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Enzymatic Degradation of H_2O_2 by *Leptospira*

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The enzymes responsible for reducing H_2O_2 were surveyed in 49 strains of *Leptospira* by using semiquantitative assays for catalase and peroxidase. The survey revealed a differential distribution of catalase and peroxidase activities between the two leptospiral complexes. The pathogenic *Leptospira interrogans* strains gave strong catalase and weak or negative peroxidase reactions. Conversely, the nonpathogenic *Leptospira biflexa* strains gave strong peroxidase and negative or weak catalase reactions. An intermediate group of four *L. biflexa* strains, which were isolated from mammals, fell into the high peroxidase, low or negative catalase group. One water isolate, H-23, gave strong reactions for both enzymes and was examined for virulence and in vitro growth parameters. Results indicate metabolic differences between pathogens and water forms in their abilities to reduce H_2O_2.

The genus *Leptospira* is composed of two complexes, the pathogenic *Leptospira interrogans* and the free-living *Leptospira biflexa* (13), each containing numerous serovars. All leptospires are aerobes and therefore might be expected to generate peroxides during respiration. Enzymatic reduction of H_2O_2 by leptospires has been reported by Faine (6) and Rao et al. (17). These authors identified the enzyme catalase in several pathogenic serovars. Rao et al. examined a single *L. biflexa* strain and found it to be a low catalase producer. Faine reported that a catalase-inactive strain of serovar *icterohaemorrhagiae* did not produce peroxidase. Green et al. (8) found catalase present in the pathogenic *canicola* Moulton and *pomona* S-91 strains but absent in the pathogenic *icterohaemorrhagiae* RGA and *L. biflexa* strains *patoc* I and Waz. Baseman and Cox (2) reported a pyrogallol-oxidizing activity in the aquatic isolate *L. biflexa* B-16. Subsequent unpublished work performed in this laboratory revealed that catalase was present in all *L. interrogans* but absent in most *L. biflexa* strains examined. The absence of catalase and the paucity of evidence for peroxidases in the *L. biflexa* complex appears to be anomalous, since these organisms not only respire aerobically but have catabolic pathways (1) and terminal electron transport mechanisms (2) similar to the *L. interrogans* complex.

In this study, a survey of the peroxidative enzymes of both leptospiral complexes was conducted. The aims of the survey were to investigate the fate of H_2O_2 in members of the *L. biflexa* complex and to define possible peroxidative distinctions between pathogenic and free-living leptospires.

MATERIALS AND METHODS

Bacteria. The B and H strains of *L. biflexa* were isolated in this laboratory from surface water samples and carried as stock cultures at 30°C in synthetic medium SM-4 (10). All other strains had been obtained from various laboratories (11) and maintained as stock cultures at 30°C in 0.2% tryptose-phosphate broth (Difco Laboratories, Detroit, Mich.) containing 10% nonimmune rabbit serum and 0.2% agar (Difco). All strains were cloned by the method of Cox and Larson (5) before being put into stock culture and used in experiments.

Media, cultivation, and harvest. Cells were grown in a synthetic medium, SM-7, composed of the following: NaCl, 8.5 mM; KCl, 5.4 mM; $MgSO_4$, 0.4 mM; $CaCl_2$, 0.34 mM; $FeSO_4$, 5×10^{-3} mM; salt-free casein hydrolysate (acid), 1.0 mg/ml; vitamin B_{12}, 0.02 μg/ml; thiamine, 1.0 μg/ml; biotin, 0.1 μg/ml; Na_2HPO_4, 14.2 μg/ml; sodium acetate, 200 μg/ml; glycerol, 200 μg/ml; and sodium pyruvate, 200 μg/ml. The sources of fatty acids were: 0.01% (wt/vol) Tween 80 for all B and H strains, *canicola* Moulton, K-6, and K-22; 0.01% Tween 80 and 0.2% bovine serum albumin fraction V (Miles Laboratories Inc., Elkhart, Ind.) for strains *illini* 3055, *sejroe* M-84, *pomona* Wickard, *pomona* Riggs, *pomona* Pomona, *hardjo* Hardjo, *javanica* Veldrat Bataviae, *ballum* M-127, *tarassovi* Tarassovi, *pyrogenes* Salinem, *fort-bragg* Fort-Bragg, *sentot* Sentot, *grippotyphosa* Moskva V, *copenhageni* M-20, *sarmin* Sarmin, and *patoc* I; and a 10:1 ratio of Tween 60 and 80 in a combined final concentration of 0.01% for *schueffneri* Vleermuis 90C, *canicola* Benjamin, *canicola* Hond Utrecht, *pomona* STH-262, *grippotyphosa* 1540, *grippotyphosa* 1545, *celledoni* Celledoni, *andamana, kazachstanika, sao paulo, semaranga, czekalowski,* CDC, LT430, wa Rieden, and Waz. Cells were grown without shaking at 30°C in 10-ml volumes in test tubes (20 by 150 mm) with Morton-type caps. Bacteria were harvested when the growth achieved a turbidity of 40 to 50 nepholometer units

using a Coleman 9 nephocolorimeter adjusted to 20 nepholometer units with a Coleman 81 nepholos standard, which corresponded to approximately 10^8 cells/ml. A 20-ml culture of each strain was harvested by centrifugation at 17,300 × *g* for 20 min at 4°C. The supernatant fluids were discarded, and each pellet was suspended in 0.1 to 0.2 ml of residual supernatant fluid. Cells were enumerated with a Petroff-Hausser chamber.

Growth inhibition studies. Growth inhibition of leptospires by $CuSO_4$ (7), 8-azaguanine (15), and normal rabbit serum (14) were performed as described previously except that SM-7 and SM-4 with 0.01% Tween 80 and SM-4 and 0.2% tryptose-phosphate broth with 10.0% rabbit serum were used as growth media.

Enzyme assays. Catalase (EC 1.11.1.6) was assayed by a semiquantitative procedure that consisted of adding 0.05 ml of sample to the well of a Linbro microtiter tray (IS-MRC-96, Linbro Chemical Corp.) and then adding 0.05 ml of a 3.0% H_2O_2 solution to the well and observing for bubble evolution under a dissecting microscope at ×10 magnification for 5 min. Peroxidase (EC 1.11.1.7) was assayed by an adaptation of the procedure of Herzog and Fahimi (12). This consisted of observing color development after adding 0.03 ml of each of the following to the well of a Linbro microtiter tray: 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, Mo.) and gelatin (Difco), each at a concentration of 1.0 mg/ml suspended in phosphate-citric acid buffer (17) at a pH of 4.8, 0.3% H_2O_2, and sample.

Virulence studies. The virulence studies were performed on hamsters as previously described (L. A. Baker, Ph.D. thesis, University of Massachusetts, Amherst, 1974). Groups of two animals were used for each cardiac bleeding. Groups of six animals, which had not been bled, were used for mortality determinations. Livers and kidneys were aseptically removed from exsanguinated animals 8 days postinfection, ground in SM-7 medium supplemented with 0.2% bovine serum albumin fraction V, and serially diluted in the same medium. Cultures were incubated without shaking at 30°C and were monitored by dark-field microscopy for 60 days before being considered negative.

Reagents. All chemicals used in this study were of reagent grade. Horseradish peroxidase type III (185 U/mg) and purified bovine liver catalase (2,200 U/mg) were obtained from Sigma. All of the assay reagents were freshly prepared for each experiment.

RESULTS

Assay procedures. In our system, three levels of catalase activity were discerned. The data in Table 1 indicate that as few as 2.4×10^8 catalase-producing cells or 10^{-4} mg of purified bovine liver catalase per ml was detected.

The peroxidase assay depends upon the ability of whole cells to utilize DAB as the hydrogen donor in a peroxidase reaction. DAB was added in its reduced form, which is colorless at the concentration used. DAB has an absorbance maximum at 465 nm and appears brown when oxidized. In this reaction, H_2O_2 is the oxygen donor and reduces to water. This procedure is also semiquantitative, with three levels of activity discernable. Readings for the peroxidase assay were made over a light box with a groundglass plate. An incubation time of 40 min was selected because it was sufficient for cells with low levels of peroxidase to develop a weak reaction but was not enough time for apparent DAB autooxidation. A brown homogeneous color developed throughout the well when DAB was oxidized by a solution of purified enzyme or by autooxidation. A darker precipitate, presumably from leptospires, was also seen when a leptospiral suspension was used. This observation could aid in distinguishing weak whole-cell peroxidase reactions from autooxidation of the substrate. Table 1 demonstrates that 3.9×10^8 peroxidase-producing cells or 10^{-6} mg of horseradish peroxidase per ml could be detected. Catalase did not catalyze DAB oxidation, nor did peroxidase cause bubble evolution; thus each assay was specific. Also, the presence of both enzymes in the same mixture did not obscure the results of either assay.

TABLE 1. *Assays for catalase and peroxidase*

Sample	Enzyme activity	
	Catalase	Peroxidase
Catalase 1.0 mg/ml	++[a]	0[b]
Catalase 10^{-2} mg/ml	++	0
Catalase 10^{-4} mg/ml	+[c]	0
Catalase 10^{-5} mg/ml	0	0
Horseradish peroxidase 1.0 mg/ml	0	++
Horseradish peroxidase 10^{-4} mg/ml	0	++
Horseradish peroxidase 10^{-6} mg/ml	0	+
Horseradish peroxidase 10^{-7} mg/ml	0	0
Horseradish peroxidase 10^{-4} mg/ml and catalase 10^{-2} mg/ml	++	++
L. biflexa B-16 3.9×10^8 cells/ml	0	+
L. biflexa B-16 3.9×10^{10} cells/ml	0	++
L. interrogans pomona Riggs 2.4 × 10^8 cells/ml	+	0
L. interrogans pomona Riggs 2.4 × 10^{10} cells/ml	++	0

[a] Strong activity.
[b] No activity.
[c] Weak activity.

Survey. The results of the survey are presented in Table 2. A total of 49 strains are included, comprising 23 pathogens, 4 intermediate strains that were isolated from mammals but resemble water leptospires in other respects (*czekalowski, andamana, semaranga,* and *kazachstanika*), and 22 water isolates. Members of the four genetic groups described by Brendle et

TABLE 2. *Catalase and peroxidase distribution in Leptospira*

Leptospiral strain	Enzyme activity	
	Catalase	Peroxidase
L. biflexa LT430, Waz, Wa Rieden, CDC, B-2, B-5, B-6, B-7, B-10, B-16, B-17, H-13, H-21, K-6, K-22, *patoc* I, *sao paulo,* and *czekalowski*	0[a]	++[b]
L. biflexa B-3, B-9, H-3, *andamana, kazachstanika,* and *semaranga*	+[c]	++
L. interrogans grippotyphosa 1540, *pomona* Riggs, *javanica* Veldrat Bataviae, *sejroe* M-84, *fort-bragg* Fort-bragg, and *copenhageni* M-20	++	0
L. interrogans celledoni Celledoni, *sentot* Sentot, *sarmin* Sarmin, *ballum* M-127, *grippotyphosa* 1545, *grippotyphosa* Moskva V, *canicola* benjamin, *canicola* Hond Utrecht, *canicola* Moulton, *hardjo* Hardjo, *tarrasovi, pomona* STH 262, *pomona* Pomona, *pomona* Wickard, *pyrogenes* Salinem and *djasiman* Djasiman	++	+
L. interrogans schueffneri Vleermuis and *L. biflexa illini* 3055 and H-23	++	++

[a] No activity.
[b] Strong activity.
[c] Weak activity.

al. (3) are included. Table 2 shows that the *L. biflexa* and *L. interrogans* complexes can indeed be distinguished on the basis of their peroxidative enzymes. The pathogens characteristically gave a strong catalase and a weak or negative peroxidase reaction. Conversely, the water isolates characteristically gave a strong peroxidase and a negative or weak catalase reaction. The intermediate group of mammalian isolates in the *L. biflexa* complex all fell into the high peroxidase, low catalase group.

L. biflexa H-23 was the only water isolate with strong catalase activity. Based upon this observation further studies were conducted to determine whether H-23 shared other features characteristic of pathogenic leptospires. Unlike members of the *L. interrogans* complex, H-23 has simple nutritional requirements and was isolated and routinely grown upon the synthetic medium SM-4. Concentrations of $CuSO_4$ sufficient to inhibit the growth of pathogens but not water isolates had no effect on the growth of H-23. However, the presence of 8-azaguanine, at 225 μg/ml, which completely inhibited the growth of pathogens but had no effect on the growth of the water isolates, did have an inhibitory action on the growth of H-23. A lag was produced such that approximately one-half yields were seen at a time when control cultures lacking 8-azaguanine achieved full growth. With extended incubation, the cultures with 8-azaguanine also realized full growth. The lag phenomenon from small inocula in 8-azaguanine further indicated a possible connection between H-23 and pathogenic leptospires. In addition, unpublished data show H-23 to be more resistant to the killing effects of nonimmune rabbit serum than other water isolates and yet are more sensitive than pathogenic forms. Finally, strain H-23 was unable to cause morbidity or mortality, was rapidly cleared and failed to appear in the blood, and was unable to colonize the livers or kidneys of hamsters as determined by our virulence studies.

Similar tests were performed on *L. interrogans schueffneri,* and this strain behaved as did other pathogenic leptospires that were used as controls in these experiments. The possibility that our *schueffneri* culture was contaminated with a water leptospire was also considered. This possible explanation was dispelled by performing microscopic agglutination tests upon our strain of *schueffneri* and a reference strain of *schueffneri* obtained from the Center for Disease Control, Atlanta, Ga., against antiserum for *canicola* Hond Utrecht, a closely related member of the same serogroup. Both cultures gave the same microscopic agglutination titer.

DISCUSSION

Catalase and peroxidase assays were rapid and sensitive, and they required small amounts of cellular material. Both tests can be standardized by including the appropriate dilutions of catalase and peroxidase solutions.

The pattern of low or no catalase and high peroxidase activities in the *L. biflexa* complex and high catalase and low or no peroxidase activities for the *L. interrogans* complex held true for most strains examined. Since there were only three exceptions to the pattern (strains *schueffneri, illini* 3055, and H-23) and the peroxidative tests were facile, we propose that these techniques may be useful in taxonomic and diagnostic evaluations of leptospires.

Given the plethora of leptospiral types and strains, the presence of exceptions to the peroxidative pattern is not an unexpected result. The finding of exceptions from each complex that gave strong catalase and peroxidase reactions is of possible interest from an evolutionary perspective. One might speculate that a physiological continuum exists between pathogens and

nonpathogens, and that H-23 and *schueffneri* are closely related to ancestral leptospires that were in transition from a free-living to a parasitic mode of existence. This contention is further supported by the partial growth inhibition of H-23 by 8-azaguanine and the increased resistance of this strain to the toxic effects of nonimmune rabbit serum. The virulence studies indicate that H-23 is not pathogenic for hamsters. This strain may possess some of the physiological attributes of pathogens that may be necessary but not sufficient for the expression of pathogenicity. The aberrant reactions of *illini* 3055 are not inconsistent with the atypical nutritional, antigenic, and genetic features of this serovar (9).

No correlation between genetic group (3) and the peroxidative capabilities of leptospires was apparent. No differences were observed when leptospires were grown with aeration. There were also no correlations between the various growth media or serovars and the peroxidative reactions of an individual strain. This last point is clearly demonstrated by *L. biflexa* B-7 and *L. biflexa* H-23, which are antigenically indistinguishable (11) but quite different by our peroxidative criterion. Whether the difference is genotypic or phenotypic is not known, but the data are sufficient to show that leptospires of apparently identical antigenic structures can have significant metabolic differences.

Our observations might indicate a critical distinction between the metabolism of pathogenic and free-living leptospires with respect to mechanisms for generation and reduction of H_2O_2. Canale-Parola (4) has speculated that pathogenic spirochetes are derived from free-living and commensal forms. If this holds for leptospires, the results of our survey would make peroxidase the more ancient H_2O_2-degrading enzyme. Leptospiral peroxidases are currently being studied to clarify the relationship between this enzyme and leptospiral catalase. This line of research may be of significance in understanding the general ecology and pathogenic capabilities of spirochetes.

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