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# Determination of Catalase, Peroxidase, and Superoxide Dismutase Within the Genus *Legionella*

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**We examined 40 strains of *Legionella* for reduced-oxygen scavenging enzymes. Using a simple reaction chamber with a Swinney filter for the Beers and Sizer assay, we determined the catalase activity of live cells grown on buffered charcoal-yeast extract agar. For 29 strains of *Legionella pneumophila*, the apparent first-order rate constants for catalase ranged from 0.000 to 0.005. Similarly, low values ranging from 0.001 to 0.005 were observed for *Legionella wadsworthii*, *Legionella oakridgensis*, and *Legionella gormanii*. High catalase activities were found for *Legionella jordanis*, *Legionella longbeachae*, *Legionella micdadei*, and *Legionella bozemanii*, with first-order rate constant values of 0.010 to 0.035. Cell-free extracts were analyzed for catalase, peroxidase, and superoxide dismutase. Cell-free extracts of all strains had superoxide dismutase levels ranging from 8.2 to 30.5 U per mg of protein. The species could be characterized by their catalase and peroxidase since *L. pneumophila* and *L. gormanii* had only peroxidase (relative molecular weight [$M_r$], 150,000); *L. dumoffii* had a peroxidase ($M_r$, 150,000) plus a catalase ($M_r$, 174,000); and all remaining species had catalase only ($M_r$, 300,000, 220,000, or 150,000).**

Members of the genus *Legionella* are characterized on the basis of cellular morphology, Gram staining characteristics, the inability to grown on common laboratory media such as Trypticase soy (BBL Microbiology Systems) or blood agar, and the requirement for complex media having either starch or charcoal, cysteine, and added iron (10, 27, 28). All presently recognized species show excellent growth on an Aces-buffered yeast extract medium containing charcoal and cysteine (BCYE) (28), and with the exception of *Legionella oakridgensis*, all species fail to grow or exhibit marginal growth if cysteine is deleted (5, 27). However, specific identification of each species rests primarily upon serological reactivity (15), fatty acid and ubiquinone composition (8, 18, 26), and DNA homology (4, 15, 27). There are few physiological characteristics which serve to identify a species, although to date, *Legionella pneumophila* is well differentiated from all other species by its ability to hydrolyze hippurate (14).

*L. pneumophila* is described as being catalase positive (32), but tests involving cells grown on complex organic agar media or in chemically defined broth have shown that catalase activity is either extremely weak or nonexistent. (29). Subsequently, we analyzed the whole-cell catalase activities of numerous strains of *L. pneumophila* and those of newly described *Legionella* species. Although catalase activity was evident in *L. pneumophila*, this activity was very slight and short-lived compared with the vigorous and continued release of oxygen obtained with the other species of *Legionella*. It has been recognized that media can greatly affect the production of catalase (12, 13, 36), but with the limited media which supported the growth of *Legionella* species, our tests consistently demonstrated quantitative differences between the catalase activity of *L. pneumophila* and that of other *Legionella* species. These presumptive differences between species were further emphasized upon the examination of cell-free preparations in which no catalase was observed in extracts from *L. pneumophila*, but strong activity was found in those from *Legionella bozemanii*, *Legionella dumoffii*, and *Legionella micdadei* (17).

Previously, we observed that charcoal had a catalase-like activity in the BCYE medium, and because the presence or absence of catalase could be associated with the inability of these organisms to grow on conventional laboratory media (17), we made a systematic appraisal of the catalase contents of the whole cells of numerous strains of *L. pneumophila* and the other *Legionella* species. These results led to the examination of cell-free systems for the presence of catalase, peroxidase, and superoxide dismutase (SOD), all of which deal with the destruction of potentially toxic reduced forms of oxygen (11). We found that all species contained SOD, but peroxidase and catalase were not uniformly distributed among the species. *L. pneumophila* and *Legionella gormanii* had only peroxidase, and *L. dumoffii* had both peroxidase and catalase. All of the other species contained only catalase, this catalase apparently differing among some of the species according to its relative molecular weight. Details of these experiments are presented here.

## MATERIALS AND METHODS

**Bacterial strains, media, and cultivation.** All legionellae were recognized strains, identified and obtained from laboratories of the Center for Infectious Diseases, Atlanta, Ga. Cultures of *Escherichia coli* O111:B4 and K-12:Su65-42 were obtained from Lois Britt, Center for Infectious Diseases, and *Pseudomonas aeruginosa* B6045 was obtained from R. E. Weaver, Center for Infectious Diseases. All *Legionella* cultures were grown and maintained on BCYE agar at 35°C until confluent surface growth was obtained, and then were stored at 5°C. Cultures used for experimental purposes were stored for no longer than 7 days. Cultures of other genera were grown on blood agar base at 35°C in deep agar stabs and stored at room temperature (22°C). For experimental purposes, all cultures were grown on BCYE agar plates; all plates were examined for contamination by dark-field microscopy and by streaking on blood and Trypticase soy agar

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plates (BBL Microbiology Systems). The cells were removed from the plate surface with 2 ml of sterile distilled water, centrifuged at 2,000 × *g* for 30 min, suspended in water at approximately 30 times the packed-cell volume, and recentrifuged. The pellet was either frozen at −60°C or was immediately suspended in deionized water to give a standardized suspension with an absorbance of 2.0, using a 1.8-cm light path at a wavelength of 660 nm.

**Determination of live whole-cell catalase activity.** Quantitative determination of whole-cell catalase activity was accomplished by using a simple reaction chamber prepared from a 60-ml plastic syringe. This was fitted with a Swinney 25-mm Swinnex adapter (Millipore Corp.) having a 0.45-μm type HA filter. A 5-mm entry port was drilled at the 30-ml mark, and a small Teflon-coated magnetic stirring bar was placed within the syringe. The plunger was inserted up to the entry port, and the reaction chamber was fixed to the surface of a magnetic stirrer with modeling clay. Then 20 ml of 26.4 mM $H_2O_2$ in 0.05 M $K_2HPO_4$ (pH 7.0) was pipetted into the entry port, 0.5 to 2.0 ml of cell suspension was added, and the plunger was inserted just beyond the entry port to seal the chamber. The magnetic stirrer was immediately started, and samples (ca. 1.5-ml) were expressed through the filter into 2-ml quartz curvettes at different times, usually 1, 3, 5, and 7 min. Catalase activity, as measured by the loss of absorbance at 240 nm, was determined by the procedure of Beers and Sizer (3); rate constants were determined according to Herbert (16).

**Preparation of toluene-treated cells or cell-free extracts.** The frozen pellets (see above) were thawed rapidly in warm water, and 2.5 volumes of 0.01 M phosphate buffer (pH 7.2) were added. These cell suspensions were passed twice through a French press, the chamber and cells being kept cold with ice. Alternatively, the cells were disintegrated by using a 20-KC Sonifier operating at approximately 100 W for 2 min. Exposure to sonic energy was at 30-s intervals in an alcohol-ice bath with 2-min cooling periods between bursts. When foaming prevented sonification, the suspension was diluted with increments of buffer in which the final ratio of cell volume to buffer was no less than 1:8. Whole cells and cell debris were removed by centrifugation at 10,000 for 30 min. The supernatants were centrifuged at 5°C at 41,000 × *g* for 2 h or at 100,000 × *g* for 1 h. The clear supernatants were dispensed in 1- to 2-ml portions and stored at −60°C.

Cells were treated with toluene as described previously for the determination of catalase (35) and SOD (33).

**Enzyme determinations.** Catalase activity was assayed by adding 0.1 ml of cell extracts to 1.4 ml of freshly prepared 13.2 mM $H_2O_2$ in 0.05 M $K_2HPO_4$ (pH 7.0) (0.15 ml of 30% $H_2O_2$ per 100 ml). The reaction was initiated by adding the enzyme list. Procedures in which the $H_2O_2$ concentrations were greater or in which the $H_2O_2$ was added last to initiate the reaction caused a loss of enzyme activity. The solution was mixed, and a loss of absorbance was determined at 240 nm (3) by using a Gilford 2000 recording spectrophotometer for 1 to 3 min. Rates (16) were calculated on the basis of the slopes obtained during the first 30 s. Units of catalase were calculated by using a molar absorbance index for $H_2O_2$ of 43.6 (35). Peroxidase was determined by using *o*-dianisidine (35). To 1.5 ml of 0.01 M phosphate buffer (pH 6.0), 0.01 ml of *o*-dianisidine (1% in methanol) was added and mixed; 0.1 ml of enzyme was added and mixed. To this was added 0.1 ml of 0.3% freshly prepared $H_2O_2$ in deionized water; the solutions were mixed, and the change of absorbance was recorded at 460 nm for 3 to 5 min. The sequence of the addition of reagents could seriously affect the determination (see below). Units of peroxidase were calculated by using a molar absorbance of $11.3 \times 10^3$ (35). SOD was determined by the procedure of Marklund and Marklund (24). The relationship of percent inhibition versus concentration of enzyme was linearized by a log-log conversion, the equation for the curve was determined by linear regression, and the unit of enzyme was calculated as the amount of protein (in milligrams) causing 50% inhibition of the rate of oxidation of pyrogallol under the test conditions. To determine the relative SOD activities of fractions eluted from a gel filtration column, we used the reduction of nitroblue tetrazolium in the presence of light and riboflavin (34). Cyanide was not used in the EDTA buffer for this procedure.

**Analytical gel filtration.** Relative molecular weights ($M_r$s) of catalase, peroxidase, and SOD extracted from the various species were determined by gel filtration, using Sephadex G-200 (Fine) (Pharmacia Fine Chemicals). Approximately 10 mg of protein in a volume of 1.0 to 2.0 ml was added to a column (1.5 by 90 cm). The proteins were eluted with 0.05 M $K_2HPO_4$ (pH 7.2)–0.1 M NaCl buffer at a rate of 7.4 ml $h^{-1}$; 2-ml fractions were collected. All filtrations were done at 5°C. To determine the elution volume of the standards, mixed solutions of 1 mg of each of the various standards were added to the column in 1.5 ml of elution buffer; we determined their concentration in eluted fractions by absorbance at 257 or 280 nm or by respective enzymatic activities. Void volume was determined with dextran blue, and total volume was determined with histidine. Calculation of the $M_r$ was done on the basis of fraction number or $K_{av}$ (elution volume − void volume)/(total volume − void volume). The curves relating log molecular weight to tube number or $K_{av}$ were determined by regression analysis, and the $M_r$s were calculated. Both calculations gave identical values. When multiple peaks of SOD were observed, the means of the peak elution values were used for the calculation of $M_r$.

Molecular weight (MW) standards were thyroglobulin (MW, 669,000), ferritin (MW, 540,000), ferritin (MW, 440,000), catalase (MW, 232,000), aldolase (MW, 158,000), bovine serum albumin (MW, 60,000), horseradish peroxidase (MW, 40,000), beef erythrocyte SOD (MW, 32,500), and cytochrome *c* (MW, 12,500). All standards were obtained from Pharmacia, except for ferritin, which was obtained from Pierce Chemical Co., and SOD and peroxidase, which were obtained from Sigma Chemical Co.

After isolation by gel filtration as described above, peroxidase fractions of Knoxville were purified further by chromatofocusing. A column (1 by 40 cm) was packed with 35 ml of Polybuffer exchange resin-94 (Pharmacia) in 0.025 M imidazole-hydrochloride buffer (pH 7.4). Enzyme solution (3.2 mg of protein, dialyzed against imidazole buffer) was applied, and the column was eluted with Polybuffer-74–hydrochloride (pH 4.5). A total of 110 fractions (3.2 ml each; pH 7.8 to 4.5) were collected at a rate of 12.8 (ml $h^{-1}$). All manipulations were done at 5°C.

**Acrylamide gel electrophoresis.** SODs were separated and localized on acrylamide gels, using the procedure of Beauchamp and Fridovich (2). Cells (0.7 to 1.0 g [wet weight]) were ruptured by two passes through a French pressure cell and were centrifuged at 10,000 × *g* for 30 min to remove cellular debris; the supernatant was centrifuged at 100,000 × *g* for 90 min to remove particulate material. The presence of SOD in crude cell-free extracts of *Legionella* species was also determined by electrofocusing, using an LKB 2117 Multiphor system and LKB Ampholine PAG plates (pH range, 3.5 to 9.5). Samples were dialyzed against three changes of 1% glycine, and 0.025-ml (50- to 200-μg) samples

were applied to the gel on Whatman 3MM Paratex filter paper. The anode electrode solution was 1 M $H_3PO_4$; the cathode electrode solution was 1 M NaOH. The separation was done at 15 W of constant power and maximum settings of 1,400 V and 30 mA for 2 h at 10°C. The gels were stained for protein by using Coomassie blue and for SOD activity as described above. Protein was determined by the method of Lowry et al. (23).

Catalase and peroxidase were separated and localized on 4% polyacrylamide gels as described by Claiborne and Fridovich (6), using an LKB 2117 Multiphor system. A 3% stacking gel was used, and a 30 min pre-electrophoresis was run at 20 mA and 10°C. The enzymes were dialyzed against 0.025 M bicine-imidazole buffer, and 10 μl samples containing 50 to 150 μg of protein were applied to each well; electrophoresis was done at 50 mA for 55 min at 10°C.

## RESULTS

**Development of assay procedures.** In certain tests for the determination of catalase, the reactions showed an initial high rate of reaction lasting but 10 to 15 s. In several cases, initial reaction rates showed an increase in absorbance instead of the expected decrease. These problems were reconciled when it was recognized that when the enzyme was added first to the buffer, subsequent addition of $H_2O_2$ could result in fine precipitation of the enzyme, causing an increase in absorbance and loss of enzyme activity. However, when the hydrogen peroxide was added to the buffer first, followed by enzyme, precipitation did not occur and enzyme activity appeared to be maximal. As suggested by the literature (36), the recommended concentration (60 mM) of $H_2O_2$ was inhibitory and the final concentration of the reaction mixture was reduced to approximately 14 mM.

For strains of *L. dumoffii*, the peroxidase test (35) showed high rates of *o*-dianisidine oxidation, which terminated abruptly. It was determined that the final concentration of $H_2O_2$ should be increased from 1 to 6 mM and that we should (i) add the enzyme to the *o*-dianisidine buffer solution first, (ii) mix the solution well, and (iii) add the $H_2O_2$ to start the reaction. Addition of $H_2O_2$ first, followed by mixing and then addition of *o*-dianisidine, could result in total inhibition or erratic reading of the peroxidase reaction.

Difficulties encountered with both the catalase test and the peroxidase test with the crude cell-free extracts were explained in part, retrospectively, with the recognition that *L. pneumophila* strains contained only peroxidase having only minor catalatic activity, whereas *L. dumoffii* strains had both catalase and peroxidase. The rapid loss of peroxidatic activity by extracts of *L. dumoffii* strains was reversed by fresh addition of $H_2O_2$, suggesting that the inhibition of peroxidatic activity was due to the depletion of $H_2O_2$ by catalase. Thus, an extract of WIGA strains having only catalase inhibited the peroxidase activity of Knoxville by 50%, the latter having only peroxidase. Claiborne and Fridovich (6) have described the powerful inhibition by *o*-dianisidine of the catalatic activity of peroxidase (Hydroperoxidase I), and this could explain in part the need to add the *L. pneumophila* enzyme first with mixing to *o*-dianisidine buffer solution and then to add the $H_2O_2$ to initiate the peroxidase reaction. In this way, the catalatic activity would be inhibited without affecting the peroxidase reactions. Catalase would not, therefore, deplete the substrate or form oxygen bubbles which interfered with the spectrophotometric readings. The final protocols (see above) for catalase and peroxidase gave reliable and reproducible results with crude *Legionella* extracts; with extracts purified by gel filtration, no difficulties have been encountered.

**Evaluation of whole-cell catalatic activity in *Legionella* species.** Although the results obtained with the microcatalase test were entirely consistent with the conclusion that strains of *L. pneumophila* and *L. gormanii* had much lower enzyme activities than did the other species, there was sufficient variation to impose a requirement for more quantitative determinations. Use of the reaction chamber fitted with a Swinney filter showed good reproducibility, with bacterial strains having very low or very high activity, and the first-order reaction rates ($K$) showed excellent agreement between the calculated $K$ value and the relative concentration of cells. Application of the procedure to a diverse group of bacterial strains, including *Legionella* species, differentiated three groups of organisms based upon their relative catalatic activities (Fig. 1).

An examination of some 40 *Legionella* strains (Table 1) gave $K$ values ranging from 0.000 to 0.035, with the highest value being found for *L. dumoffii*, *Legionella jordanis*, *Legionella longbeachae*, *L. micdadei*, and *L. bozemanii*. The lowest values were found for *L. gormanii* ($K$, 0.0012), and 90% of the *L. pneumophila* strains ranged in $K$ from 0.000 to 0.0027. Two strains of *L. pneumophila*, Pontiac and Orlando, gave $K$ values comparable to those found for *L. oakridgensis* ($K$, 0.0044) (Table 1). Reactions obtained with *L. dumoffii*, TEX-KL, and YN-23 were variable, ranging from 0.0 to 0.0095 with different lots of cells. Their $K$ values, therefore, showed little differences between the catalatic activities of some *L. pneumophila* strains and those of certain of the other species.

**Enzyme activities of toluene-treated whole cells.** Recognition of the localization of the enzymes and the total potential enzyme content of the cell may be determined by the treatment of the whole cell with reagents that rupture the cytoplasmic membrane. When *Legionella wadsworthii* was treated with toluene, the residual cell debris showed 230 and 241% of the original catalase and SOD activities, respectively. No catalase activity was found in the supernatant of treated cells suspensions, but 238% of the original whole cell SOD activity was found in this fraction. These results were similar to those obtained with TATLOCK and TEX-KL, but Knoxville showed no treated whole-cell catalase activity.

Thus, only SOD activities were found in the supernatants, and total catalase activity was restricted to the treated cell residue, albeit this activity was greater than that found for the untreated cells. Peroxidase activity could not be assessed quantitatively with whole cells, toluene-treated cells, or the supernatants from toluene-treated cells. What peroxidase activity that was evident was limited to the cell sufaces; no color was formed in filtered reaction mixtures. It is apparent that the whole live-cell catalase shown in Table 1 represents but a limited enzyme potential of the whole cell and does not necessarily express the enzyme content of the cell.

**Catalase, peroxidase, and SOD activities of cell-free preparations.** In several direct comparisons, the enzyme preparations obtained by sonic energy had specific activities of the three enzymes greater than or equal to those obtained with the French press. However, protein yields were greater when the French press was used. Although tests with the crude cell extracts of Knoxville and Bellingham showed vigorous release of oxygen and strong catalatic activity on $H_2O_2$, calculations of the specific activities of catalase from *L. pneumophila* strains and from *L. gormanii* showed these to be the lowest values when compared with preparations of

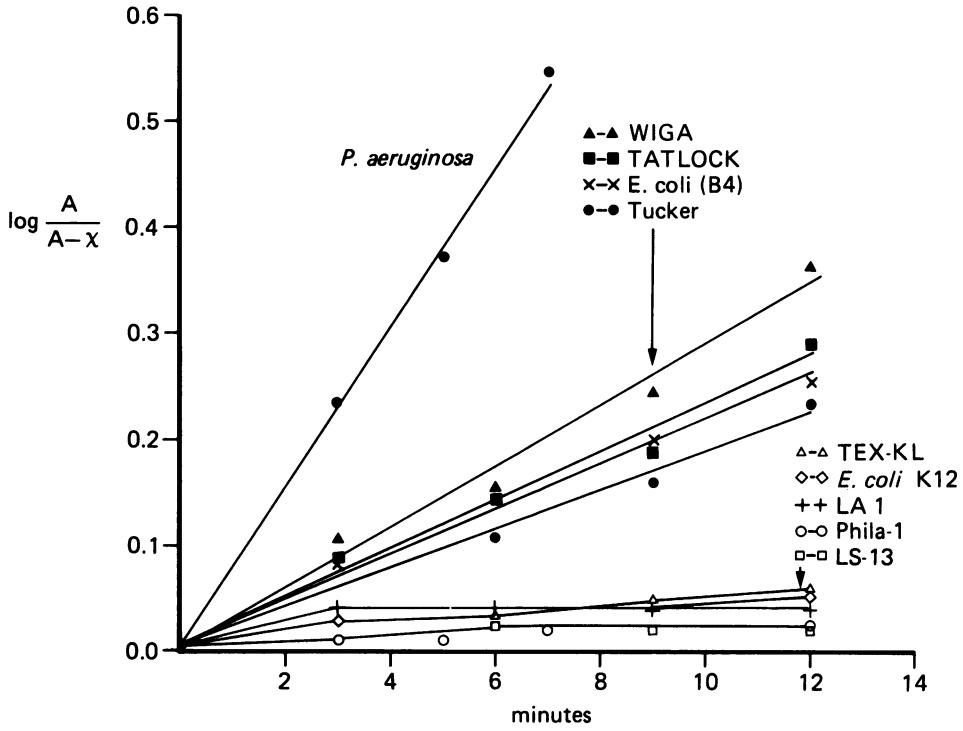


FIG. 1. Three levels of catalase activity observed with live-cell suspensions of *Legionella*, *E. coli*, and *P. aeruginosa*.

the remaining species (Table 2). The values for these two species ranged from 0.00 to 3.6, whereas those for the other species were from 7.2 to 17.5.

Only three species, *L. pneumophila*, *L. gormanii*, and *L. dumoffii*, showed peroxidase activity. All strains showed SOD activity in a wide range of concentrations which did not characterize any one species.

Gel filtration of the crude extracts of *L. pneumophila* showed only a single enzymatically active peak; this contained both the catalatic and peroxidatic activities of a given strain. Based upon the elution pattern in which the catalase and peroxidase activities were virtually superimposed upon one another (Fig. 2), and based upon the descriptions of hydroperoxidase I and II (HP I and HP II) of *E. coli* B (6, 7), it appeared that the *L. pneumophila* strains had only a single hydroperoxidase which had both catalatic and peroxidatic activities. The presence of a single hydroperoxidase in *L. pneumophila* was supported by the fact that when cells were

TABLE 1. First-order reaction rates ($K$) of live-cell catalase of *Legionella* strains[a]

| Species | Strain (serogroup) | No. of strains | $K$ range × $10^2$ (mean) |
|---|---|---|---|
| *L. pneumophila* | Albuquerque (1), Baltimore (1), Burlington (1), Detroit 1 (1), Flint 1 (1), Gastonia (1), Knoxville 1 (1), Memphis (1), New York 1 (1), Rochester (1), Philadelphia 2 (1), LA 1 (4), Dallas 4 (5), Cambridge 2 (5), Chicago 8 (7), IN23 (9) | 16 | 0.00–0.10 (0.019 ± 0.035) |
| | Buffalo (1), Berkeley (1), Miami Beach (1), Philadelphia 1 (1), Bellingham (1), Togus (2), Bloomington (3), Dallas 17 (5), Chicago 2 (6), Houston 2 (6), Concord 3 (8), Pontiac (1), Orlando (1) | 11 | 0.14–0.27 (0.224 ± 0.049) |
| | | 2 | 0.38–0.46 |
| *L. dumoffii* | New York 23, TEX-KL | 2 | 0.00–0.95 (variable) |
| *L. gormanii* | LS 13 | 1 | 0.12 |
| *L. jordanis* | BL 540, ABB-9 | 2 | 0.80–1.05 |
| *L. longbeachae* | Long Beach 4 (1), Tucker (2) | 2 | 1.40–2.23 |
| *L. micdadei* | HEBA, Tatlock | 2 | 2.12–2.30 |
| *L. bozemanii* | WIGA | 1 | 3.50 |
| *L. oakridgensis* | Oak Ridge 10 | 1 | 0.44 |
| *L. wadsworthii* | 81–716 | 1 | 0.77 |
| *P. aeruginosa* B6045 | | 1 | 7.62–14.30 |
| *E. coli* K-12:Su65-42 | | 1 | 0.42 |
| *E. coli* O111:B4 | | 1 | 3.25 |

[a] $K$ is the apparent first-order reaction rate calculated on the basis of 1 ml of whole-cell suspension (absorbance at 1.8 cm, 2.0) added to 20 ml of $H_2O_2$ buffer solution.

TABLE 2. Catalase, peroxidase, and SODs activities of 39,000 × *g* supernatants of sonic extracts of *Legionella* species

| Strain (serogroup) | Protein (mg/ml) | Activity (U per mg of protein × $10^3$)[a] of: | | |
|---|---|---|---|---|
| | | Catalase | Peroxidase | SOD |
| *L. pneumophila* | | | | |
| Philadelphia 1 (1) | 6.4 | 0.0 | 0.2 | 13.4 |
| Knoxville 1 (1) | 3.6 | 2.5 | 2.7 | 9.1 |
| Bellingham 1 (1) | 20.2 | 2.8 | 2.3 | 13.6 |
| Burlington 1 (1) | 29.2 | 0.3 | 1.1 | 18.3 |
| Atlanta 4 (2) | 15.3 | 0.8 | 0.9 | 18.9 |
| Bloomington 2 (3) | 28.6 | 3.6 | 1.4 | 14.7 |
| Baltimore 1 (4) | 28.0 | 0.9 | 1.2 | 10.8 |
| Los Angeles 1 (4) | 14.0 | 1.6 | 1.1 | 15.8 |
| Cambridge (5) | 8.7 | 0.7 | 1.1 | 9.3 |
| Dallas 17 (5) | 23.3 | 1.4 | 0.7 | 12.0 |
| Chicago 2 (6) | 14.3 | 0.9 | 0.5 | 14.8 |
| *L. bozemanii* | | | | |
| WIGA | 1.1 | 9.6 | 0.0 | 16.0 |
| *L. dumoffii* | | | | |
| TEX-KL | 1.4 | 17.5 | 3.8 | 8.2 |
| NY-23 | 1.8 | 7.8 | 2.0 | 29.3 |
| *L. gormanii* | | | | |
| LS-13 | 11.2 | 2.3 | 1.2 | 18.8 |
| *L. jordanis* | | | | |
| BL-540 | 8.5 | 7.2 | 0.0 | 8.4 |
| *L. longbeachae* | | | | |
| LB-4 (1) | 3.0 | 7.5 | 0.0 | 22.5 |
| Tucker 1 (2) | 12.6 | 17.2 | 0.0 | 24.7 |
| *L. micdadei* | | | | |
| TATLOCK | 2.3 | 16.6 | 0.0 | 30.5 |
| HEBA | 2.1 | 16.7 | 0.0 | 21.7 |

[a] Catalase and peroxidase activities are expressed in international units (35); for SOD, 1 unit is that amount of enzyme (milligrams) giving 50% inhibition of the spontaneous oxidation of pyrogallol under test conditions (24).

grown on BCYE, harvested at different times, and analyzed for their catalase and peroxidase, quantitative changes in enzyme activities were identical. This was not true for TEX-KL, as discussed below, in which the peroxidase activity increased as catalase decreased and in which both enzymes were found. Also, when the peroxidase-containing fractions of Knoxville, isolated by molecular filtration, were purified further by chromatofocusing, a single active peak consisting of six fractions of peroxidatic activity was eluted at pH 6.5; the catalatic activity of these fractions coincided with the peroxidase activity. Similarly, polyacrylamide gel separation of the enzymes of five *L. pneumophila* strains, including Philadelphia I and Knoxville, showed a single peroxidase band which also had catalatic activity. Strains of *L. dumoffii*, TEX-KL and NY-23, showed this same band but had, in addition, a second band having only catalatic activity. The crude extracts of *L. gormanii* which had both catalase and peroxidase activities also showed a single active hydroperoxidase elution peak of strong peroxidatic activity with extremely weak catalatic activity. The elution pattern of the enzymes of NY-23 and TEX-KL from Sephadex 200 showed two hydroperoxidases, one containing only catalatic activity but the second, as in *L. pneumophila*, having both catalatic and peroxidatic activities (Fig. 2). All other species exhibited only a single hydroperoxidase peak; this was a catalase without any oxidative activity on *o*-dianisidine (Fig. 2).

In contrast to the elution profiles observed for catalase and peroxidase, which covered approximately 8 to 10 2-ml fractions, the elution patterns of all of the SOD activities covered approximately 20 tubes (Fig. 2). This suggested the presence of multiple SOD enzymes; this was confirmed by polyacrylamide gel electrophoresis and by isoelectric focusing in which two or more bands were observed for the 10 species tested. With the exception of *L. pneumophila* and *L. oakridgensis* (not shown), all species exhibited a fast-moving SOD between $R_f$ 0.54 and 0.55, which corresponded to the iron-containing SOD of *E. coli* (Fig. 3). No effort was made to determine the metal cofactor for the various SODs. With fresh extracts, a slow-migrating SOD corresponding to that of the mangano-SOD of *E. coli* were seen for *L. pneumophila*, *L. gormanii*, and *L. oakridgensis*; lower activities of this band were observed for the other species, except for *L. jordanis*, which had none. Extracts frozen at −20°C lost 50% of their activity, and when they were electrophoresed and stained for SOD activity, variable results were obtained, including multiple banding and streaking of activity within lanes. No attempt was made to study these enzymes further.

Significant differences in the elution profiles of catalase of the various species were evident, and the $M_r$s of the three different enzymes were determined (Fig. 4 and Table 3). As a consequence of these determinations and the presence or absence of catalatic or peroxidatic activities, each of the species could be characterized by its enzymatic content. Recognizing that the elution peaks of peroxidase activity could differ by as much as two tubes within the strains of *L. pneumophila*, we found this species had no catalase, but had a peroxidase with catalatic activity and an $M_r$ of approximately 150,000. *L. dumoffii* strains appeared to have this same enzyme plus a catalase of $M_r$ 274,000. Among the species which demonstrated catalase only, at least three groups of catalases were apparent. These were *L. jordanis* and *L. wadsworthii* with a catalase of $M_r$ 300,000, *L. bozemanii* and *L. oakridgensis* with an $M_r$ of 220,000, and *L. micdadei* and *L. longbeachae* with an $M_r$ of approximately 150,000.

## DISCUSSION

Hassan and Fridovich (13) described the production of two catalases by *E. coli* B; one was inducible, and formation was repressed by glucose, whereas the second was constitutive. Subsequently, Claiborne and Fridovich (6) and Claiborne et al. (7) isolated and biochemically characterized both enzymes. These enzymes, which utilize hydrogen peroxide as electron acceptors in oxidative metabolism, are classified as hydroperoxidases; the hydroperoxidases of *E. coli* B were designated hydroperoxidase I (HP I) and hydroperoxidase II (HP II). Enzymatically, HP I was a peroxidase demonstrating catalatic activity. The catalatic activity was substantial, having a $K_m$ of 3.9 mM and an $H_2O_2$ turnover number of $9.8 \times 10^5$ $min^{-1}$. But the enzyme also had very strong peroxidatic activity, attacking *o*-dianisidine, guiacoll, pyrogallol, *p*-phenylenediamine, and catechol. Furthermore, an oxidized product of *o*-dianisidine which formed during the peroxidatic reaction strongly inhibited the peroxidatic activity. This autoinhibition did not occur with the other substrates. Furthermore, *o*-dianisidine itself strongly inhibited the catalatic reaction so that in the presence of *o*-dianisidine, the evolution of $O_2$ was strongly inhibited.

We discuss our results below in the context of a peroxidase having both catalatic and peroxidatic activities and a catalase having only catalatic activity. Final conclusions on the enzymatic activity and chemical nature of the *Legionella* hydroperoxidases must depend upon the isolation, purification, and characterization of the respective enzymes. Although the enzymes were strongly active in our purified fractions, we have not recovered significant amounts of

protein in these experiments to determine other biochemical or chemical characteristics of either enzyme. In one experiment, the catalase fractions of strain Tucker, obtained by gel filtration at 5°C, were concentrated and purified further by chromatofocusing. Strong catalase activities were observed with isoelectric peaks at pH 5.35 and 5.55, but no absorbance spectrum could be taken. Heated preparations had no activity.

Throughout these experiments, we used *P. aeruginosa* and *E. coli* for comparative purposes. Based on the results of Fridovich and collaborators (6, 7, 19, 37), the $M_r$ values that we found for the peroxidase (HP I) and for the SOD of *E. coli*

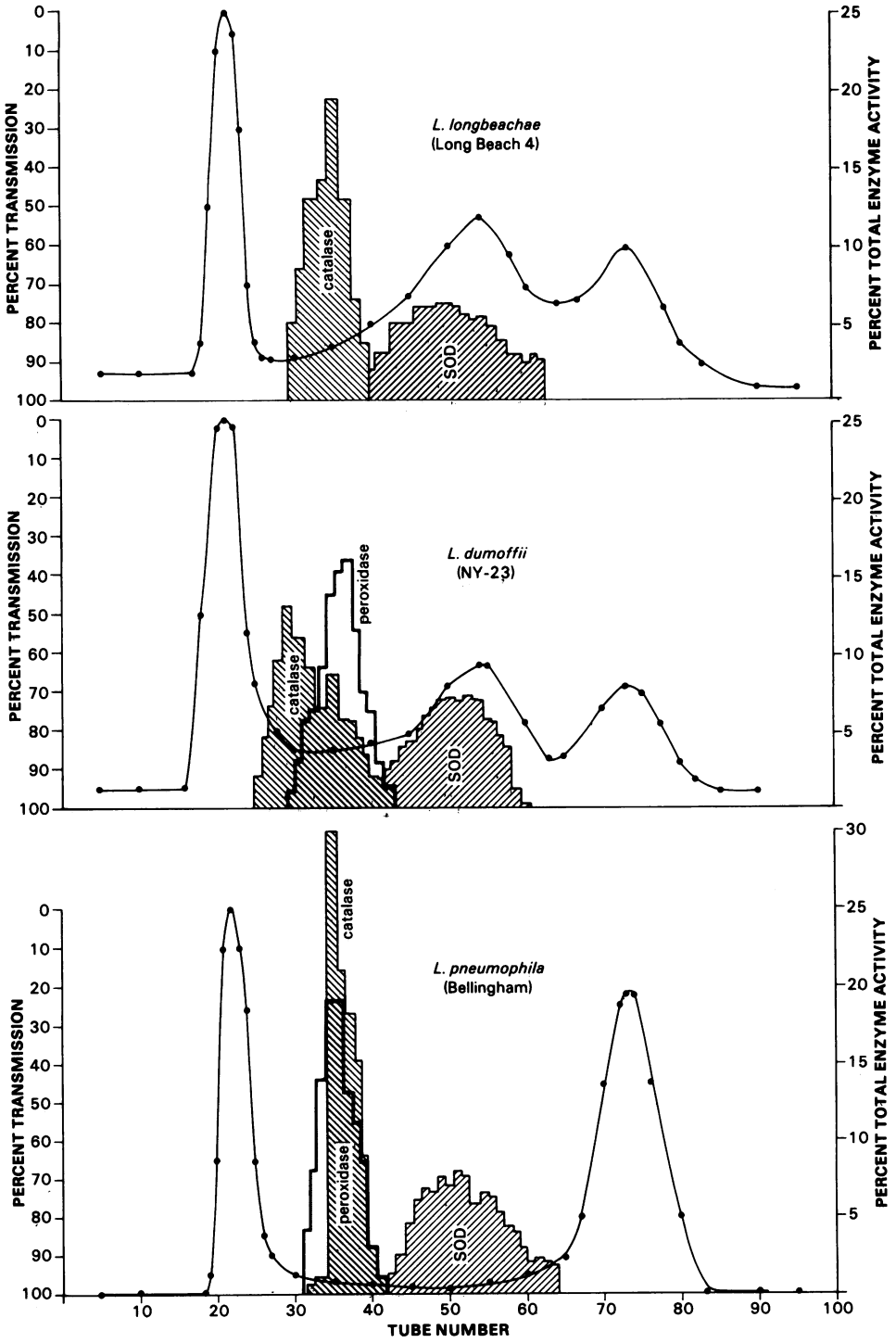


FIG. 2. Elution patterns of catalase, peroxidase, and SOD activities of *L. pneumophila*, *L. dumoffii*, and *L. longbeachae*. Bellingham (bottom) shows a single peak of hydroperoxidase with catalatic and peroxidatic activity. NY-23 (center) shows two hydroperoxidase peaks, one with catalatic activity only. Long Beach 4 shows a single hydroperoxidase with catalatic activity only.

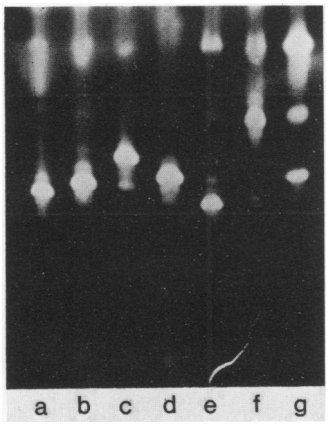


FIG. 3. Separation of *Legionella* SODs by electrophoresis in 7.5% acrylamide gel. Lanes a, b, c, d, e, f, and g contained fresh extracts of *L. bozemanii* (WIGA), *L. dumoffii* (TEX-KL), *L. micdadei* (HEBA), *L. jordanis* (ABB-9), *L. gormanii* (LS-13), *L. pneumophila* (Philadelphia 1), and *E. coli* B, respectively.

B were low (Table 3). The reported values were 337,000 for HP I, 38,700 for the Fe-containing SOD, and 40,000 for the Mn-containing SOD. Failure to observe catalase, i.e., HP II, in either strain of *E. coli* is attributed either to differences in our strains or, more probably, to the fact that all strains were grown on the BCYE agar. This medium was found to strongly inhibit catalase formation when tested with live cells of *E. coli* (data not shown).

The primary motivation for the analyses of whole-cell catalase in *L. pneumophila* stemmed from recent observations that *L. penumophila* was highly sensitive to external $H_2O_2$; also, there appeared to be a correlation between the cellular sensitivity of different *Legionella* species to $H_2O_2$ and the catalase contents of their cell-free extracts (17). Furthermore, charcoal was found to have a catalase-like function in the medium that supported the best growth of *Legionella*, i.e., the BCYE agar. However, with the analyses of the crude cell-free extracts, we were stimulated to the examination of the genus as a whole for the presence of the enzymes catalase, peroxidase, and SOD, not only for taxonomic purposes, but also for potential relationships of these enzymes to the growth and pathogenicity of the different species. As a result of these investigations, we have been able to devise a whole-cell peroxidase-catalase test (30) which serves as an excellent adjunct to the hippurate hydrolysis test (14) used for the identification of *L. penumophila*. The results reported here also serve to characterize the other *Legionella* species on a phylogenetic basis not previously reported.

Based on the overall observations of the catalase, peroxidase, and SOD contents of many strains, we found that *L. pneumophila* is a physiological group in which the live cells exhibit low catalase-like activity. This catalatic activity is secondary to the peroxidatic function of the enzyme, which is strongly expressed in the cell-free extracts of the various

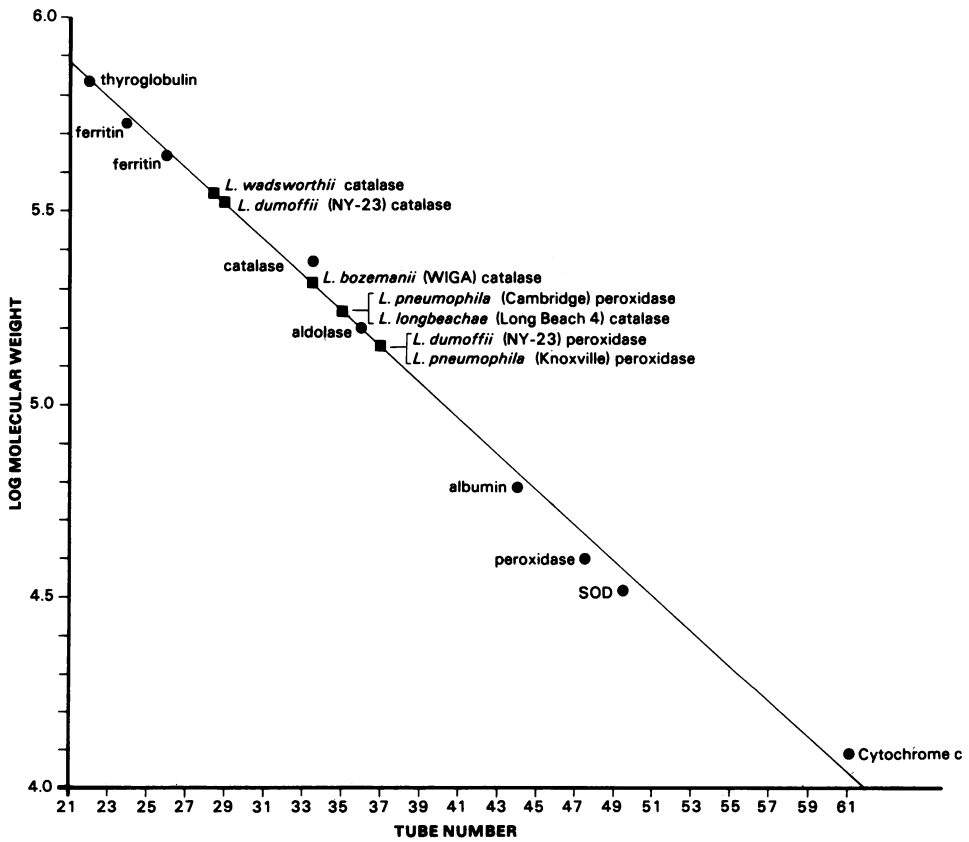


FIG. 4. Relative molecular weights of catalase and peroxidase of *Legionella* species determined by gel filtration with Sephadex G-200 (Fine). Approximately 10 mg of *Legionella* protein was added to a column (1.5 by 90 cm), and 2-ml fractions were eluted with 0.05 M $K_2HPO_4$ (pH 7.2)–0.1 M NaCl buffer.

TABLE 3. Relative molecular weights of catalase, peroxidase, and SOD of *Legionella* species

| Species | Strain (serogroup) | $M_r$ of: | | |
|---|---|---|---|---|
| | | Catalase[a] | Peroxidase[b] | SOD |
| *L. dumoffii* | NY-23 | 321,000 | 137,000 | 31,000 |
| | TEX-KL | 321,000 | 130,000 | 31,000 |
| *L. jordanis* | BL-540 | 304,000 | — | 31,000 |
| | ABB-9 | 288,000 | — | 34,000 |
| *L. wadsworthii* | 81-716 | 338,000 | — | 31,000 |
| *L. oakridgensis* | Oak Ridge 10 | 246,000 | — | 28,000 |
| *L. bozemanii* | WIGA | 199,000 | — | 32,000 |
| | D-62 | 199,000 | — | 32,000 |
| *L. micdadei* | TATLOCK | 144,000 | — | 32,000 |
| | HEBA | 130,000 | — | 29,000 |
| *L. longbeachae* | Tucker 1 | 130,000 | — | 29,000 |
| | Long Beach 4 | 170,000 | — | 36,000 |
| *L. gormanii* | LS-13 | — | 137,000 | 32,000 |
| *L. pneumophila* | Philadelphia 1 (1) | — | 137,000 | 25,000 |
| | Knoxville 1 (1) | — | 137,000 | 33,000 |
| | Bellingham 1 (1) | — | 144,000 | 33,000 |
| | Cambridge 1 (1) | — | 170,000 | 32,000 |
| | Burlington (1) | — | 170,000 | 38,000 |
| | Togus 1 (2) | — | 123,000 | 29,000 |
| | Atlanta 1 (2) | — | 161,000 | 38,000 |
| | Bloomington 2 (3) | — | 137,000 | 38,000 |
| | Los Angeles 1 (4) | — | 144,000 | 34,000 |
| | Dallas 17 (5) | — | 144,000 | 36,000 |
| | Chicago 2 (6) | — | 161,000 | 34,000 |
| *E. coli* | O111:B4 | — | 288,000 | 34,000 |
| | K-12:Su65-42 | — | 321,000 | 34,000 |
| *P. aeruginosa* | B6045 | 179,000 | 111,000 | 42,000 |

[a] No catalase activity exhibited peroxidatic activity. —, No activity.
[b] All peroxidase peak activities showed concomitant catalatic activity.

strains but which can also be expressed under acid conditions of the whole cell (30). With the possession of this single hydroperoxidase, *L. pneumophila* differs from all of the other species tested, with the exception of *L. gormanii*. *L. gormanii* differs from *L. pneumophila* in having virtually no whole-cell peroxidatic activity, no ability to hydrolyze hippurate, and what appears to be very limited catalatic activity in cell-free preparations. A more newly named species, *Legionella feelei* (16a) appears to be similar to these two species in having live cells which express strong peroxidatic activity with limited catalatic activity. All other species have been found to have a catalase with $M_r$s falling in one of three classes of 300,000, 220,000, or 150,000.

The biochemical or chemical relationships of these catalases to one another or to the peroxidase of $M_r$ 150,000 are unknown, and there is no immediate recognition of a growth advantage had by any one of these species with its particular hydroperoxidase content. The presence or absence of a given catalase has not been related to growth advantages in other genera (38), although the production of catalase has most recently been related to the survival of *Lactobacillus plantarum* in the stationary phase of its growth cycle (20). An investigation of the breakdown of $H_2O_2$ by leptospira showed that pathogenic *Leptospira interrogans* gave a strong catalase and weak or negative peroxidase reaction, whereas the nonpathogenic *Leptospira biflexa* showed strong peroxidase but negative or weak catalase reactions (9). *Treponema pallidum* had strong SOD and catalase activities but lacked peroxidase, whereas cultivatible *Treponema* strains had none of these enzymes (1). Middlebrook reported that the acquisition of isoniazide resistance by *Mycobacterium tuberculosis* was accompanied by a loss of catalase and a loss of virulence (25).

*L. pneumophila* is responsible for 85% of human infections caused by *Legionella* species, with serogroups 1 and 6 accounting for 52 and 23%, respectively (31). Thus, there does not appear to be a direct association of the peroxidase with pathogenicity because the peroxidase appears equally distributed among all of the serotypes (Tables 1 to 3). *L. pneumophila* appears highly sensitive to externally added $H_2O_2$ (17, 22), to in vitro products of the mycloperoxidase system, and to the products of the xanthine oxidase system (21, 22). As indicated by the fact that killing of the cells in these systems is reversed by the addition of catalase, SOD, and mannitol, it appears that $H_2O_2$, $O_2^-$, and OH° were the toxic products or functioned to form them. Nevertheless, comparative results with virulent and avirulent strains lead to the conclusion that sensitivity to these products is not the basis for virulence or avirulence (21, 22). Our observations of the distribution of SOD and catalase among the species do not suggest that these enzymes play a role in pathogenesis, although there is positive evidence that the ability or lack of ability of these enzymes to deal with the external environment can seriously affect the growth of *Legionella* strains. It is therefore obvious that factors not related to the elimination of externally toxic $H_2O_2$ are in some manner restricting growth.

It is emphasized that all cells tested in our study were grown on BCYE agar. This medium has been found to give the maximum production by *Legionella* cells of both catalase and peroxidase, although *Escherichia* and *Pseudomonas* strains which have been grown on this medium have shown their lowest production of these enzymes. Furthermore, *Legionella* species grown in chemically defined broth have strongly repressed peroxidase and catalase production (L. Pine and P. S. Hoffman, unpublished data). Strains showing

no production of catalase might produce catalase if grown on an appropriate medium.

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