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Production of Superoxide and Hydrogen Peroxide in Medium Used To Culture *Legionella pneumophila*: Catalytic Decomposition by Charcoal

P. S. HOFFMAN,[1]* L. PINE,[2] AND S. BELL[3]

Microbiology Section, Department of Biology, Memphis State University, Memphis, Tennessee 38152[1]; Bacteriology Branch, Bacterial Diseases Division, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]; and Department of Microbiology, University of Georgia, Athens, Georgia 30602[3]

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The difficulties associated with the growth of *Legionella* species in common laboratory media may be due to the sensitivity of these organisms to low levels of hydrogen peroxide and superoxide radicals. Exposure of yeast extract (YE) broth to fluorescent light generated superoxide radicals (3 μM/h) and hydrogen peroxide (16 μM/h). Autoclaved YE medium was more prone to photochemical oxidation than YE medium sterilized by filtration. Activated charcoals and, to a lesser extent, graphite, but not starch, prevented photochemical oxidation of YE medium, decomposed hydrogen peroxide and superoxide radicals, and prevented light-accelerated autooxidation of cysteine. Also, suspensions of charcoal in phosphate buffer and in charcoal yeast extract medium readily decomposed exogenous peroxide (17 and 23 nmol/ml per min, respectively). Combinations of bovine superoxide dismutase and catalase also decreased the rate of photooxidation of YE medium. Medium protected from light did not accumulate appreciable levels of hydrogen peroxide, and autoclaved YE medium protected from light supported good growth of *Legionella micdadei*. Various species of *Legionella* (10^4 cells per ml) exhibited sensitivity to relatively low levels of hydrogen peroxide (26.5 μM) in challenge experiments. The level of hydrogen peroxide that accumulated in YE medium over a period of several hours (>50 μM) was in excess of the level tolerated by *Legionella pneumophila*, which contained no measurable catalase activity. Strains of *L. micdadei*, *Legionella dumoffii*, and *Legionella bozmanii* contained this enzyme, but the presence of catalase did not appear to confer appreciable tolerance to exogenously generated hydrogen peroxide.

The addition of activated charcoal to some bacteriological media substantially enhances the isolation and growth of many bacterial pathogens (2, 9, 23). Activated charcoal (Norit A and Norit SG)-supplemented yeast extract medium (CYE medium) is widely used for the isolation and culture of *Legionella pneumophila* and related species (5, 6). *L. pneumophila* does not grow in autoclaved yeast extract medium (YE medium) in the absence of added charcoal; however, if the YE medium is sterilized by filtration, the medium supports good growth from large inocula (13, 25). Ristroph et al. (25) suggested that unknown toxic products may be released into the medium during autoclaving. It is generally assumed that these toxic compounds may be fatty acids since charcoal can adsorb and thereby detoxify free fatty acids (2, 28). In this regard, Pine et al. (22) reported that in a chemically defined medium, oleic acid is toxic for *L. pneumophila* unless it is added with starch. However, starch does not substitute for charcoal in autoclaved YE medium, suggesting that there are additional mechanisms for the stimulatory action of charcoal.

Activated charcoals have good adsorptive properties and are commonly used for adsorbing pollutants out of water and air (27). Charcoal is also a weak reducing agent and can participate in oxidation-reduction reactions (27, 29). Most of the studies on the mechanism of action of charcoal have centered on adsorptive properties, and little is known concerning reactions with free radicals of oxygen.

Work in our laboratories has suggested that *L. pneumophila* strain Philadelphia-1 possesses little or no catalase and therefore might be susceptible to damage by low levels of hydrogen peroxide (12a, 22). Low levels of hydrogen peroxide inhibit growth of a variety of bacteria, including

Neisseria gonorrhoeae (19), *Campylobacter jejuni* (11), and *Spirillum volutans* (20). Autooxidation and/or photochemical oxidation of complex culture media, solutions of amino acids, and vitamins are known to generate appreciable levels of hydrogen peroxide (3, 4, 11, 19) and superoxide radicals (3, 4, 11, 16). Cysteine, which is required in apparent excess of the level necessary for nutrition of legionellae (7, 8, 22, 26, 30), is known to form hydrogen peroxide upon autooxidation (3, 4). When present in solutions of amino acids, cysteine accelerates the production of peroxide (19). Moreover, photooxidation or free radical-mediated oxidation of membrane lipids is known to produce toxic lipid peroxides and hydroperoxides (15, 28). The idea that hydrogen peroxide may form in the medium used for isolation and culture of *L. pneumophila* is further supported by the observation of Waterworth (31), who found that of several media tested for photochemical production of peroxide, only the Mueller-Hinton medium failed to accumulate hydrogen peroxide. Mueller-Hinton medium supplemented with starch supports good growth of large inocula of *L. pneumophila* in the absence of added charcoal (7).

Our preliminary studies have also indicated that addition of peroxide and superoxide radical scavengers, including enzymes such as catalase and superoxide dismutase (SOD), stimulate growth in the absence of charcoal. Since nutritional studies have not resolved the difficulties associated with the growth of legionellae, we decided to investigate further the possibility that the legionellae are sensitive to low levels of hydrogen peroxide and superoxide and that charcoal may act as a protective agent in culture media. In this paper we describe additional mechanisms for charcoal; these mechanisms may explain the failure of many laboratory media to support growth of legionellae in the absence of charcoal.

MATERIALS AND METHODS

Bacterial strains and medium. *L. pneumophila* strains Philadelphia-1, Knoxville-1, and Bellingham-1, *Legionella micdadei* strain TATLOCK, *Legionella bozmanii* strain WIGA, and *Legionella dumoffii* strains LB4 and TEX KL were obtained from the culture collection of the Centers for Disease Control and were maintained on Aces-buffered charcoal yeast extract agar (BCYE agar) (21) at 35°C. YE medium was prepared by dissolving 10 g of yeast extract (Difco Laboratories, Detroit, Mich.) in 1 liter of distilled water. The pH was adjusted to 7.0 before autoclaving or sterilization by filtration. For sterilization by filtration, the broth was first passed through a Whatman no. 1 filter and then through a membrane filter (pore size, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.). Charcoal (0.15%; Sigma Chemical Co., St. Louis, Mo.) was added to the medium before autoclaving or, for the medium sterilized by filtration, was autoclaved as a powder and then added after filtration. Where required, solutions of cysteine and ferric pyrophosphate, prepared as described previously (6), were added to the YE medium after autoclaving or sterilization by filtration.

Chemicals. Catalase (beef liver grade III), SOD (bovine erythrocyte), *o*-dianisidine (ODD), Nitro Blue Tetrazolium (NBT), and horseradish peroxidase (HRPD) were purchased from Sigma Chemical Co. Each enzyme was tested for activity; catalase had no appreciable SOD activity, and SOD had no detectable catalase or peroxidase activity. Stock solutions of ODD (5 mg/ml) and NBT (5 mg/ml) were prepared in distilled water, whereas SOD (1 mg/ml), catalase (2 mg/ml), and HRPD (1 mg/ml) were prepared in 50 mM potassium phosphate buffer (pH 7.0). Where required, all solutions were sterilized by filtration and added aseptically to YE medium. The following charcoals were tested: Norit A (American Norit Co., Jacksonville, Fla.), Norit SG and graphite (Sigma Chemical Co.), BPL (Calgon, St. Louis, Mo.), and CPG (Pittsburgh Chemical Co., Pittsburgh, Pa.).

Photochemical production of hydrogen peroxide and superoxide in YE medium. Superoxide radicals were detected in YE broth (without added cysteine and ferric pyrophosphate) exposed to fluorescent light (15 W) by using the NBT reduction method (11). In this procedure, NBT (0.1 mg/ml) was added to two flasks of YE broth, and to one of the flasks 50 to 75 U of SOD was added. Both flasks were exposed to fluorescent light at a distance of 10 cm. The light intensity at this distance was 1.34 mW per cm^2 (determined with an energy meter). Samples (2 ml) were withdrawn from the flasks at different times over a 6-h period, and the amount of NBT reduced was determined spectrophotometrically at 540 nm (3-ml cuvettes; 1-cm light path) by using a molar extinction coefficient of 9,500 for NBT, as determined from a standard curve. The difference in reduction of NBT between flasks containing SOD and flasks without SOD was used to determine the rate of superoxide radical formation. Photochemical production of hydrogen peroxide was determined by the ODD-HRPD assay (11). HRPD (20 μg/ml) and ODD (80 μg/ml) were added to YE broth. Control flasks were stored in the dark and protected from light. Since superoxide radicals reduce oxidized ODD (16) and thus would cause a low determination of the hydrogen peroxide produced, we added SOD to some flasks of YE medium. There was no oxidation of ODD in the absence of HRPD. The flasks were also exposed to fluorescent light as described above for the superoxide radical experiment. Samples were taken at different times, and the amount of oxidized ODD was determined spectrophotometrically at 460 nm by using a molar extinction coefficient of 5,400 (3-ml cuvettes; 1-cm light path). The molar extinction coefficient determined in this study was obtained for ODD in YE broth. We found that the extinction coefficient varied depending on the pH or the type of buffer or at the extremes of the concentration range, as described elsewhere (10).

Determination of oxygen consumption. A 1.35-ml water-jacketed cell equipped with a YSI oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) was used to measure oxygen consumption (30°C). Oxygen consumption by YE broth was

first determined in the dark to obtain a steady rate, and then the YE broth was illuminated with a fluorescent lamp. Traces of oxygen consumption were recorded with a strip chart recorder or a microcomputer interfaced with the YSI oxygen monitor. Rates of oxygen consumption were determined by linear regression analysis. Various enzymes, such as SOD and catalase, as well as free radical scavengers such as histidine and mannitol, were added to the chamber to determine their effects on the rate of oxygen consumption. Boiled enzymes, bovine serum albumin, and starch served as controls. In addition, various types of activated charcoals, graphite, and the CYE medium prepared as described by Feeley et al. (6) were examined. Except where indicated, all media used in this study were prepared 24 h before the assay.

We tested the ability of various charcoals to decompose hydrogen peroxide by adding 1.5 mM hydrogen peroxide to the chamber. The contents of the chamber contained charcoal plus YE medium (autoclaved and unautoclaved), charcoal suspended in 50 mM potassium phosphate buffer (pH 7.0), or CYE medium. To simplify measurement of oxygen evolution, we sparged the samples with argon to remove some of the dissolved oxygen. The starting oxygen concentration was between 60 and 80% of air saturation. The rate of oxygen evolution was corrected to take into account that 1 mol of oxygen is generated from decomposition of 2 mol of hydrogen peroxide.

Growth studies. YE broth and brucella broth, both containing cysteine and ferric pyrophosphate, were protected from light during preparation, sterilization by autoclaving, and incubation. Unprotected medium exposed to room light served as a control. The media were inoculated with 0.1 ml of a suspension of *L. micdadei* washed from a BCYE agar plate per 100 ml of medium, and the flasks were incubated statically at 37°C for 24 h. Flasks exhibiting growth were then shaken at 100 rpm on a rotary shaker.

The stimulatory effects of starch and charcoal in chemically defined liquid medium (CDM) were determined by using the medium of Reeves et al. (24). This medium was supplemented with combinations of 1% starch and 1 μg of oleic acid per ml or with 0.15% charcoal. A cell suspension of *L. pneumophila* containing approximately 2.7×10^7 CFU, as determined on BCYE agar, was diluted through the 10^{-6} dilution, and 0.1 ml of each dilution was added to duplicate plates of CDM. After incubation at 35°C for 5 days, growth was scored visually.

Sensitivity to peroxide challenge. Cells of *Legionella* strains grown in BCYE agar were suspended in 10 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 0.85% NaCl (PBS). The optical density at 660 nm was adjusted to between 0.05 and 0.11 (18- by 160-mm test tubes), as determined with a Beckman model B spectrophotometer. Such suspensions were used directly or diluted 100-fold in PBS. At zero time, 1-ml samples were removed from the two suspensions and diluted (10^{-1} to 10^{-4} dilutions), and 0.1-ml samples were plated onto duplicate plates of BCYE agar for determination of colony-forming units. Hydrogen peroxide was then added to the remaining cell suspensions (26.5 or 55.5 μM) at 25°C, and samples were removed and plated as described above. The plates were incubated at 35°C for 5 days, at which time the colony-forming units were scored. In one experiment where the number of colonies was too numerous to count, the colonies were washed from the plates with PBS, and the optical density of a 10-ml suspension was determined as described above.

Catalase activities. Catalase activity was measured by the method of Beers and Sizer (1). Various strains of *Legionella* grown on BCYE agar for 2 to 3 days were washed from the surface with PBS, disrupted by sonication (12a), and centrifuged first at 37,000 × *g* to remove cell debris and then at 100,000 × *g* for 90 min to obtain the high-speed supernatant. Protein was estimated by the method of Lowry et al. (14), using bovine serum albumin as the standard.

RESULTS

Substantial levels of hydrogen peroxide and superoxide radicals were detected in YE medium exposed to fluorescent light. The intensity of the light was approximately twice that found in a well-illuminated laboratory. As Fig. 1 shows, photochemical oxidation of YE broth (without cysteine and ferric pyrophosphate) generated superoxide radicals, as evidenced by the reduction of NBT. SOD inhibited NBT reduction, and the amount of superoxide produced was 3 nmol/ml per h. Since superoxide radicals spontaneously dismutate to form hydrogen peroxide, we also examined YE medium for accumulation of hydrogen peroxide. Figure 2 shows that hydrogen peroxide was rapidly generated in YE medium exposed to light, as evidenced by the oxidation of ODD. The superoxide radicals generated photochemically in the medium also affected the estimation of hydrogen peroxide, and when SOD was added, a marked increase in the rate of ODD oxidation was observed (Fig. 2). The amount of hydrogen peroxide generated was 16.6 nmol/ml per h (16.6 μM). In 3 h, the amount of hydrogen peroxide accumulated ap-

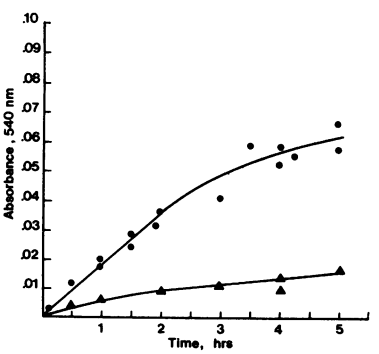

FIG. 1. Photochemical generation of superoxide radicals in YE broth. YE medium was exposed to fluorescent light, and NBT reduction was measured spectrophotometrically at 540 nm. Symbols: ●, YE medium + NBT; ▲, YE medium + NBT + SOD (75 U).

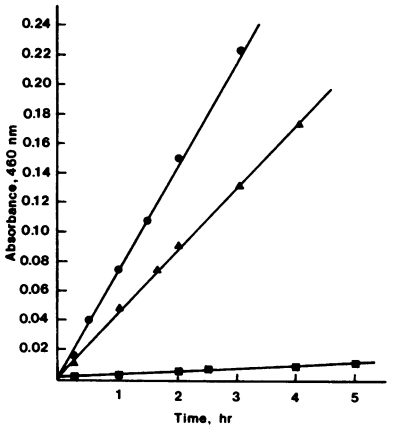

FIG. 2. Photochemical production of hydrogen peroxide in YE broth. Hydrogen peroxide production was determined with ODD and HRPD in the light (▲) and in the dark (■). SOD was added to YE medium containing ODD and HRPD in the light (●).

proached 50 μM. The hydrogen peroxide was stable in YE medium since added hydrogen peroxide remained at nearly constant levels for 8 h, as determined by the ODD-HRPD assay (data not shown).

Photochemical oxidation of YE medium can also be monitored by an oxygen probe, and we used this method to test the effects not only of charcoal, but also of added SOD and catalase on the rate of photochemically initiated oxygen consumption. Boiled catalase and SOD, as well as bovine serum albumin and starch, served as controls. If photochemical oxygen consumption proceeds by the following reaction sequence:

$$4O_2^- + 4H^+ \rightarrow 2H_2O_2 + 2O_2 \quad (1)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

then we would predict that with SOD and catalase present, 75% of the oxygen consumed photochemically should be regenerated. As Table 1 shows, photochemical oxidation of YE medium occurred at a rate of 5 to 10 nmol of oxygen consumed per min per ml of YE broth. The higher rate of superoxide and peroxide formation observed with the oxygen electrode system compared with the rates observed with NBT reduction or ODD oxidation appeared to be due to more efficient illumination of the medium (smaller volume and stirred). Addition of SOD to YE medium caused a 45% decrease in the rate of oxygen consumption, suggesting that superoxide radicals were produced photochemically. Our results are consistent with equation 1, which predicts 50% recovery of the oxygen consumed in the formation of superoxide. Catalase was less effective, as predicated from equation 2. Addition of catalase caused a decrease in oxygen consumption of approximately 28%. When SOD and catalase were added to YE medium, a decrease in oxygen consumption of 68% was achieved, which is within 7% of the theoretical value of 75%. A portion of the oxygen may have been present as hydroxyl radicals or other reduced forms since mannitol had a slight effect on the rate of oxygen consumption (less than 2% decrease). Histidine had no effect on oxygen consumption. Boiled SOD and catalase, as well as bovine serum albumin and starch, had no effect on the photochemical oxidation of YE medium.

Activated charcoal completely inhibited photochemically initiated oxygen consumption in YE medium (Table 2). All of the charcoals used in this study had a similar effect on oxygen consumption. Charcoal in buffer or charcoal freshly prepared in BCYE medium consumed oxygen. The rate of oxygen consumption by charcoal in the dark decreased with time and after 24 h varied between 0.1 and 0.28 nmol of oxygen per min per ml. In the photochemical studies, graphite was the poorest supplement in preventing photochemical oxygen consumption (2 nmol of oxygen consumed per min per ml of YE medium). However, with the activated charcoals only a slight increase in oxygen consumption was observed in the light (0 to 0.3 nmol of oxygen consumed per min per ml of YE medium). We could not distinguish between prevention of photochemical oxidation by charcoal and replenishment of oxygen back into the medium by removal of reduced forms of oxygen. Probably both mechanisms were involved. When 1.5 mM hydrogen peroxide was added to the YE broth containing the various types of carbon, all of the charcoals decomposed hydrogen peroxide at rates between 2 and 9 nmol/ml per min. The graphite was less active in decomposing hydrogen peroxide (0.9 to 1 nmol/ml per min). Unsup-

TABLE 1. Photochemical oxidation of YE medium[a]

YE medium supplement(s)	O_2 consumption (nmol/ml)	% O_2 regenerated
Unsupplemented (autoclaved)	5.2–6.9	0
Catalase	4.02	28
SOD	3.56	45
Mannitol	4.6	2
Histidine	7.2	0
SOD + catalase	2.3	68
Boiled catalase	5.53	0
Boiled SOD	5.71	0
Bovine serum albumin	5.06	0.3
Starch	6.5	0

[a] Autoclaved YE medium was exposed to fluorescent light, and oxygen consumption and evolution were measured with an oxygen electrode.

TABLE 2. Oxygen consumption in the dark and in the light and H_2O_2 decomposition[a]

Medium	O_2 consumption (nmol/min)[b]		H_2O_2 decomposition (nmol/min)[c]
	Dark	Light	
YE medium (filtered)	0.39	1.0	<0.5
YE medium (autoclaved)	0.12	5.5	<0.5
YE medium (autoclaved) + Norit BPL	<0.1	<0.3	5.0
YE medium (autoclaved) + Norit A	<0.1	<0.3	6.44
YE medium (autoclaved) + Norit CPG	<0.1	<0.3	4.52
YE medium (autoclaved) + Norit SG	<0.1	<0.3	5.92
YE medium (autoclaved) + Graphite	<0.1	0.92	2.29
CYE	4.4	<0.1	23.6

[a] Oxygen consumption was measured with an oxygen electrode.
[b] Oxygen consumed in the light was corrected for the rate measured in the dark.
[c] Hydrogen peroxide decomposition was determined by measuring oxygen evolution in media to which 1.5 mM hydrogen peroxide had been added.

plemented YE medium did not decompose hydrogen peroxide, suggesting that hydrogen peroxide was stable in YE medium.

The CYE medium also decomposed hydrogen peroxide. Despite the presence of cysteine and ferric pyrophosphate that might react to form hydrogen peroxide, this medium rapidly decomposed hydrogen peroxide (23 nmol/ml per min). The high rate of peroxide decomposition by CYE medium appeared to be partly due to the action of cysteine, which is peroxidized to cysteine and water. The ferric pyrophosphate supplement appeared to have no effect on the decomposition of hydrogen peroxide. Charcoal prepared in potassium phosphate buffer also decomposed hydrogen peroxide (17.6 nmol/ml per min), indicating that components of YE medium (including cysteine and ferric pyrophosphate) were not required for the observed activity of the charcoal. In addition, fresh suspensions of charcoal in YE broth decomposed hydrogen peroxide more rapidly than suspensions stored for 24 h (20 to 40 versus 10 nmol/ml per min). The rate of hydrogen peroxide decomposition was also dependent on the concentration of charcoal (data not shown). The latter experiments with CYE medium and phosphate buffer were performed with 0.2% charcoal rather than the 0.15% charcoal used in the other experiments. These results demonstrated that activated charcoals in suspension both decompose hydrogen peroxide and superoxide radicals and decrease the accumulation of these compounds in complex media exposed to light.

The addition of charcoal to YE medium also prevented light-accelerated oxidation of cysteine. Freshly prepared YE broth containing 0.04% cysteine autooxidized in the dark at a rate of 40 nmol of O_2 consumed per min. In the presence of fluorescent light, the rate doubled. When charcoal was included in this medium, cysteine oxidation in the dark decreased slightly (30 nmol/ml per min), but more significantly, charcoal prevented light-accelerated oxidation of the cysteine.

Growth studies. If photochemical oxidation of autoclaved YE medium supplemented with cysteine and ferric pyrophosphate renders the medium inhibitory for growth, then medium protected from light should not be inhibitory. *L. micdadei* grew poorly in autoclaved YE medium (from a large inoculum) exposed to room light (growth at 48 h), whereas rapid growth occurred within 24 h in YE medium protected from light. The unprotected medium eventually reached a similar turbidity by 72 h. A more dramatic difference was observed with brucella broth. Medium exposed to light was completely inhibitory, whereas the protected medium supported good growth by 48 h. These results suggest that the difficulties associated with growing legionellae in autoclaved media (without charcoal) may be due to light-induced formation of hydrogen peroxide.

Since starch-supplemented media reportedly enhanced the growth of *L. pneumophila* (6, 13), we examined the effect of starch in CDM that contained no fatty acids. CDM supported growth to a 10^{-4} dilution. However, when starch or starch and oleic acid were tested, growth was slightly inhibited (10^{-3} dilution). In contrast, charcoal-supplemented CDM supported growth at a 10^{-7} dilution. This medium contained no fatty acids. These results are consistent with our finding that starch had no effect on photochemical oxidation reactions or on decomposition of hydrogen peroxide and superoxide radicals. This experiment diminishes the probability that toxic fatty acids are the predominant factor in preventing growth in most media.

Hydrogen peroxide sensitivity and catalase activities. *L. pneumophila* was acutely sensitive to low levels of hydrogen peroxide (Table 3). Challenge with 26.5 μM hydrogen peroxide rapidly killed strains of legionellae when low cell numbers were used (10^4 CFU). However, at higher

TABLE 3. Effect of hydrogen peroxide on viability of *Legionella* spp.

Strain	H_2O_2 concn (μM)	Optical density of cell inoculum	Colony counts (× 10^4) at:				Optical density of cell suspension at:[a]			
			Zero time	0.5 h	1 h	2 h	Zero time	0.5 h	1 h	2 h
L. pneumophila Philadelphia-1	26.5	0.11×10^{-2}	48	0	0	0				
L. pneumophila Knoxville-1	26.5	0.06×10^{-2}	51	0.8	0	0				
L. bozmanii WIGA	26.5	0.10×10^{-2}	100	20	0	0				
L. micdadei TATLOCK	26.5	0.07×10^{-2}	610	0.13	0	0				
L. pneumophila Philadelphia-1	55.5	0.10					1.65	0	0	0
L. bozmanii WIGA	55.5	0.095					7.0	3.2	4.3	5.2
L. micdadei TATLOCK	55.5	0.09					0.41	0.33	0.65	0.82

[a] Growth was determined optically at 660 nm as described in the text. Zero-time cells and cells exposed to hydrogen peroxide were plated onto BCYE medium. After 48 h the resulting colonies were washed from the surface, and the optical density of each suspension was determined as described in the text. The values given are the averages of duplicate plates.

cell densities (10^6 CFU) and at 55.5 μM hydrogen peroxide, *L. micdadei* and *L. bozmanii* remained viable (Table 3). Thus, the range of sensitivity of these strains to hydrogen peroxide fell within the levels of hydrogen peroxide detected in YE broth exposed to light. Moreover, it might be anticipated that individual cells may be even more sensitive to these concentrations. *L. pneumophila* appeared to be slightly more sensitive to the effects of low levels of hydrogen peroxide than the other species. These results may be explained in part by the fact that *L. pneumophila* strains had no detectable catalase activity (Table 4), whereas the other species had measurable levels of catalase, with *L. micdadei* having the highest level (61 U/mg of protein).

DISCUSSION

We recently reported that *L. pneumophila* contains considerable SOD (25 to 30 U/mg of protein), but has little or no catalase activity (12a). The apparent deficiency in catalase suggests that these bacteria might be sensitive to low levels of hydrogen peroxide. Because nutritional studies have failed to explain the difficulties associated with the isolation and cultivation of the legionellae, sensitivity to low levels of hydrogen peroxide and superoxide radicals represented a viable alternative explanation. Nutritional studies have revealed that the legionellae require no vitamins and only a few amino acids for growth (7, 8, 23, 25, 30). Thus, these bacteria should be potentially capable of growing in rather simple media. However, many simple and complex culture media fail to support growth of *Legionella* spp. from small inocula unless the media are supplemented with charcoal. All of these media appear to share a common problem that is relieved by charcoal, but not by starch. Thus, determination of the mechanism of action of charcoal could greatly aid in the resolution of problems associated with growth of *Legionella* species.

TABLE 4. Catalase activity of *Legionella* strains

Strain	Protein concn (mg/ml)	Catalase activity (U/mg of protein)
L. pneumophila Philadelphia-1	9.9	±[a]
L. pneumophila Knoxville-1	10.0	±
L. pneumophila Bellingham-1	5.1	±
L. bozmanii WIGA	4.8	17.9
L. dumoffii LB4	5.0	13.8
L. dumoffii TEX KL	1.1	33.2
L. micdadei TATLOCK	0.6	61.6

[a] ±, Less than 0.1 U of catalase per mg of protein.

Our studies suggest that the difficulties associated with primary isolation of *Legionella* from clinical samples, as well as environmental samples, may be explained as follows: (i) legionellae are sensitive to relatively low levels of hydrogen peroxide and superoxide; (ii) these agents are rapidly generated in media exposed to light by photochemical oxidation reactions; (iii) activated charcoals enhance growth by preventing photochemical oxidation reactions and by detoxifying media of reduced forms of oxygen; and (iv) charcoal also prevents free radical-accelerated oxidation of cysteine, an essential growth factor. Exposure of YE medium to room light (particularly after autoclaving) initiates photochemical oxidation reactions that lead to accumulation of toxic levels of hydrogen peroxide. In addition, we found that the level of peroxide generated in YE medium exposed to light (50 μM in 3 h) was in excess of the level tolerated by suspensions of legionellae (26.5 μM). It is reasonable to assume that with fewer cells ($<10^4$ cells), as might be present in clinical specimens, even lower con-

centrations of hydrogen peroxide would be inhibitory. In contrast to the legionellae, *Escherichia coli* reportedly can tolerate millimolar levels of hydrogen peroxide (32).

The results of this study also demonstrate that activated charcoals prevent photochemical oxidation reactions in complex media and decompose hydrogen peroxide and superoxide radicals. Activated charcoals are widely used for purification of water and air, and the sorptive properties of these preparations with regard to detoxification of free fatty acids have been well studied (2, 9, 17, 23). However, we have found little information on detoxification of peroxide and various free radicals of oxygen by activated charcoals. Charcoals may prevent photochemical oxidation reactions by adsorbing the reactant compounds, by decomposing reduced forms of oxygen, or by acting as a trap for free radicals in general. Although we could demonstrate detoxification of hydrogen peroxide by charcoal, no attempt was made to study the other possibilities. Activated charcoals are commonly used to adsorb free fatty acids (2, 17, 23), but adsorption may not be a major mechanism for permitting growth of *Legionella*. Starch, which also adsorbs free fatty acids (22, 28), is only slightly effective as a replacement for charcoal. Johnson et al. (13) reported that starch-supplemented YE medium sterilized by filtration (but not by autoclaving) supported good growth of various strains of *Legionella*. However, the beneficial effects of this medium may be due to the inclusion of peroxide scavengers, such as heme and α-ketoglutarate, instead of the added starch. In addition, starch could not replace charcoal in our CDM. These observations appear to diminish fatty acid toxicity as the major problem associated with growth of the legionellae.

Peroxide toxicity is further implicated since *L. micdadei* could be successfully grown in autoclaved YE medium protected from light during preparation and incubation. The effect was even more dramatic in brucella broth, in which growth only occurred in the protected medium. Both media, when protected from light, did not accumulate appreciable levels of hydrogen peroxide (11). *L. micdadei* was found to have the highest level of catalase (60 U/mg of protein), yet these bacteria did not exhibit an appreciable increase in tolerance compared with the other strains of *Legionella* in peroxide challenge experiments. Since catalase and SOD are located in the cytoplasm (12a), the results of this study suggest that targets for damage by peroxide may be located external to and including the cytoplasmic membrane. We further believe that activated charcoal acts outside the cell, since it is unlikely that powdered charcoal passes through the cytoplasmic membrane.

The problems associated with an acute sensitivity to hydrogen peroxide are not unique to the legionellae, but have been reported for other fastidious bacteria, including *N. gonorrhoeae* (19), *C. jejuni* (11, 12), *S. volutans* (20), and *Treponema pallidum* (18). Interestingly, *N. gonorrhoeae* reportedly grows on nutrient agar only if it is supplemented with charcoal (9). The obligate microaerophile *C. jejuni* could be grown aerobically (21% oxygen, air atmosphere) on brucella agar medium when the medium was either protected from light or supplemented with SOD or catalase (11). Padgett et al. (20) also demonstrated that another microaerophile, *S. volutans*, could be grown on vitamin-free casein hydrolysate agar under 12% oxygen when the medium was protected from light. Moreover, if the medium was exposed to room light for as little as 15 s, the medium was rendered so inhibitory that the bacteria failed to grow even under microaerophilic conditions. Preliminary studies with *L. pneumophila* indicated that addition of SOD, catalase, or HRPD enhanced growth on brucella agar and to a lesser extent on YE medium in the absence of charcoal. The results of the enzyme study, as well as studies on the effects of various chemical agents on the growth of legionellae, will be presented elsewhere. Although such results implicate hydrogen peroxide and superoxide radicals in growth inhibition, our preliminary results with enzymes do not indicate that they are as efficient or as practical as the charcoal supplement. The results of our studies on the mechanism of action or charcoal may have far-reaching implications for a variety of fastidious bacteria whose growth is also greatly enhanced on media supplemented with activated charcoal. Knowledge of the sensitivity of legionellae to low levels of hydrogen peroxide and to other reduced forms of oxygen may be useful for development of improved primary isolation media.

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