INFECTION AND IMMUNITY, July 1991, p. 2239–2244
0019-9567/91/072239-06$02.00/0

Vol. 59, No. 7

Elimination of Hydrogen Peroxide by *Haemophilus somnus*, a Catalase-Negative Pathogen of Cattle

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Received 26 December 1990/Accepted 5 April 1991

***Haemophilus somnus* is a catalase-negative, gram-negative pathogen of cattle which is refractory to killing by bovine neutrophils. In this report, we showed that *H. somnus* rapidly inhibited Luminol-dependent chemiluminescence of bovine neutrophils costimulated with opsonized zymosan or phorbol myristate acetate. We have postulated that this inhibition resulted in part from *H. somnus* preventing the accumulation of hydrogen peroxide (H_2O_2) during the oxidative burst. In support of this hypothesis, we have demonstrated that when stimulated with viable *H. somnus*, bovine neutrophils accumulate lower levels of H_2O_2 than did neutrophils stimulated with heat-killed *H. somnus* or opsonized zymosan. We have presented evidence that four separate strains of *H. somnus*, despite being catalase negative by conventional criteria, removed H_2O_2 from solution. Viable cells of *H. somnus* were required for the removal of H_2O_2 from solution; little or no activity was observed when suspensions of heat-killed, formalin-killed, or sonicated cells of *H. somnus* were incubated with H_2O_2. In addition, the elimination of H_2O_2 occurred only in the presence of carbon sources that could be utilized by *H. somnus*, indicating that elimination of H_2O_2 was an energy-dependent process. The amount of H_2O_2 that could be eliminated by 10^7 cells of *H. somnus* was greater than 10 nmol, an amount comparable to that produced by a similar number of stimulated bovine neutrophils. Thus, we suggest that the ability of *H. somnus* to remove H_2O_2 from solution may be an important virulence mechanism that contributes to the survival of the organism following ingestion by bovine neutrophils.**

Haemophilus somnus is a gram-negative pathogen that causes a variety of clinical and subclinical manifestations in cattle, collectively referred to as the *H. somnus* complex (4). *H. somnus* was initially isolated and described in the early 1960s as the etiological agent of thromboembolic meningoencephalitis, a rapidly fatal form of septicemia and meningitis (2, 24). Subsequently, the organism has been isolated from cases of pneumonia (1, 4, 9, 27, 40), abortion (6, 14, 39), arthritis (22), and mastitis (18) and has been implicated in chronic infertility problems (8). The epizootic nature of *H. somnus* infections is illustrated by the fact that the organism can also be readily isolated from the urogenital tract of apparently healthy cattle (21, 28). These isolates are biochemically and serologically indistinguishable from strains isolated from thromboembolic meningoencephalitis or pneumonia cases (15, 22), although urogenital isolates are more likely to be serum sensitive (7). At this time, it is not clear whether *H. somnus* is an opportunistic pathogen and these urogenital isolates represent a reservoir for disease outbreaks or whether there exist distinct populations of commensal and pathogenic strains.

Exposure to *H. somnus* is not uncommon, and it appears that most cattle are able to clear the infection before clinical symptoms appear. The specific means by which the host controls *H. somnus* infection is not clear. Although specific antibody is partially protective in experimentally challenged cattle, the organism can persist despite the presence of circulating antibody (16, 19). The role of neutrophils in the pathogenesis of the disease has been inferred largely from histopathological examination of infected tissues. *H. somnus* lesions typically exhibit a large influx of neutrophils associated with vasculitis and thrombosis (1, 16). Despite the presence of neutrophils, *H. somnus* continues to persist within the lesion, suggesting that the neutrophils are ineffectual in controlling the initial infection. These descriptions, along with in vitro observations that bovine neutrophils ingest but do not kill *H. somnus* (10), suggest that its ability to resist killing by neutrophils is critical to the establishment and progression of clinical disease. It has been demonstrated that the organism can inhibit phagocytosis and iodination of particulate stimuli by bovine neutrophils (20). These inhibitory properties were associated with the release of purine nucleotides and the presence of a high-molecular-weight RNA moiety on the cell surface (5).

Although originally classified as a member of the genus *Haemophilus* because of its requirement for hemin and an increased carbon dioxide atmosphere, it is now clear that *H. somnus* is not a true member of the genus *Haemophilus* and awaits reclassification (38). The organism lacks detectable catalase activity, a feature which, along with its characteristic yellow pigment, makes it easily identified in the laboratory (15, 38). In other organisms, levels of catalase have been correlated with virulence (17, 26, 37) and with increased resistance to the oxidative burst of professional phagocytes (3, 11). In this respect, it is interesting that *H. somnus*, a catalase-negative bacteria, is able to avoid the destructive effects of bovine neutrophils. However, in this report we have presented evidence that *H. somnus* removes hydrogen peroxide (H_2O_2) from solution in a manner inconsistent with a catalase-mediated mechanism. Thus, *H. somnus* may be able to inactivate H_2O_2 generated by neutrophils during the oxidative burst. This could explain, in part, the survival of *H. somnus* following ingestion by bovine neutrophils.

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MATERIALS AND METHODS

Media and reagents. All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Hanks balanced salt solution (HBSS) and Dulbecco's phosphate-buffered saline (PBS) were purchased from GIBCO (Grand Island, N.Y.). Brain heart infusion broth and yeast extract were obtained from Difco (Detroit, Mich.). Sheep blood agar (SBA) plates were purchased from the BBL division of Becton Dickinson (Cockeysville, Md.), and Percoll was obtained from Pharmacia LKB Biotechnology (Piscataway, N.J.).

Cultivation of bacteria. *H. somnus* 8025 (ATCC 43625) and CAHL (ATCC 43626) were described in a previous study as laboratory and field strains, respectively (10). Strains 2336 (pneumonia isolate) and 127P (preputial isolate) were previously described and generously provided by Lynette Corbeil (23). Bacteria were grown for 18 to 24 h in brain heart infusion broth containing 0.5% yeast extract at 37°C under an atmosphere of 5% CO_2. Bacteria were opsonized for 30 min with antiserum raised in cattle against formalin-killed organisms (10) and were harvested by centrifugation at 5000 × *g* for 15 min, washed twice, and resuspended in an appropriate buffer. Organisms were enumerated by serial dilution and spread plating on SBA incubated at 37°C under 5% CO_2 atmosphere. Heat-killed bacteria were prepared by incubating suspensions of *H. somnus* at 60°C for 1 h. Formalin-killed bacteria were incubated with 0.5% formaldehyde for 2 h at 37°C. Killed bacteria were washed extensively in PBS prior to use in experiments. In some experiments, bacteria were disrupted by sonication prior to use.

Preparation of bovine neutrophils. Bovine neutrophils were prepared from peripheral blood of healthy Holstein cattle as previously described (2). Blood was centrifuged for 20 min (1,000 × *g*), and the resulting buffy coat was discarded. The remaining erythrocyte portion was lysed by brief exposure to hypotonic conditions; after reestablishing isotonicity, the neutrophils were harvested by centrifugation at 300 × *g*. Neutrophils were further purified by centrifugation through a 65% Percoll cushion for 15 min at 300 × *g*, washed twice, and resuspended in HBSS prior to use. This procedure consistently gives suspensions of at least 95% neutrophils and greater than 98% viability.

Luminol-dependent chemiluminescence assay. Luminol-dependent chemiluminescence (LDCL) was measured as previously described (33, 34) with a Pico-Lite luminometer (Packard Instrument Co., Rockville, Md.). Chemiluminescence was determined by using 10^7 neutrophils per tube in 0.2 ml of HBSS containing 8 μM Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). Opsonized bacteria or other stimuli were suspended in HBSS and added to neutrophils at the beginning of each experiment.

Superoxide and hydrogen peroxide generation assays. Production of superoxide anion (O_2^-) was measured by the cytochrome *c* reduction assay, and H_2O_2 was measured by the horseradish peroxidase-dependent phenol red oxidation assay of Pick and Mizel (31). These assays were slightly modified as previously described (33, 34).

Hydrogen peroxide elimination assay. Scavenging of H_2O_2 was measured by using a modification of the horseradish peroxidase-mediated phenol red oxidation microassay of Pick and Keisari (30). A similar approach has been described for the determination of H_2O_2 generated in bacterial cultures (25). Briefly, *H. somnus* was incubated in micro-ELISA (enzyme-linked immunosorbent assay) wells with 10 nmol of H_2O_2 at 39°C in a final volume of 0.2 ml. After a 1-h incubation, the amount of H_2O_2 remaining was determined by adding 0.05 ml of 0.1% phenol red containing 100 U of horseradish peroxidase per ml (PRS/HRP). The pH was adjusted to approximately 11.0 by the addition of 0.02 ml of 1.0 N NaOH, and the absorbance was measured with a micro-ELISA-plate reader (model MR580; Dynatech Instruments, Torrence, Calif.). The amount of H_2O_2 remaining was calculated on the basis of a standard curve generated with known amounts of H_2O_2.

For kinetic experiments, H_2O_2 scavenging by *H. somnus* was terminated at the indicated times by the addition of PRS/HRP. Phenol red oxidation was rapid, and virtually all remaining H_2O_2 was converted within 15 s of the addition of PRS/HRP (data not shown). For experiments investigating the role of different energy sources in H_2O_2 elimination by *H. somnus*, all reagents were prepared in buffers containing the indicated carbohydrates.

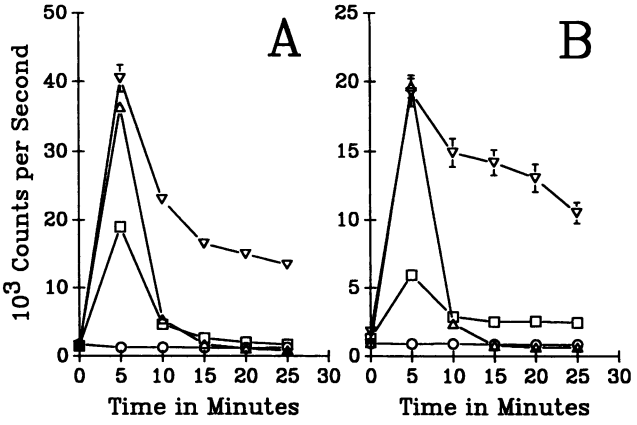

FIG. 1. Interference by *H. somnus* with the chemiluminescence response of bovine neutrophils. The LDCL responses of bovine neutrophils stimulated with 0.1 mg of opsonized zymosan (A) or 100 ng of PMA per ml (B) are shown in the presence (□) and absence (▽) of 10^7 opsonized *H. somnus* organisms. The responses of neutrophils incubated only with opsonized *H. somnus* (△) or HBSS (○) are also shown. Data are means ± standard errors of counts per second of triplicate determinations from a representative experiment (one of three).

RESULTS

Chemiluminescence response of bovine neutrophils. *H. somnus* stimulates an oxidative burst of limited duration in comparison with other particulate stimuli, such as *Escherichia coli* (10) or opsonized zymosan (Fig. 1A). In addition, we have demonstrated that viable *H. somnus* reduced the duration of zymosan-stimulated LDCL (Fig. 1A). Costimulation of bovine neutrophils with *H. somnus* and opsonized zymosan resulted in a transient burst of LDCL, which rapidly returned to baseline levels. The kinetics of this response was similar to that elicited by *H. somnus* alone. The altered response is not likely due to competition between opsonized *H. somnus* and zymosan for Fc receptors on the neutrophils, since a similar inhibition was observed when phorbol myristate acetate (PMA) was used as a costimulus with *H. somnus* (Fig. 1B).

The rapid abatement of the LDCL response suggested to us that *H. somnus* interfered with the ability of reactive oxygen species to interact with Luminol. Since it has previously been reported that *H. somnus* does not affect super-

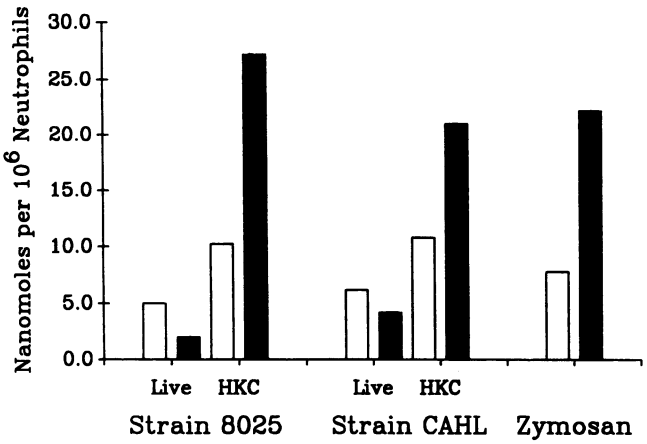

FIG. 2. Generation of O_2^- and H_2O_2 by bovine neutrophils stimulated with live and heat-killed *H. somnus*. The generation of O_2^- (□) and H_2O_2 (■) by bovine neutrophils was determined following stimulation with live or heat-killed cells (HKC) of *H. somnus* (two different strains) or opsonized zymosan. Data are presented as nanomoles of O_2^- and H_2O_2 generated per 10^6 neutrophils after 2 h and are the results of a representative experiment.

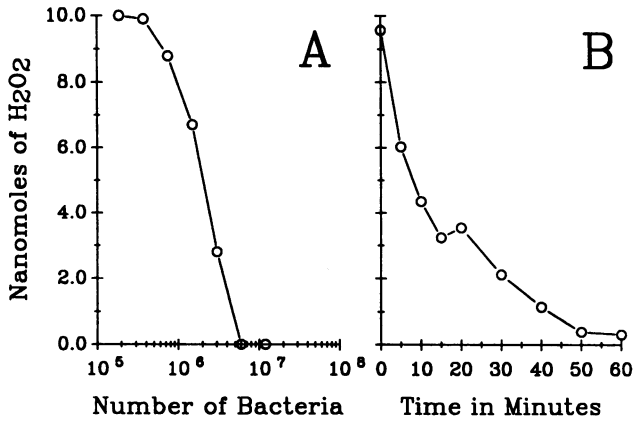

FIG. 3. Ability of *H. somnus* to remove H_2O_2 from solution in a dose- and time-dependent manner. *H. somnus* were incubated with 10 nmol of H_2O_2 in HBSS at 37°C for the indicated times, and the remaining H_2O_2 was measured as described in Materials and Methods. (A) Amount of H_2O_2 remaining after incubation for 1 h with increasing concentrations of *H. somnus*; (B) amount of H_2O_2 remaining at various times of incubation at 39°C with 10^7 viable organisms. Data are from a representative experiment (one of four).

oxide (O_2^-) production by bovine neutrophils (20), we investigated the possibility that *H. somnus* inhibits LDCL by influencing the generation of H_2O_2 by neutrophils.

Stimulation of O_2^- and H_2O_2 production by *H. somnus*. Stimulation of bovine neutrophils with either heat-killed *H. somnus* or opsonized zymosan resulted in the generation of more than 20 nmol of H_2O_2 per 10^6 neutrophils (Fig. 2). Stimulation with the same number of live *H. somnus* resulted in the detection of greatly reduced levels of H_2O_2, less than 5 nmol of per 10^6 neutrophils. Although live *H. somnus* also stimulated slightly reduced levels of superoxide anion, the amount of reduction was not nearly as great as that seen with H_2O_2.

Since H_2O_2 is derived directly from O_2^-, these results suggested that live *H. somnus* may actually be interfering with our ability to detect H_2O_2 being generated by neutrophils. We examined the possibility that, despite being catalase negative, *H. somnus* is still able to destroy H_2O_2 and make it unavailable for the oxidation of phenol red by horseradish peroxidase.

***H. somnus* removes hydrogen peroxide from solution.** Despite lacking catalase activity as defined by conventional tests (15, 38), *H. somnus* 8025 eliminated H_2O_2 from solution when suspended in HBSS. At least 10 nmol of H_2O_2 was removed by 5×10^6 cells of *H. somnus*, in a final volume of 0.2 ml, within 1 h (Fig. 3A), while lower cell numbers removed proportionately less. The removal of H_2O_2 was a time-dependent process which required about 1 h for maximal scavenging of H_2O_2 by 10^7 cells (Fig. 3B). The ability of *H. somnus* to eliminate H_2O_2 could not be blocked with antisera directed against the bacteria (data not shown). Removal of H_2O_2 occurred in the presence of normal bovine sera, antisera raised against formalin-killed *H. somnus* (described in reference 10), or convalescent sera from experimentally infected cattle (generously provided by L. Corbeil). In all cases, greater than 90% of the added H_2O_2 was removed, irrespective of the addition of antisera.

H_2O_2 removal requires metabolically active *H. somnus*. Live *H. somnus* at 3×10^7 per well were able to efficiently scavenge H_2O_2 from PBS containing 0.2% glucose (Table 1). A heat-killed preparation of bacteria at the same concentration was unable to remove significant amounts of H_2O_2. Likewise, sonicated cell preparations and formalin-killed *H. somnus* were unable to eliminate appreciable amounts of H_2O_2. To show that elimination of H_2O_2 required the presence of *H. somnus*, and to rule out the possibility that H_2O_2 removal was the result of a metabolic by-product or pH change during the assay, bacteria were incubated in PBS containing 0.2% glucose for 1 h and subsequently removed by centrifugation. The cell-free supernatant did not scavenge H_2O_2 (Table 1), whereas the pelleted bacteria were able to remove most of the H_2O_2 from solution when resuspended in an equal volume of fresh PBS containing glucose.

In contrast to the results presented above, when viable cells of *H. somnus* were suspended in PBS without added glucose, scavenging of H_2O_2 was not observed, even with greater than 10^7 cells (Fig. 4A). All four strains of *H. somnus* that we tested were able to remove H_2O_2 at similar cell densities in the presence but not the absence of glucose (Fig. 4B). To test the specific role of glucose in the removal of H_2O_2 from solution, other carbohydrates were compared for

TABLE 1. Evidence that elimination of H_2O_2 by *H. somnus* 8025 requires viable organisms

Bacterial prep[a]	% Viability[b]	Amt (nmol) of H_2O_2 remaining[c]
Untreated cells	100.0	0.7
Heat-killed cells	0.01	9.4
Formalin-killed cells	<0.001	9.8
Sonicated cells	0.3	8.7
Cell-free supernatant	<0.001	9.4

[a] *H. somnus* was suspended in PBS containing 0.2% glucose, and aliquots were treated as described in Materials and Methods.

[b] Determined by serial dilution and spread plating on SBA. The untreated cell suspension contained 3×10^7 per well and was assumed to be 100% viable.

[c] Suspensions were incubated with 10 nmol of H_2O_2 at 37°C; data indicate the amount of H_2O_2 remaining after 1 h.

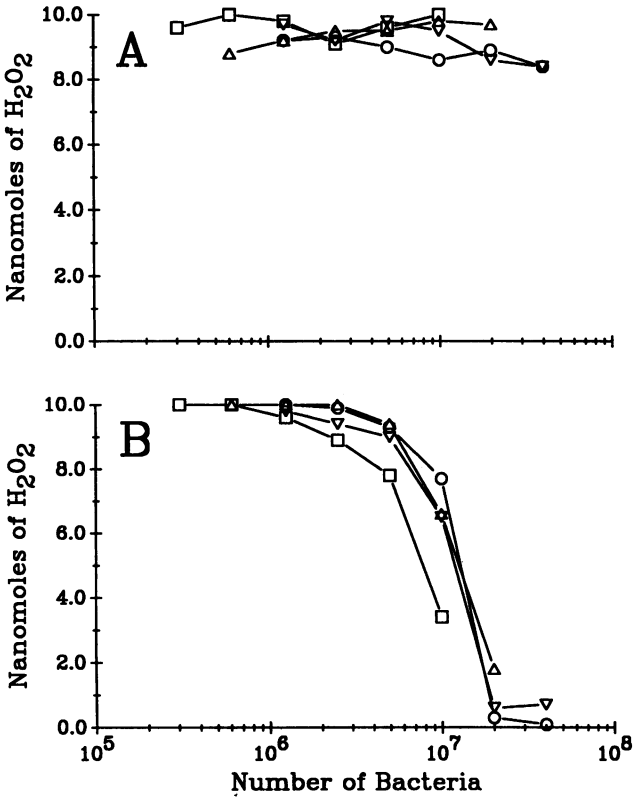

FIG. 4. Evidence that removal of hydrogen peroxide is dependent on glucose. Strains of *H. somnus* were resuspended in PBS (A) or PBS containing 0.2% glucose (B) and incubated with 10 nmol of H_2O_2. Data show the amount of H_2O_2 remaining following incubation with increasing concentrations of strains 8025 (○), CAHL (△), 2336 (□), and 127P (▽) for 1 h at 39°C. Data are from a representative experiment (one of three).

their ability to support scavenging of H_2O_2 by *H. somnus* (Table 2). Four compounds (glucose, lactose, sucrose, and mannitol) were chosen on the basis of the ability of *H. somnus* to utilize them as energy sources. Both glucose and mannitol, which are fermented by *H. somnus*, allowed the bacteria to remove H_2O_2 from solution. In contrast, *H. somnus* did not eliminate H_2O_2 in the presence of lactose and sucrose, which do not support the growth of *H. somnus*.

TABLE 2. Evidence that elimination of H_2O_2 by *H. somnus* 8025 requires a utilizable energy source

Carbon source[a]	Fermentation by *H. somnus*[b]	Amt (nmol) of H_2O_2 remaining[c]
None		10.0
Glucose	Yes	1.1
Mannitol	Yes	1.3
Lactose	No	9.1
Sucrose	No	8.8

[a] The indicated carbohydrate was added to PBS at a final concentration of 0.2%. Each well contained 10^7 viable cells of *H. somnus*.

[b] The ability of *H. somnus* to ferment the indicated carbohydrates was previously described (15, 38).

[c] Bacterial suspensions were incubated with 10 nmol of H_2O_2 at 37°C; data indicate the amount of H_2O_2 remaining after 1 h.

DISCUSSION

Previous work from this laboratory examined the interactions of *H. somnus* with bovine neutrophils (10). Opsonized *H. somnus* elicited an LDCL response from neutrophils which was of markedly shorter duration than the LDCL response stimulated by the same number of *E. coli*. From these experiments, it was not clear whether *H. somnus* was a poor stimulus for bovine neutrophils or whether the organism interfered with the oxidative burst. In this report, we have shown that viable *H. somnus* reduced both the magnitude and the duration of LDCL costimulated by either zymosan or PMA. We cannot rule out the possibility that the decrease in peak LDCL is the result of a rapid stimulation which occurred before the 5-min time point. However, it is clear that the presence of viable *H. somnus* greatly reduced the duration of the LDCL response to zymosan and PMA. These results suggest that the organism is able to either inhibit the oxidative burst of bovine neutrophils or interfere with our ability to measure it with LDCL.

Suppression of bovine neutrophil functions by *H. somnus* has been previously described (20), and the bacterial components responsible for the inhibition have been partially characterized (5). Although myeloperoxidase-mediated iodination of target cells was inhibited by *H. somnus*, no effect on nitroblue tetrazolium reduction by neutrophils was observed. Since the latter is an indirect measure of O_2^- generation, the authors concluded that *H. somnus* had no direct effect on the oxidative response of bovine neutrophils. Furthermore, they concluded that the inhibition of iodination, an H_2O_2-mediated event, was due to either suppression of granule fusion or inhibition of the myeloperoxidase enzyme (20). In contrast, our LDCL data suggested that *H. somnus* does interfere with the oxidative burst of bovine neutrophils. In an attempt to reconcile these seemingly disparate results, we examined the possibility that *H. somnus* may interfere with or prevent the accumulation of H_2O_2 during the oxidative burst.

When bovine neutrophils were stimulated with viable *H. somnus*, we observed a striking reduction in the amount of H_2O_2 detected compared with levels stimulated by opsonized zymosan. Although the organism may exert some inhibition of neutrophil functions, it is unlikely that the reduction in H_2O_2 is due solely to quelling of the oxidative response, since O_2^- production was only mildly affected. This reduction in H_2O_2 levels was not seen with heat-killed organisms, suggesting that the interference with H_2O_2 generation was dependent on the viability of the bacteria. It is also clear that the organism is not necessarily a poor stimulus for bovine neutrophils, since heat-killed organisms elicited a response equal to that seen with opsonized zymosan. A likely explanation for these results is that live *H. somnus* is able to eliminate H_2O_2 as it is being generated by the neutrophil and preclude it from serving as a substrate for horseradish peroxidase in the detection system.

Although *H. somnus* lacks catalase activity as measured by typical diagnostic tests (29), we have demonstrated that it is capable of removing H_2O_2 from aqueous solution. Consumption of H_2O_2 required viable organisms; we did not observe scavenging of H_2O_2 by nonviable *H. somnus*, whether they had been heat killed, formalin killed, or disrupted by sonication. In addition, *H. somnus* did not remove H_2O_2 unless supplied with a suitable energy source. The ability of various carbohydrates to support the removal of H_2O_2 correlated with their ability to be fermented by *H. somnus* (15, 38). Neither lactose nor sucrose has been

reported to be fermented by any strains of *H. somnus*, and neither sugar supported H_2O_2 consumption. Likewise, both glucose and mannitol are utilized by *H. somnus*, and either carbohydrate enabled the organism to eliminate H_2O_2. Taken together, these data suggest that the mechanism of H_2O_2 removal requires a metabolic intermediate, possibly an adequate adenylate phosphate charge or reducing power, in order to occur.

When we incubated *H. somnus* in PBS containing glucose for 1 h and then removed the bacteria, no scavenging of H_2O_2 was observed in the cell-free supernatant. Therefore, it is unlikely that elimination of H_2O_2 by *H. somnus* was due to metabolic by-products produced during fermentation. It is also unlikely that the removal of H_2O_2 by *H. somnus* resulted from levels of catalase not detected by conventional diagnostic assays. Since purified catalase does not require glucose or any other energy source for its enzymatic action, one would expect sonicated organisms to retain the ability to scavenge H_2O_2 if the process was mediated by catalase (26).

At this time it is not clear what mechanism is used by *H. somnus* to eliminate H_2O_2 from solution. However, a similar phenomenon has been described in another catalase-negative organism, *Streptococcus faecalis* (12, 13, 35, 36). The ability of *S. faecalis* to eliminate H_2O_2 has been ascribed to a peroxidase which converts H_2O_2 to H_2O in the presence of NADH (32). This NADH peroxidase is a tetrameric flavoprotein enzyme that fails to utilize the usual substrates for hematin peroxidases, such as horseradish peroxidase (13). Likewise, our attempts to demonstrate direct peroxidase activity in *H. somnus* by using phenol red and ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] have been unsuccessful (data not shown). We are currently addressing the possibility that *H. somnus* contains an NADH peroxidase similar to that seen in *S. faecalis*.

Although a lower amount of H_2O_2 is scavenged by *H. somnus* than by catalase-positive organisms, this amount may be biologically relevant. In a previous report we have shown that 10^6 bovine neutrophils produce between 5 and 20 nmol of H_2O_2 when stimulated with opsonized zymosan or PMA (33). Thus, a single cell of *H. somnus* would be able to remove most of the H_2O_2 generated by a single neutrophil. In situations in which *H. somnus* outnumber neutrophils, the bacteria would most likely be able to scavenge virtually all of the H_2O_2 being generated by stimulated neutrophils. This hypothesis would readily explain the observations that *H. somnus* is able to repress iodination by bovine neutrophils without concomitantly affecting O_2^- generation (20). It has also been postulated that hypochlorous acid (HOCl) and associated chloramines play a key role in microbial killing mechanisms of neutrophils (41). In addition to having direct bactericidal properties, HOCl activates at least some of the proteolytic enzymes located in neutrophil granules, including elastase, collagenase, and gelatinase. Since HOCl is generated from H_2O_2 via myeloperoxidase, it is reasonable to suggest that any mechanism by which a bacterium can compete for H_2O_2 with myeloperoxidase would reduce the net amount of potentially toxic HOCl generated by neutrophils. This in turn could reduce the effectiveness of antibacterial enzymes released into the phagolysosome following granule fusion.

The data presented here provide evidence of a catalase-independent mechanism of H_2O_2 scavenging by at least four strains of *H. somnus*. Unlike catalase-mediated breakdown of H_2O_2, the mechanism employed by *H. somnus* was energy dependent and strictly cell associated; little or no activity could be detected in nonviable cells or from viable cells in the absence of a fermentable carbon source. At this time we have not elucidated the pathway(s) involved in H_2O_2 scavenging, nor have we been able to determine the fate of ıe H_2O_2 removed from solution. Because of interference by atalase, it will be difficult to determine whether this mechanism is common to other pathogens or is unique to *H. somnus*. However, it is tempting to postulate that elimination of H_2O_2, in conjunction with suppression of neutrophil functions, may be an important virulence attribute which allows *H. somnus* to survive the oxidative burst of bovine neutrophils.

ACKNOWLEDGMENTS

We thank Lynette Corbeil for supplying strains of *H. somnus* and the convalescent sera used in these experiments.

This work was supported by funds from the U.S. Department of Agriculture (86-CRSR-2-2847, 87-CRCR-1-2308, and 90-37265-5524), the Office of Naval Research (contract N00014-87-K-0319), the National Institutes of Health (grant AI-21343), and the University of Wisconsin School of Veterinary Medicine.

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