JOURNAL OF BACTERIOLOGY, Apr. 1983, p. 520–523
0021-9193/83/040520-04$02.00/0

Vol. 154, No. 1

Superoxide Anion Production and Superoxide Dismutase and Catalase Activities in *Coxiella burnetii*

EMMANUEL T. AKPORIAYE AND OSWALD G. BACA*

Department of Biology, The University of New Mexico, Albuquerque, New Mexico 87131

Received 4 October 1982/Accepted 3 January 1983

Coxiella burnetii was examined for superoxide anion (O_2^-) production and superoxide dismutase and catalase activities. The organism generated O_2^- at pH 4.5 but not at pH 7.4. The rickettsia displayed superoxide dismutase activity distinguishable from that of the host cell (L-929 mouse fibroblast). Catalase activity was maximal at pH 7.0 and diminished at pH 4.5. These enzymes may account, in part, for the ability of this obligate intracellular parasite to survive within phagocytes.

Univalent reduction of molecular oxygen results in the production of the transitory oxygen radical superoxide anion (O_2^-) (4, 12). Reduced flavins and flavoproteins in aerobic bacteria have been directly implicated as the potential sites of superoxide radical formation during electron transport (25). *Coxiella burnetii*, the etiological agent of Q fever, possesses some enzymes of the tricarboxylic acid cycle (30, 31) and uses oxygen during its metabolism of glutamate and glucose (30). *Coxiella* Sp. might therefore be expected to generate superoxide radicals during its utilization of oxygen. By itself O_2^- may be toxic (2, 16, 21); toxicity may also be due to H_2O_2 resulting from the dismutation of O_2^- (1, 19, 20, 28). Superoxide dismutase (SOD), an enzyme which catalyzes the disproportionation of O_2^-, is considered indispensable to bacteria which reduce oxygen (27) and, according to some investigators, protects against oxygen toxicity (14, 15).

We show that *C. burnetii* generates superoxide anions, exhibits catalase activity, and possesses SOD distinguishable from that of the host cell in which it was propagated. These enzymes may afford protection to the parasite from the toxic oxygen metabolites of the host.

C. burnetii, strain Nine Mile, phase I, was propagated in embryonated eggs or mouse fibroblast cells (L-929) and purified as previously described (3). Rickettsial concentrations were determined by the methods of Silberman and Fiset (34). Purified rickettsiae were stored at −70°C until required. *Escherichia coli* B was grown to the late log phase in brain heart infusion broth. Cells were harvested and frozen at −70°C.

To assay for superoxide production, freshly isolated or frozen pellets of *C. burnetii* and *E. coli* B were suspended in P-25 buffer (50 mM potassium phosphate, 152.5 mM potassium chloride, 15 mM sodium chloride, 100 mM glycine) at the appropriate pH. Superoxide production was measured by the reduction of cytochrome *c* (26).

SOD was assayed with *C. burnetii* (1.8×10^{11} to 2.3×10^{12} cells) and *E. coli* B (1.2×10^{10} cells) suspended in a minimal volume of 50 mM potassium phosphate buffer (pH 7.8 or 10.0 [pH 4.5 for *C. burnetii* only]) containing 1 mM EDTA. Cells held at 0 to 4°C were subjected to sonic treatment with a S75 Sonifier (Branson Instruments Co., Stamford, Conn.; *C. burnetii*, 4 min, 30-s pulses, 1-min cooling; *E. coli*, 2 min, 30-s pulses, 1-min cooling). Sonic extracts were centrifuged (57,000 × *g*, 17 min), and the resulting supernatants (pH 7.8 or 10.0 [pH 4.5 for *C. burnetii* only]) were dialyzed as previously described (27). Supernatants were frozen at −70°C until needed. L-929 cells (2×10^7 to 5×10^7) were suspended in 50 mM potassium phosphate buffer (pH 7.8 or 10.0) containing 1 mM EDTA. Cells were lysed in 0.2% (wt/vol) Triton X-100 (33). This was followed by sonication (30 s), ultracentrifugation (57,000 × *g*, 17 min), and dialysis in buffer (pH 7.8 or 10.0). Supernatants were frozen at −70°C until needed. SOD activity in the supernatants was measured by the method of McCord and Fridovich (26).

Catalase was assayed in cell extracts spectrophotometrically essentially by the method of Beers and Sizer (7). Protein concentrations were determined by the method of Bradford (8).

C. burnetii generated superoxide at pH 4.5 but not at 7.4 (Table 1). This correlates with the observation by Hackstadt and Williams (17) of increased oxygen uptake and metabolism by *C. burnetii* at acid pH. Glutamate, which is catabolized by intact *C. burnetii* at pH 4.5 (17), did not enhance superoxide production. Superoxide

TABLE 1. Superoxide anion generation by *C. burnetii* and *E. coli* B[a]

Organism/ expt	Superoxide production at pH:			
	4.5	4.5 + glutamate	7.4	7.4 + glutamate
C. burnetii				
Expt 1	2.40	2.28	0.0	0.0
2	0.95 (0.38)[b]	1.00	0.0	0.56
3	1.42	1.20 (0.38)	0.29	0.0
4	6.58 (0.57)	2.86 (0.19)	0.0	0.96
5	2.00 (0.86)	2.10 (0.86)	0.0	0.0
	2.76 ± 1.01[c]	*1.89 ± 0.35*	*0.06 ± 0.06*	*0.30 ± 0.20*
E. coli B				
Expt 1	0.4	0.18	1.34	3.62
2	3.40	0.96	3.80 (2.1)	4.84
3	6.30	3.70	8.59	8.59 (3.30)
	3.37 ± 1.70	*1.61 ± 1.07*	*4.58 ± 2.13*	*5.68 ± 1.50*

[a] Superoxide production measured as nanomoles of cytochrome *c* reduced per 60 min/2.5 × 10^{10} *C. burnetii* and 1.0 × 10^9 *E. coli* B. *C. burnetii* (ca. 2.5 × 10^{10}; fresh or frozen) or 10^9 *E. coli* B cells were contained in a final reaction volume of 2 ml. Paired reaction tubes contained 80 μM ferricytochrome *c* (horse heart, type III; Sigma) in P-25 buffer (pH 4.5 or 7.4) with or without 5 mM glutamic acid and also with or without 50 μg of SOD per ml (bovine blood, type 1; Sigma). Controls included ferricytochrome *c* alone or ferricytochrome *c* and SOD. All tubes were incubated at 37°C for 60 min with constant rotation in the dark, after which they were centrifuged (16,000 × *g*, 10 min) and the pellets were discarded. The absorbance of the supernatants was determined at 550 nm. The amount of cytochrome *c* reduced was used as an indication of superoxide generation and was calculated by using the extinction coefficient of 2.1 × 10^4 M^{-1} cm^{-1} at 550 nm (26).

[b] Figures in parentheses represent superoxide production measured as nanomoles of cytochrome *c* reduced in the presence of 50 μg of SOD per ml.

[c] Italicized values indicate the mean ± standard error of the mean.

production in the absence of glutamate is probably due to endogenous metabolism (30) resulting in oxygen uptake. To determine whether or not the reduction of cytochrome *c* was due to O_2^-, bovine blood SOD was included in some of the reaction tubes (Table 1). Its inclusion significantly affected the reduction of cytochrome *c*. Furthermore, varying the amount of exogenous SOD at pH 4.5 affected the amount of cytochrome *c* that was reduced. In one experiment, inhibition of cytochrome *c* reduction ranged from 92.4% with 50 μg of SOD to 56.2% with 0.1 μg of SOD. *E. coli* B generated O_2^- optimally at pH 7.4, and its production was enhanced in the presence of glutamate. The variability in the amount of superoxide detected may be attributed to the different batches of organisms employed in the assays and which were grown and harvested on different occasions.

SOD activity was found in extracts of *C. burnetii* (Table 2). This activity was greater (per milligram of protein) than that detected in *E. coli* B extracts at the pH 7.8 optimum (Table 2). *C. burnetii* extracts did not exhibit SOD activity at pH 4.5. The lack of detectable SOD activity at this pH is probably due to several factors including (i) the inherent instability of O_2^- at acid pH, which results in its rapid conversion to hydroperoxy radical (HO_2 [5, 11]); (ii) the known inhibition of xanthine oxidase at acid pH in the presence of excess purine substrate (13); and (iii) the instability of SOD below pH 4.8 (24). Thus, the lack of detectable *Coxiella* SOD activity at acid pH may well be due to its acid lability or to the inaccessibility of O_2^- resulting from its spontaneous disproportionation at this pH or both. However, by using purified bovine blood SOD (Sigma Chemical Co., St. Louis, Mo.) to ascertain the effect of pH on enzyme activity, some residual activity was detected at pH 4.5 with 22.5 μg of SOD. This activity was 27% of the activity at pH 10.0. It appears that eucaryotic SOD may function in disproportionating O_2^- generated by xanthine oxidase at pH 4.5, although at reduced efficiency. *C. burnetii* SOD was not significantly inhibited by cyanide (Table 2), suggesting the presence of a manganese-containing enzyme reported for bacteria (9, 18, 22). In contrast, SOD derived from L-929 cells had a pH optimum of 10.0 and was inhibited by cyanide, suggesting that a cupro-zinc enzyme would occur in eucaryotic cells as previously described (26, 33, 35). Electrophoretic analysis would be required to unequivocally characterize the *Coxiella* SOD.

Significant catalase activity with a pH opti-

TABLE 2. SOD activity in extracts of *C. burnetii*, *E. coli* B, and L-929 mouse fibroblasts[a]

Source	U/mg of protein[b]			KCN inhibition[c]
	pH 4.5	pH 7.8	pH 10	
C. burnetii	0.0	6.61 ± 0.75	4.74 ± 0.18	−
E. coli B	ND[d]	3.01 ± 0.08	2.06 ± 0.66	−
L-929 cells	ND	1.71 ± 0.14	2.80 ± 0.43	+

[a] The assay was performed at 23°C in 1.0-cm cuvettes in a final volume of 2.5 ml of buffer (pH 4.5, 7.8, or 10.0; 50 mM potassium phosphate containing 1 mM EDTA). The reaction mixture contained 10 μM ferricytochrome *c* and 50 μM xanthine oxidase (buttermilk; Sigma) to produce a rate of reduction of ferricytochrome *c* at 550 nm of 0.024 absorbance units per min. At pH 4.5, 250 μM xanthine and about 50 to 60 times more xanthine oxidase were required to generate the same reaction rate. To distinguish between manganese- and cupro-zinc-containing enzymes (6), 1 mM cyanide was added to the reaction mixture. Values are means ± standard error of the mean of five determinations of *C. burnetii*, two determinations of *E. coli* B, and four determinations of L-929 cell extracts.

[b] One unit is the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

[c] Determined at pH 7.8 for *C. burnetii* and *E. coli* B and at pH 7.8 and 10.0 for L-929 cells.

[d] ND, Not done.

TABLE 3. Catalase activity in extracts of *C. burnetii* and *E. coli* B[a]

Source	U/mg of protein[b]	
	pH 4.5	pH 7.0
C. burnetii	5.29 ± 1.19[c]	23.39 ± 1.74
E. coli B	6.40 ± 1.56	21.14 ± 1.98

[a] Extracts were derived from previously frozen *E. coli* and freshly isolated *C. burnetii*. Assays were performed at 24°C in 1.0-cm cuvettes in 3.0 ml of H_2O_2 phosphate buffer (50 mM phosphate buffer, 13.8 mM H_2O_2; pH 7.0 or 4.5). Reactions were initiated by adding 500 μl of *C. burnetii* or 100 μl of *E. coli* B cell extract. Activity was calculated by using the extinction coefficient for H_2O_2 of 43.6 M^{-1} cm^{-1} at 240 nm (36).

[b] One unit is the amount of enzyme catalyzing the decomposition of 1.0 μmol of H_2O_2 per min at 24°C.

[c] Values are means ± standard error of the mean of eight determinations at pH 7.0 and three determinations at pH 4.5 of *C. burnetii*; three and two determinations at pH 7.0 and 4.5, respectively, of *E. coli* B cell extracts.

mum of 7.0 was found in *C. burnetii* (Table 3); the level of activity detected was comparable to that found in *E. coli* B.

Recently, we demonstrated that lysosomes fuse with rickettsia-containing phagosomes within a murine macrophage-like tumor cell line, J774 (unpublished data). This agrees with a similar observation by Burton et al. (10) for mouse fibroblasts (L-929). In spite of phagosome-lysosome fusion, the parasite replicates within the phagolysosome. *C. burnetii* SOD may participate in the elimination of O_2^-, generated by the host cell in the phagolysosome, which may diffuse into the rickettsial cell. It is well documented that the generation of toxic oxygen metabolites (including O_2^-) is a prime mechanism used by phagocytes for defending against invading bacteria (23, 32). That *C.* burnetii SOD operates optimally at neutral pH is suggestive of a cytoplasmic enzyme that may also function to eliminate internal parasite-generated O_2^-. Still, the SOD may be important during transient exposures of the parasite to oxygen during its sojourn outside a host. Such a protective role of SOD has even been proposed for anaerobic bacteria which do not use oxygen but which nevertheless possess significant SOD activity (18). By possessing SOD activity, *C. burnetii* represents an example of an obligate intracellular parasite that generates superoxide anions and possesses the complementary O_2^--detoxifying enzyme. Catalase activity in *C. burnetii* with a pH 7.0 optimum may function in the destruction of H_2O_2 generated by parasite cytoplasmic SOD which is also operating at physiological pH. Conceivably, the catalase may also function in eliminating host-generated H_2O_2. Myers et al. (29) have reported that *Rickettsia prowazeki*, which proliferates intracytoplasmically, lacks catalase activity and fails to produce hydrogen peroxide. Unlike *C. burnetii*, the typhus agent probably does not require catalase for survival because of the apparent lack of host-generated hydrogen peroxide in the surrounding cytoplasm (29).

Studies are currently in progress to assess the importance, if any, of SOD and catalase to the fate of *C. burnetii* in in vitro killing assays and in the macrophage-like tumor cell line J774 in which this organism establishes a persistent infection (3).

The technical assistance of Jim Rowatt and Adam Aragon is gratefully acknowledged.

This study was supported by National Science Foundation grant PCM8010633 and by Public Health Service grant RR-08139 from the National Institutes of Health.

LITERATURE CITED

1. **Amin, V. M., and N. F. Olson.** 1968. Influence of catalase activity on resistance of coagulase-positive staphylococci to hydrogen peroxide. Appl. Microbiol. 16:267-270.
2. **Babior, B. M., J. T. Curnutte, and R. S. Kipnes.** 1975. Biological defense mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. J. Lab. Clin. Med. 85:235-244.
3. **Baca, O. G., E. T. Akporiaye, A. S. Aragon, I. L. Martinez, M. V. Robles, and N. L. Warner.** 1981. Fate of phase I and phase II *Coxiella burnetii* in several macro-

phage-like tumor cell lines. Infect. Immun. 33:258–266.
4. **Ballou, D., G. Palmer, and V. Massey.** 1969. Direct demonstration of superoxide anion production during the oxidation of reduced flavin and of its catalytic decomposition by erythrocuprein. Biochem. Biophys. Res. Commun. 36:898–904.
5. **Baxendale, J. H.** 1962. The flash photolysis of water and aqueous solutions. Radiat. Res. 17:312–326.
6. **Beauchamp, C. O., and I. Fridovich.** 1973. Isozymes of superoxide dismutases from wheat germ. Biochim. Biophys. Acta 317:50–64.
7. **Beers, R. F., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195:133-140.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
9. **Britton, L., D. P. Malinowski, and I. Fridovich.** 1978. Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. J. Bacteriol. 134:229–236.
10. **Burton, P. R., J. Stueckemann, R. M. Welsh, and D. Paretsky.** 1978. Some ultrastructural effects of persistent infections by the rickettsia *Coxiella burnetii* in mouse L cells and green monkey kidney (Vero) cells. Infect. Immun. 21:556–566.
11. **Czapski, G., and L. M. Dorfman.** 1964. Pulse radiolysis studies. V. Transient spectra and rate constants in oxygenated aqueous solutions. J. Phys. Chem. 68:1169–1177.
12. **Fridovich, I.** 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J. Biol. Chem. 245:4053–4057.
13. **Fridovich, I., and P. Handler.** 1958. Xanthine oxidase. IV. Participation of iron in internal electron transport. J. Biol. Chem. 233:1581–1585.
14. **Gregory, E. M., and I. Fridovich.** 1973. Oxygen toxicity and the superoxide dismutase. J. Bacteriol. 114:1193–1197.
15. **Gregory, E. M., and I. Fridovich.** 1973. Induction of superoxide dismutase by molecular oxygen. J. Bacteriol. 114:543–548.
16. **Gregory, E. M., F. J. Yost, Jr., and I. Fridovich.** 1973. Superoxide dismutases of *Escherichia coli*: intracellular localization and functions. J. Bacteriol. 115:987–991.
17. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. U.S.A. 78:3240–3244.
18. **Hewitt, J., and J. G. Morris.** 1975. Superoxide dismutase in some obligately anaerobic bacteria. FEBS Lett. 50:315–318.
19. **Ismail, G., W. D. Sawyer, and W. S. Wegener.** 1977. Effect of hydrogen peroxide and superoxide radical on viability of *Neisseria gonorrhoeae* and related bacteria. Proc. Soc. Exp. Biol. Med. 155:264–269.
20. **Jackett, P. S., V. R. Arber, and D. B. Lowrie.** 1978. Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. 104:37–45.
24. **Johnston, R. B., B. B. Keele, H. P. Misra, J. E. Lehmeyer, L. S. Krebb, R. L. Baehner, and K. V. Rajagopalan.** 1975. The role of superoxide anion generation in phagocytic bactericidal activity: studies with normal and chronic granulomatous diseases leukocytes. J. Clin. Invest. 55:1357–1372.
22. **Keele, B. B., J. M. McCord, and I. Fridovich.** 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. J. Biol. Chem. 245:6176–6181.
23. **Klebanoff, S. J.** 1975. Antimicrobial mechanism in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. 12:117–142.
24. **Klug, D., J. Rabani, and I. Fridovich.** 1972. A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. J. Biol. Chem. 247:4839–4842.
25. **Massey, V., S. Strickland, S. G. Mayhew, L. G. Howell, P. L. Engel, R. G. Matthews, M. Schuman, and P. C. Sullivan.** 1969. The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. Biochem. Biophys. Res. Commun. 36:891–897.
26. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase, an enzymic function of erythrocuprein (hemocuprein). J. Biol. Chem. 244:6049–6055.
27. **McCord, J. M., and I. Fridovich.** 1971. An enzyme based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. 68:1024–1027.
28. **Murray, H. W., and Z. A. Cohn.** 1979. Macrophage oxygen dependent antimicrobial activity. I. Susceptibility of *Toxoplasma gondii* to oxygen intermediates. J. Exp. Med. 150:938–949.
29. **Myers, W. F., L. E. Warfel, and C. L. Wisseman, Jr.** 1978. Absence of hydrogen peroxide production by or catalase action in *Rickettsia prowazeki*. J. Bacteriol. 136:452–454.
30. **Ormsbee, R. A., and M. G. Peacock.** 1964. Metabolic activity in *Coxiella burnetii*. J. Bacteriol. 88:1205–1210.
31. **Paretsky, D., C. M. Downs, R. A. Consigli, and B. K. Joyce.** 1958. Studies on the physiology of rickettsiae. I. Some enzyme systems of *Coxiella burnetii*. J. Infect. Dis. 103:6–11.
32. **Roos, D.** 1980. The metabolic response to phagocytosis, p. 337–385. *In* G. Weissman (ed.), The cell biology of inflammation. Elsevier/North Holland Biomedical Press, N.Y.
33. **Salin, M. L., and J. M. McCord.** 1974. Superoxide dismutases in polymorphonuclear leukocytes. J. Clin. Invest. 54:1005–1009.
34. **Silberman, R., and P. Fiset.** 1968. Method for counting rickettsiae and chlamydiae in purified suspensions. J. Bacteriol. 95:259–261.
35. **Weisiger, R. A., and I. Fridovich.** 1973. Superoxide dismutase: organelle specificity. J. Biol. Chem. 248:3582–3592.
36. **Worthington Biochemicals Corporation.** 1972. Worthington enzyme manual, p. 41–42. Worthington Biochemicals Corp., Freehold, N.J.