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Comparative Cytochrome Oxidase and Superoxide Dismutase Analyses on Strains of *Azotobacter vinelandii* and Other Related Free-Living Nitrogen-Fixing Bacteria

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Quantitative *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase and superoxide dismutase (SOD) analyses were performed on representative organisms of the family *Azotobacteraceae*. *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Azotobacter paspali*, and *Derxia gummosa* exhibited high quantitative TMPD oxidase activities, and their extracts possessed very active and electrophoretically homogeneous (single gel band) Fe-type SODs. *Azomonas macrocytogenes* extracts had similar single Fe-type SODs, and their cells exhibited no TMPD-dependent cytochrome oxidase activity. Nitrogen-fixing cells of *Beijerinckia indica*, *Beijerinckia derxii*, and *Beijerinckia mobilis* exhibited minimal TMPD oxidation capabilities (rates equivalent to the TMPD autooxidation reaction), and these extracts also possessed very active SODs but only of the Mn metallotype.

Azotobacter vinelandii and related organisms of the family *Azotobacteraceae* are unique in that they fix N_2 and concomitantly respire by using atmospheric O_2. Of interest is that nitrogen-fixing cells of *Azotobacter vinelandii* possess one of the highest respiratory rates known (6, 15–17), and it was proposed that this high metabolic rate "scavenges" molecular O_2 (20). This allows for the maintenance of a low redox potential within the cell which would be required for biological dinitrogen reduction ($N_2 \rightarrow NH_3$). Reduction of molecular O_2 by *Azotobacter vinelandii* at such a high metabolic rate indicates that this organism must generate large amounts of superoxide free radical. The superoxide free radical is a potentially toxic intermediate, and the enzyme superoxide dismutase (SOD) would be required by this organism as a detoxifying enzyme (4, 9, 11, 19). Yet little is known about the SODs of *Azotobacter vinelandii* or any other free-living nitrogen-fixing organisms. One report (citing unpublished studies) notes the presence of an Fe-containing SOD in *Azotobacter vinelandii* (2), and another study reports on the isolation and purification of an Mn-containing SOD from *Azotobacter chroococcum* (5). Thus the oxygen-metabolizing or scavenging enzymes or both are of prime interest in studies of *A. vinelandii* and other organisms of the family *Azotobacteraceae*. In this report, a study was undertaken which comparatively analyzed cytochrome oxidase activity and SOD activities and types in a representative group of these free-living nitrogen fixers. All cells analyzed were grown on atmospheric N_2 and glucose. A quantitative colorimetric *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase assay was used to determine cytochrome oxidase activity levels in whole cells, whereas analyses for SOD activity and metalloenzyme type were performed with cell-free extracts obtained from these same organisms.

It is important to cite that recent DNA-RNA hybridization studies have shown that there is substantial diversity between organisms of the family *Azotobacteraceae* (8). Although species within *Azotobacter*, *Azomonas*, *Beijerinckia*, and *Derxia* are taxonomically homogeneous (8), there are large intergeneric differences among the genera themselves. The fact that organisms of the family *Azotobacteraceae* may no longer be taxonomically related does raise some very fundamental questions with regard to O_2 metabolism within this group. For example, do all these aerobic free-living nitrogen fixers have high respiratory metabolic rates as does *Azotobacter*? Do all these taxonomically diverse nitrogen fixers have a similar TMPD-dependent type of cytochrome oxidase? Finally, which SOD metalloenzyme types are found, and how active are they relative to the activity levels found in other bacteria?

The organisms examined were *Azotobacter vinelandii* O (ATCC 12518) as well as strains A and S5, *Azotobacter chroococcum* WAG-2 (NCIB 8004), *Azomonas macrocytogenes* (NCIB 8702), *Beijerinckia indica*, and *Escherichia coli* (ATCC 25922). All nitrogen-fixing bacteria were grown on a rotary shaker in liquid media at 30°C, using modified Burk mineral salts with 1% (wt/vol) glucose (12, 14–17). *E. coli* was grown similarily at 35°C but on nutrient broth (GIBCO) supplemented with 0.5% (wt/vol) glucose and 0.1% (wt/vol) yeast extract (Difco). SOD analyses were performed on centrifugally clarified (144,000 × *g*, 120 min) cell-free extracts (S_3 fraction) of sonically disrupted resting cells (12, 14). SOD activity was assayed by the standard spectrophotometric assay of McCord and Fridovich (19). Protein was determined by the biuret method (10). Electrophoretic analyses were performed on 10% polyacrylamide gels by the method of Davis (7), modified for a slab gel apparatus. Before gel staining for SOD activity, some gels were treated with H_2O_2 (1, 3, 4). The colorimetric TMPD-dependent cytochrome oxidase analyses were performed on resting cells (13) grown on the same media but on plates containing 1.8% (wt/vol) agar (Difco). The cytochrome oxidase assays were performed by monitoring Wurster's blue formation (Σ mM = 12.0) at 610 nm, and specific activities were reported on the basis of cellular dry weight as previously described (13, 18).

Figure 1 shows the SOD electrophoretic gel patterns of extracts obtained from *Azotobacter vinelandii* strains O, A, and S5, *Azotobacter chroococcum*, *Azomonas macrocytogenes*, *B. indica*, and an *E. coli* control. Noteworthy is that only one predominant SOD type was detected in extracts of nitrogen-fixing cells. The electrophoretic mobilities of the

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TABLE 1. Cytochrome oxidase and SOD analyses on *Azotobacter vinelandii* strains and related free-living nitrogen-fixing organisms

Organism	Cytochrome oxidase[a] sp act (μmol/h per mg of dry wt)	SOD[b]		
		Sp act (U/mg of protein)	Enzyme type	Relative mobility (10% gels)
Azotobacter vinelandii O (ATCC 12518)	2.8	73.2	Fe-SOD	0.60
Azotobacter vinelandii A	2.3	32.3	Fe-SOD	0.60
Azotobacter vinelandii S5	3.1	86.3	Fe-SOD	0.49
Azotobacter chroococcum (NCIB 8004)	4.3	49.1	Fe-SOD	0.59
Azomonas macrocytogenes (NCIB 8702)	0.0	40.9	Fe-SOD	0.47
Beijerinckia indica	0.1	52.4	Mn-SOD[c]	0.49
Escherichia coli (ATCC 25922)	0.0	14.0	Fe-SOD, Fe-SOD, Mn-SOD[c]	0.48, 0.35, 0.24
Pseudomonas aeruginosa	4.7	ND[d]	ND	

[a] TMPD-dependent cytochrome oxidase analyses with turbidimetrically standardized whole cell suspensions.
[b] Analyses performed on the centrifugally clarified (144,000 × *g* for 120 min) sonic extract.
[c] Mn-SOD determined by its insensitivity to H_2O_2 pretreatment of polyacrylamide gels.
[d] ND, Not done.

single bands shows that there is some heterogeneity among the SODs found in nitrogen-fixing organisms. The SOD metallotype was detected by H_2O_2 pretreatment of the polyacrylamide gels (Fig. 2). As shown, two types were detected, the peroxide-sensitive Fe-SOD, found in extracts of all *Azotobacter* spp. analyzed and in *Azomonas macrocytogenes*, and the peroxide-insensitive Mn-SOD, found predominantly in extracts of *B. indica*.

Table 1 summarizes all of the experimental findings of this preliminary study. All *Azotobacter* spp. exhibited high TMPD-dependent cytochrome oxidase activity levels, with values ranging from 2.3 to 4.4. Although a high TMPD oxidase value of 4.7 was noted for *Pseudomonas aeruginosa* cells, this organism (as all other oxidase-positive bacteria) never expressed this high metabolic rate in natural substrate oxidation studies as do *Azotobacter vinelandii* strains (15–17). Surprisingly, no TMPD oxidase activity was detected in *Azomonas macrocytogenes* cells (like *E. coli*). The TMPD-dependent cytochrome oxidase activity levels were barely detectable in *B. indica* cells; the rates recorded were equivalent to the TMPD autooxidation value (13).

Table 1 also shows the SOD activity analyses performed on extracts of these same organisms. All nitrogen-fixing organisms examined possessed very high SOD activity levels; the values ranged from 32.3 to 86.3. The comparative value for the *E. coli* control was 14.0 activity units per mg of protein (Table 1). This latter value is representative of the activity levels reported for other common bacterial species

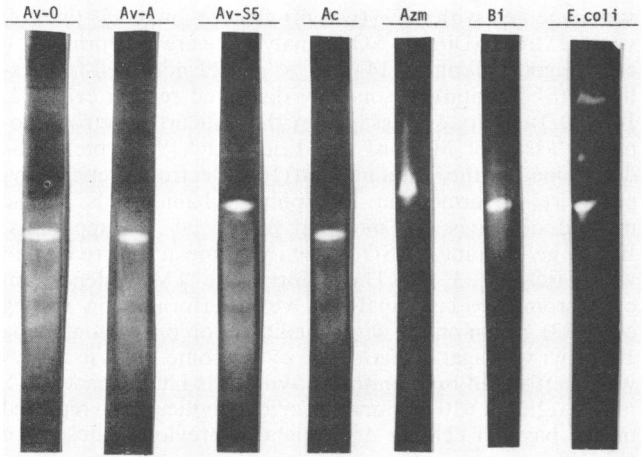

FIG. 1. Electrophoretic polyacrylamide gels stained for SOD activity found in sonic cell-free extracts of aerobic nitrogen-fixing bacteria of the family *Azotobacteraceae* and the facultative anaerobe *E. coli*. The organisms examined were *Azotobacter vinelandii* strains, O, A, and S5 (Av-O, Av-A, and Av-S5, respectively), *Azotobacter chroococcum* (Ac), *Azomonas macrocytogenes* (Azm), *Beijerinckia indica* (Bi), and the control *E. coli*.

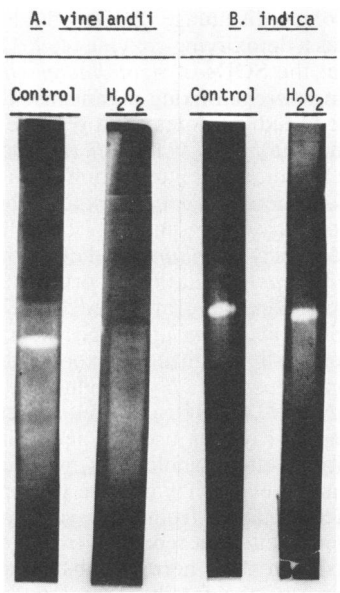

FIG. 2. Analytical polyacrylamide gels of SODs found in extracts of *Azotobacter vinelandii* O and *B. indica*. Both a control and H_2O_2-pretreated gel are shown. One Fe-SOD metalloenzyme, inactivated by H_2O_2 pretreatment, was found in extracts of all *Azotobacter* spp. analyzed, whereas the peroxide-insensitive Mn-SOD type was found in extracts of *B. indica* as well as four other *Beijerinckia* spp. analyzed.

analyzed by Britton et al. (4). Also shown in Table 1 are the SOD types found. Fe-SOD was the predominant metalloenzyme in all the *Azotobacter* spp. analyzed as well as in *Azomonas macrocytogenes*. Mn-SOD was the predominant metalloenzyme in *B. indica*. To date, four *Beijerinckia* spp., representing six strains, had only Mn-SOD. Unlike the SODs of *E. coli* (see Fig. 1), multiple isoenzymes could not be consistently detected in extracts of any of these nitrogen-fixing species. The SOD electrophoretic migration patterns, expressed as relative mobilities, are also shown. As noted in Table 1, the migration pattern of the Fe-SOD of *Azotobacter vinelandii* S5 differed markedly from those of strains O and A. More recent analyses on *Azotobacter vinelandii* (five strains), *Azotobacter chroococcum* (two strains), *Azotobacter beijerinckii* (two strains), *Derxia gummosa* (two strains) and *Azotobacter paspali* (one strain) showed that all of these organisms predominantly had a single Fe-containing SOD. Mn-SOD was the predominant metalloenzyme in *B. indica* (Fig. 2); more recent analyses on extracts of *Beijerinckia mobilis*, *Beijerinckia derxii*, *B. indica* subsp. *lacticogenes*, and *B. derxii* subsp. *venezuelae* all showed the presence of a single Mn-containing SOD band.

Our preliminary findings indicate that there will be great heterogeneity in the oxidases and SODs in organisms of the family *Azotobacteraceae*. Among these obligate aerobic nitrogen fixers are (i) the highly reactive oxidase-positive organisms like *Azotobacter vinelandii* and *Azotobacter chroococcum* (also [not shown] *Azotobacter beijerinckii*, *Azotobacter paspali*, and *Derxia gummosa*); (ii) the minimal TMPD oxidizers like *B. indica* (also [not shown] *B. indica* subsp. *lacticogenes*, *B. derxii*, *B. derxii* subsp. *venezuelae*, and *B. mobilis*); and (iii) the completely oxidase-negative reactants like *Azomonas macrocytogenes* NCIB 8702 and 2A. The above oxidase findings confirm the published data of Thompson and Skerman (21), who used a qualitative oxidase test to survey a large number of aerobic nitrogen fixers. There were differences, however, in the data reported for the *Beijerinckia* spp.; most previous reports indicate that organisms of this genera are oxidase positive (21). Our quantitative data show *Beijerinckia* spp. to be minimal TMPD oxidizers, and thus, they would normally be considered as oxidase-negative organisms.

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