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 *Mycoplasmatales* 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 *Mycoplasmatales* 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 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 detected in all five *Acholeplasma* species studied (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 presence 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 immunoelectropherograms 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,

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TABLE 1. Occurrence of superoxide dismutases in the *Mycoplasmatales*

Organism	Strain	Source	Serological group ^a	Superoxide dismutase activity ^b	Relative mobility ^c
Bovine group 7	N-29B	J. Tully	1	—	
<i>A. axanthum</i>	S-743 ^{Td}	J. Tully	2	+	1.0
<i>A. equifetale</i>	29724 ^T	ATCC ^e	2	+	2.2
<i>A. granularum</i>	Friend	J. Tully	2	+	1.9
<i>A. hippikon</i>	29725 ^T	ATCC	2	+	1.0
<i>A. laidlawii</i>	14192	ATCC	2	+	1.3
<i>Mycoplasma gallisepticum</i>	15302	ATCC	4	—	
<i>Mycoplasma pneumoniae</i>	AP-3546	G. Kenny	5	—	
<i>Mycoplasma pulmonis</i>	63	G. Kenny	6	—	
<i>Mycoplasma arginini</i>	G-230 ^T	M. Barile	7	—	
<i>Mycoplasma gallinarum</i>	15319	ATCC	7	—	
<i>Mycoplasma hominis</i>	14027	ATCC	7	—	
<i>U. urealyticum</i>	Type 8	M. Shepard		—	

^a As defined by Kenny (3).^b +, Enzyme activity; —, no enzyme activity.^c Mobility relative to that of bovine superoxide dismutase, which was defined as 1.0.^d T = Type strain.^e ATCC, American Type Culture Collection.

suggesting that the superoxide dismutases from these two species are closely related serologically. Antiserum to *A. equifetale* formed a very faintly stained immunoprecipitin peak with the enzyme from *A. hippikon*, but no peak with the enzyme from the other heterologous species. Again, no immunoprecipitin peaks were formed with the enzymes from *A. axanthum* and *A. granularum* when they were tested against heterologous antisera.

To rule out the possibility that the enzyme activity was due to medium contaminants, 10 μ l of yeast extract-soy peptone dialysate broth and 10 μ l of agamma horse serum were similarly tested and stained for superoxide dismutase

FIG. 1. Agarose electropherograms of the superoxide dismutases of *A. axanthum* (lane A), *A. equifetale* (lane B), *A. granularum* (lane C), *A. hippikon* (lane D), and *A. laidlawii* (lane E) and of bovine superoxide dismutase (lane F). Triton X-100-solubilized cell lysates were electrophoresed for 1 h at 6 V/pcm and stained for enzyme activity as described in the text.

TABLE 2. Serological properties of the acholeplasmic superoxide dismutases^a

Antigen	Antiserum				
	<i>A. axanthum</i>	<i>A. equifetale</i>	<i>A. granularum</i>	<i>A. hippikon</i>	<i>A. laidlawii</i>
<i>A. axanthum</i>	—	—	—	—	—
<i>A. equifetale</i>	—	+	—	—	—
<i>A. granularum</i>	—	—	—	—	—
<i>A. hippikon</i>	—	±	—	+	+
<i>A. laidlawii</i>	—	—	—	+	+
Bovine superoxide dismutase	—	—	—	—	—

^a —, No precipitin peak stained for enzyme; +, strong staining of precipitin; ±, weak staining.

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.

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*. The superoxide dismutases from *A. laidlawii* 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 or heterologous antisera. The lack of serological cross-reactivity and the heterogeneous electrophoretic mobilities of the acholeplasmic superoxide dismutases indicate the

FIG. 2. Detection of superoxide dismutase in antigenic profiles. (A) *A. laidlawii* tested against homologous antiserum, Coomassie Blue stain. (B) *A. laidlawii* tested against homologous antiserum, stained for superoxide dismutase activity. The scales at the bottom indicate the relative mobilities of the peaks; 1.0 equals the mobility of bovine albumin, the peak of which is identified by the vertical bar at the top of the slide. The anode was to the left and top. The arrow indicates the superoxide dismutase peak, and the dotted curve indicates the relative position of superoxide dismutase in the Coomassie blue-stained profile.

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 dinucleotide dehydrogenases (10).

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