10,000 lx for 1 h followed by storage in the dark for 24 h yielded residual activities of 58 and 73%, respectively. Consequently, in growth experiments with enzyme-supplemented media, a proportion of the original activity of the added enzymes would have been present throughout the incubation period.

Enzyme-like activities of MPSS and CHSS broth. Both MPSS and CHSS broth possessed a low level of SOD-like activity when assayed by the method of McCord and Fridovich (20). MPSS broth exhibited an activity of 0.48 U/ml, whereas CHSS broth had an activity of 0.64 U/ml. The activity in MPSS broth was increased to 1.0 U/ml by the addition of 0.002% potassium metabisulfite. This increase was in accordance with previous results obtained by Hoffman et al. (14) using brucella broth, in which a combination of sodium metabisulfite plus an iron salt had apparent SOD-like activity (8). No catalase-like activity was detected.

Enzyme activities of cell extracts. The soluble fraction of cell extracts exhibited an SOD activity of 12 to 14 U/mg of protein, regardless of whether the cells were cultured under low oxygenation in MPSS broth or under high oxygenation in CHSS broth, indicating that the activity

TABLE 4. Ability of various supplements to prevent the inhibitory effects of illumination of MPSS agar on the growth of *S. volutans*

Incubation atmosphere	Illumination of plates before inoculation	Mean colony count[a]							
		No supplements	Potassium metabisulfite, 0.002%	SOD, 30 U/ml	Catalase, 130 U/ml	SOD, 15 U/ml + catalase, 65 U/ml	Heat-inactivated SOD	Heat-inactivated catalase	Heat-inactivated SOD + heat inactivated catalase
6% oxygen	None	166 ± 45	163 ± 39	160 ± 55	233 ± 22	200 ± 0	153 ± 8	200 ± 15	192 ± 23
	10,760 lx for 1 h	0	0	0	155 ± 15	100 ± 0	0	0	0
12% oxygen	None	135 ± 10	140 ± 7	145 ± 5	173 ± 58	150 ± 10	145 ± 30	143 ± 18	108 ± 13
	10,760 lx for 1 h	0	0	0	70 ± 20	110 ± 10	0	0	0

[a] The inoculum size was 340 cells per plate. The values given represent the mean colony count from duplicate or triplicate plates and also the range of variation.

was not inducible. The activity of ODD peroxidase was very low (0.0006 U/mg of protein) and could only be demonstrated when the protein concentration of the extracts was very high (>10 mg of protein per ml). This activity was not affected by oxygenation of the cultures. Catalase, cytochrome *c* peroxidase, and NADH peroxidase activities were not detectable under any conditions of oxygenation (<0.7 U/mg of protein, <0.5 U/mg of protein, and <0.005 U/mg of protein, respectively).

DISCUSSION

Although not related to aerotolerance, the finding that a low level of NA^+ is an absolute requirement for growth of *S. volutans* in CHSS broth is interesting in view of the low NaCl tolerance of this organism and other freshwater spirilla (18). What the function of Na^+ is for the metabolism of *S. volutans* is not known. Hylemon et al. (16) found that transport of fructose by *Aquaspirillum itersonii* was greatly stimulated by Na^+. Although *S. volutans* does not use fructose or any other carbohydrate, it is nevertheless possible that Na^+ might be required for the maintenance of a suitable membrane potential to drive transport of other substrates such as succinate.

This investigation reports the first successful cultivation of *S. volutans* on solid media, which should now make it possible to improve the isolation methods for this organism or to select various types of mutants. The most important factors involved in cultivation are (i) scrupulous protection of the media from exposure to illumination, (ii) inclusion of bisulfite, SOD, or catalase in the media (bisulfite being the most convenient to use, since it can be added before heat sterilization of the media), and (iii) incubation of the cultures in an atmosphere of high humidity.

Growth on a solid medium appears to offer more rigorous conditions for testing aerotolerance than growth in a liquid medium. This conclusion is based on the ability of *S. volutans* to grow aerobically (with static incubation) in CHSS broth, or in MPSS broth supplemented with NE, bisulfite, SOD, or catalase, whereas growth does not occur on solid media, even with supplements, under oxygen levels greater than 12%. Although liquid media were stored under an air atmosphere for 24 h before inoculation, the surface/volume ratio of such media in tubes is relatively low compared with that for solid media contained in petri dishes; therefore, liquid media may provide a less readily available or renewable supply of oxygen compared with a solid medium where the cells are in direct contact with the oxygen atmosphere. This is further supported by the failure of small inocula (2×10^3 cells per 100 ml) to grow in CHSS broth or in supplemented MPSS broth under conditions of agitation.

The addition of catalase or bovine SOD to MPSS broth allowed growth to occur under aerobic conditions (with static incubation), providing circumstantial evidence for the occurrence of superoxide radicals and hydrogen peroxide in the culture medium. The inability to demonstrate these toxic forms of oxygen in nonilluminated media suggests that their levels are very low; however, this is compatible with the finding that *S. volutans* is inhibited by extremely low levels of exogenous hydrogen peroxide. In the case of the stimulatory effects of catalase added to MPSS broth, the organisms themselves possessed no catalase activity; therefore, the added catalase might be presumed to fulfill this deficiency. However, the added catalase would have been unable to penetrate the cells and, therefore, must have exerted its action externally, presumably on the hydrogen peroxide in the culture medium. A similar situation has been reported by Blakemore et al. (2) for a magnetotactic spirillum: this organism was an obligate microaerophile and lacked catalase activity, but could be cultured aerobically when catalase was added to the medium. In this case, however, aerobic growth could be achieved on a solid medium, whereas in the present study *S. volutans* could grow on solid media only at oxygen levels of 12% or less. In a previous study, the aerotolerance of *C. fetus* subsp. *jejuni* was enhanced by catalase, even though the cells possessed catalase activity (14); this indicated that the cytoplasmic enzyme was ineffective against exogenous hydrogen peroxide. Sneath (22) reported that *Chromobacterium violaceum*, a species possessing catalase activity, grew poorly on meat digest agar unless the medium was treated with catalase or unless anaerobic conditions were used. Waterworth (23) found that a variety of catalase-positive bacteria such as *Escherichia coli* and *Staphylococcus aureus* grew poorly on agar media when used in a brightly lit laboratory, but that catalase added to the media prevented this inhibition.

The effect of bovine SOD in enhancing aerotolerant growth of *S. volutans* in MPSS broth similarly implicates superoxide radicals in the culture medium, even though the cells possess relatively high levels of SOD activity. Moreover, the synergistic enhancement of aerotolerance by a combination of SOD and catalase indicates that both hydrogen peroxide and superoxide radicals are involved in the oxygen sensitivity of the organism. However, it is also possible that hydroxyl radicals which could be formed by interaction of hydrogen peroxide and superoxide radicals in the presence of a transition metal such as iron (12) might be involved.

This could provide an alternative explanation for both the individual and synergistic effects of the two enzymes.

That exogenous hydrogen peroxide or superoxide radicals may not be solely responsible for the microaerophilic behavior of *S. volutans* is suggested by the failure of bisulfite, catalase, SOD, or a combination of catalase and SOD to allow growth of *S. volutans* on solid media under oxygen levels greater than 12%. It is possible that endogenously generated hydrogen peroxide, which would not be accessible to catalase in the culture medium, might be a contributory factor, particularly since the cells have no catalase activity and only a very low peroxidase activity. As suggested in a study of the oxygen metabolism of *C. fetus* subsp. *intestinalis*, oxygen sensitivity might be due in part to autooxidation of iron-sulfur proteins, which could give rise to hydrogen peroxide or superoxide radicals, and the beneficial effects of a combination of bisulfite and iron salts might possibly involve interactions with such proteins (13).

The effectiveness of unsupplemented CHSS broth in allowing aerobic growth, in contrast to MPSS broth which requires supplements, may be related to the higher level of SOD-like activity of the former medium. Also, the slower rate of photochemical generation of hydrogen peroxide in CHSS broth suggests that production of hydrogen peroxide may occur with greater difficulty in this medium.

The results of this study support the proposal by Hoffman et al. (14) that microaerophiles may be microaerophilic largely due to their high sensitivity to superoxide radicals or hydrogen peroxide occurring in the culture medium. Under microaerobic conditions, these toxic forms of oxygen would be produced to a lesser extent than under aerobic conditions. The chemical agents that enhance aerotolerance appear to act by quenching the exogenous toxic forms of oxygen rather than by affecting some physiological process within the cells.

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