Immunological Heterogeneity of Superoxide Dismutases in the *Acholeplasmataceae*

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The superoxide dismutases from *Acholeplasma laidlawii* and *Acholeplasma hippikon* were serologically related, whereas the superoxide dismutase from *Acholeplasma equifetale* appeared to be unique serologically. Enzymes from *Acholeplasma granularum* and *Acholeplasma axanthum* did not show any serological reactivity. Moreover, the acholeplasmic enzymes exhibited electrophoretic heterogeneity, indicating the evolutionary diversity of superoxide dismutases.

*Acholeplasma laidlawii* was the first member of the *Mycoplasmales* shown to exhibit superoxide dismutase activity (9). Kirby et al. (5) reported the presence of superoxide dismutase in *A. laidlawii* but not in *Mycoplasma pneumoniae*. To study the occurrence and serological properties of superoxide dismutase in *Mycoplasma* and *Acholeplasma* species, we examined cell lysates from 13 different species belonging to the genera *Mycoplasma, Acholeplasma,* and *Ureaplasma* for both enzymatic and antigenic activities.

A total of 13 *Mycoplasmales* species were studied (Table 1). The distribution of the species was as follows: 7 species in the genus *Mycoplasma*, which represented five different serological groups of species (3); 5 species in the genus *Acholeplasma*; and 1 serotype in the genus *Ureaplasma*. The antigens, immunogens, and antisera were prepared as described elsewhere (4).

To detect the occurrence of superoxide dismutase activity in the various organisms, cell lysates were electrophoresed in agarose (10) and then stained for enzyme activity. Rocket and two-dimensional immunoelectrophoresis were performed as described elsewhere (4). After electrophoresis, the slides were soaked in 10 mM potassium phosphate buffer (pH 7.5) for 1 h at room temperature and then immersed in the enzyme-staining solution for enzyme identification and localization. The positive activity stain for superoxide dismutase described by Misra and Fridovich (7) was used to localize enzyme activity. The staining solution contained 2 mM dianisidine and 0.1 mM riboflavin in 10 mM potassium phosphate buffer at pH 7.5. After electrophoresis, the slides were equilibrated in 10 mM potassium buffer (pH 7.5) for 1 h, immersed in the staining solution for 1 h at room temperature, rinsed briefly with distilled water, and illuminated by fluorescent light for about 15 min in an aluminum foil-lined box. Zones containing superoxide dismutase developed as brown spots or peaks. After color development was complete, the slides were washed in distilled water overnight and then dried in an incubator for photography. Peroxidase was detected by immersing the slides in a solution containing 2 mM dianisidine and 10 mM potassium phosphate buffer (pH 7.5) for 1 h, followed by a 15-min soaking in 0.1 mM H₂O₂.

Superoxide dismutase activity was not detected in the seven *Mycoplasma* species examined or in *Ureaplasma urealyticum*. On the other hand, enzyme activity was detectable in the five *Acholeplasma* species examined or in *Ureaplasma* (Table 1). Bovine superoxide dismutase (Sigma Chemical Co., St. Louis, Mo.) was tested in parallel as a positive control for enzyme staining and was used as a standard for comparing the electrophoretic mobilities of the enzymes from the various species. The superoxide dismutases from the five *Acholeplasma* species exhibited quite different electrophoretic mobilities (Fig. 1), ranging from 0.94 to 2.2 (with the mobility of bovine superoxide dismutase defined as 1.0).

The enzyme-staining system used in this study also detected the absence of peroxidase, but color development was much slower (7). To be certain that the staining was due to superoxide dismutase and not to peroxidase, the electrophoresed cell lysates were stained for peroxidases. Horseradish peroxidase (Sigma) was tested in parallel as a positive control for the staining reaction. None of the *Acholeplasma* species exhibited any peroxidase activity. Moreover, incorporation of 10 mM potassium cyanide in the staining solution for superoxide dismutase did not inhibit the formation of the stained product (peroxidase activity is suppressed by 10 mM cyanide (7)), indicating that the enzyme activity detected was indeed superoxide dismutase.

The electrophoretic mobilities of Triton X-100-solubilized cell lysates of the various *Acholeplasma* species were compared with the mobility of bovine superoxide dismutase in agarose containing 0.1% sodium deoxycholate and 0.5% Triton X-100 (charge shift conditions [2]). No changes in electrophoretic mobilities were observed, suggesting that the superoxide dismutases from the various *Acholeplasma* species were hydrophilic and likely cytoplasmic (2).

Triton X-100-solubilized cell lysates of the various species were tested against their homologous antisera in rocket immunoelectrophoresis and then stained for superoxide dismutase activity. Only the enzymes from *Acholeplasma equifetale, Acholeplasma hippikon,* and *A. laidlawii* formed specific immunoprecipitin peaks with their respective antisera (Table 2). In addition, other preparations of antisera made against *Acholeplasma axanthum* and *Acholeplasma granularum* also failed to recognize the superoxide dismutases from these two organisms. Two-dimensional immunoelectrophoregrams of the various *Acholeplasma* species with their respective homologous antisera were stained for superoxide dismutase activity (Fig. 2). Again, only the enzymes from *A. equifetale, A. hippikon,* and *A. laidlawii* formed stainable enzyme-antibody complexes.

To determine whether the superoxide dismutases from *A. equifetale, A. hippikon,* and *A. laidlawii* showed cross-reactivity, cell lysates of the different organisms were tested against heterologous antisera in rocket immunoelectrophoresis (Table 2). The enzymes from *A. hippikon* and *A. laidlawii* showed strong reactivity to antisera to these two organisms.
lysates were electrophoresed for 1 h at 6 V/pcm and stained for superoxide dismutase activity as described in the text. Antiserum to the enzyme from the other heterologous species. Again, no enzyme activity was observed; therefore, the enzyme activity detected with the cell lysates was acholeplasmic. The fact that no enzyme activity was detected in any of the Mycoplasma species grown in the same horse serum lot also ruled out medium component contamination (3). Furthermore, the differences in the electrophoretic mobilities and serological specificities of the acholeplasmic enzymes also indicate specificity.

We detected superoxide dismutase in the Acholeplasma species tested but not in the Mycoplasma species studied, which corroborated the findings of other investigators (5, 6, 8) and extended the number of species examined for this enzyme to 7 in the genus Acholeplasma, 17 in the genus Mycoplasma, and one serotype in the genus Ureaplasma. Since the Mycoplasma species chosen were representative of most serotaxonomic groups of the genus Mycoplasma (3), it appears unlikely that superoxide dismutase is present in the genus Mycoplasma.

The enzyme in A. equifetale is quite specific, showing only weak cross-reaction with A. hippikon and A. hippikon have a common specificity, whereas the superoxide dismutases from A. granularum and A. axanthum did not exhibit any antigenic activity when they were tested against homologous antisera. The lack of serological cross-reactivity and the heterogeneous electrophoretic mobilities of the acholeplasmic superoxide dismutases indicate the activity. No detectable enzyme activity was observed; therefore, the enzyme activity detected with the cell lysates was acholeplasmic. The fact that no enzyme activity was detected in any of the Mycoplasma species grown in the same horse serum lot also ruled out medium component contamination (3). Furthermore, the differences in the electrophoretic mobilities and serological specificities of the acholeplasmic enzymes also indicate specificity.

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evolutionary diversity of this enzyme. Similar antigenic and
electrophoretic diversity has been reported in *Streptococcus*
and in other microorganisms (1). The *Mycoplasmatales* also
show a large degree of antigenic variation in their adenosine
triphosphatases and reduced nicotinamide adenine dinucleo-
tide dehydrogenases (10).

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