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Periplasmic superoxide dismutases in *Aquaspirillum magnetotacticum*

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Abstract. *Aquaspirillum magnetotacticum* MS-1 cells cultured microaerobically (dissolved O_2 tension 1% of saturation), expressed proteins with superoxide dismutase (SOD) activity. The majority (roughly 95%) of total cell superoxide dismutase activity was located in the cell periplasm with little or no activity in the cell cytoplasm. Iron-type SOD (FeSOD) contributed 88% of the total activity activity detected, although a manganese-type SOD (MnSOD) was present in the periplasm as well. Cells cultured at a higher dissolved O_2 tension (10% of saturation) expressed increased activity of the MnSOD relative to that of the FeSOD.

Key words: Magnetic bacteria – Periplasm – Superoxide dismutase – Peroxidase – Microaerophiles – O_2 toxicity – *Aquaspirillum magnetotacticum*

Aquaspirillum magnetotacticum cells are obligately microaerophilic. They consume O_2 and require it for growth even when denitrifying (Bazylinski and Blakemore 1983). Cell growth is optimal when the culture dissolved O_2 tension (DOT) is at 1% of saturation and growth is not established at a DOT of > 10% with NO_3^- or with NH_4Cl as sole N source. We previously observed inhibition of NO_2^- reductase expression at elevated O_2, resulting in the accumulation of toxic quantities of NO_2^- in cultures of denitrifying cells (K. A. Short, Ph.D. thesis, University of New Hampshire, 1987). However, these findings did not explain the sensitivity to O_2 of non-denitrifying, (O_2-respiring) cells cultured with NH_4Cl as sole N source. Wild-type magnetic cells of *A. magnetotacticum*, strain MS-1, do not have measurable catalase activity (Blakemore et al. 1979) but do contain enzymes normally functioning in other bacteria to protect them from O_2 toxicity. These include c-type hemes and other compounds with peroxidase (O'Brien et al. 1987) and superoxide dismutase (SOD) activity (Short and Blakemore, Abstr. Annu. Meet. Am. Soc. Microbiol., Atlanta, GA, USA, 1987, I 123, p 193). To more completely understand the sensitivity to O_2 of this obligate microaerophile, we examined the amount and distribution of these protective enzymes at the extremes of the range of O_2 appropriate for growth.

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Periplasm selectively released from *A. magnetotacticum* cells by a freezing and thawing (F/T) method (Paoletti et al. 1987) contained large quantities of c-type hemes having peroxidase activity (O'Brien et al. 1987). Results presented here show that periplasm from this organism, obtained with F/T or with more conventional methods, also contained the majority (approximately 95%) of cellular proteins with SOD activity.

Gregory et al. (1973) and Hassan and Fridovich (1977) reported a periplasmic iron-type SOD (FeSOD) in cells of *Escherichia coli* B. These results were re-evaluated (Britton and Fridovich 1977) and it was subsequently concluded that a small quantity of SOD had been released during osmotic shock used to obtain periplasm and that the FeSOD was, in fact, cytoplasmic. We know of no other cell fractionation study which definitively identifies a periplasmic location for SOD. Most investigations of this enzyme have been conducted with "soluble" cell fractions consisting of cytoplasm combined with periplasm.

Materials and methods

Bacteria and growth conditions

Cells of *Aquaspirillum magnetotacticum* strain MS-1 and a nonmagnetic, aerotolerant mutant strain, NM-$1A_A$ (Short and Blakemore 1986), were cultured at 25°C in chemically defined medium (Blakemore et al. 1979) in 10 or 15-l carboys. The culture DOT was measured with a galvanic electrode and was maintained within approximately 50% of the desired value at 1% DOT and within approximately 20% of the desired value at 10% DOT. This was accomplished by constant stirring of the growth medium and sparging with N_2 mixed with air. The O_2 composition and delivery rate of the gas mixture was controlled by means of rotameters and was adjusted during growth to maintain a constant DOT.

Escherichia coli strain B (University of New Hampshire culture collection) was cultured at 37°C in tryptic soy broth (Difco Lab, Detroit, MI, USA) aerobically (1 l of culture in a 2.8 l Fernbach flask shaken at 220 rpm).

Cell preparation

Cells of *A. magnetotacticum*, harvested in the exponential growth phase with a Pellicon filtration system (Millipore

Corp., Bedford, MA, USA), were concentrated by centrifugation at $10{,}000 \times g$ for 15 min at 4°C. Cells of *E. coli* in exponential growth were harvested by centrifugation ($10{,}000 \times g$, 15 min, 4°C). Concentrated cells were washed once in 50 mM potassium phosphate buffer (KPB) at pH 6.8, centrifuged, and resuspended in 10–20 ml KPB. Cells were stored at −70°C prior to fractionation.

Cell fractionation

Cells of *A. magnetotacticum* were fractionated by 4 different procedures. Outer membrane proteins (OMP), cytoplasmic membrane proteins (CMP) and cytoplasmic + periplasmic proteins (CP + PP) were obtained by the method of Schnaitman (1981). Periplasm was obtained by osmotic shock (Neu and Heppel, 1965), by chloroform extraction (Ames et al. 1984), or by F/T (Paoletti et al. 1987). Cytoplasmic proteins (CP) were obtained by ultracentrifuging ($100{,}000 \times g$, 1 h, 5°C) cells disrupted in a French press (2 passages at 7×10^6 kg/m^2) after release of periplasm as described above.

Soluble proteins were concentrated by membrane dialysis (Spectrapor, 6,000–8,000 mol wt cutoff, Spectrum Medical Industries, Inc., Los Angeles, CA, USA) in polyethylene glycol (solid flake; 20,000 mol. wt; J. T. Baker Chemical Co., Phillipsburg, NJ, USA) at 4°C. Concentrated proteins were dialyzed against KPB at 4°C overnight prior to analysis for SOD activity and determination of protein content.

Enzyme analysis

SOD activity was determined at 560 nm as described by Beauchamp and Fridovich (1971). The quantity of enzyme required to inhibit the rate of nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO, USA) reduction by 50% was defined as 1 unit (U) of activity. Inhibition of SOD activity by CN^- (1.1 mM final concentration) was tested by adding inhibitor to the reaction mixture prior to initiation of the assay.

Peroxidase activity was determined as described by Clara and Knowles (1984) with 3,3′-diaminobenzidine (Aldrich Chemical Co. Inc., Milwaukee, WI, USA) as the H^+ donor. Specific activity was expressed as change in $A_{482} \cdot min^{-1} \cdot mg\ protein^{-1}$. Catalase specific activity was determined by the procedure of Beers and Sizer (1952).

After removing periplasm with chloroform extraction, by osmotic shock, or by F/T, the small pellets were resuspended in KPB. Cells were disrupted by passage through a French pressure cell and centrifuged at $8{,}000 \times g$ for 20 min at 4°C. The resultant supernatant fluid was assayed for enzyme activity. Total SOD activity in cells was determined by summing that detected in periplasm and that obtained in the remainder of cell material after this procedure. Total SOD activity in cells treated by the procedure of Schnaitman was the sum of that detected in OMP, CMP, and CP + PP.

Polyacrylamide gel electrophoresis

Soluble proteins in *A. magnetotacticum* periplasm and in cytoplasm + periplasm were separated by non-reducing tube gel (10% acrylamide; Davis 1964) and slab gel (1.5 mm, 12% polyacrylamide) electrophoresis. The upper and lower tank buffers were each at pH 8.89 (tube gels) and 7.47 (slab gels). The stack and separating gels were pH 6.9 and 8.48, respectively. CuZn type SOD from bovine erythrocytes (Sigma Chemical Co., St. Louis, MO, USA), and SOD from aerobically cultured *E. coli* were applied to this system to insure that the presence of CuZn-, Mn-, and Fe-type SOD would be detected. After electrophoresis, gels were soaked for 1 h in 50 mM KPB (pH 7.8) containing 10^{-4} M ethylenediaminetetraacetic acid (KPB/EDTA) and stained for SOD activity (Beauchamp and Fridovich 1971). To determine whether bands showing SOD activity were due to a CuZn SOD (sensitive to CN^-), similar gels were soaked in 1.0 mM KCN (Eastman Kodak) in KPB/EDTA prior to staining (Dunlap and Steinman 1986). To determine whether bands of SOD activity insensitive to CN^- were due to Fe-type SOD, gels were soaked in 5.0 mM H_2O_2 (Eastman Kodak Co., Rochester, NY, USA) prior to staining. This treatment inactivates Fe SOD but not Mn SOD (Crapo et al. 1978). Stained gels were photographed or scanned by linear densitometry at 560 nm (Beckman Instruments, Fullerton, CA, USA) and SOD quantified by integration of the area under each absorption minimum (e.g., achromatic zones).

SDS slab gel electrophoresis was performed by the method of Laemmli (1970) except that samples were not heated prior to application to the gel. Prior to staining for SOD activity, SDS gels were washed for 8 h in KPB (pH 6.8) at 4°C. Proteins in each cell fraction and molecular weight standards (Bio-Rad, Richmond, CA, USA) were solubilized prior to electrophoresis by incubation for 15 min at 24°C in 150 mM tris-HCl (pH 6.8) buffer containing 4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 20% glycerol, and 1% bromophenol blue.

Gel regions containing achromatic zones indicative of SOD activity were excised and finely diced. These samples were then washed in 50 mM KPB, (pH 6.8). Their total iron content was determined with ferrozine (Stookey 1970).

Results and discussion

Aquaspirillum magnetotacticum cell periplasm, regardless of the technique by which it was obtained, contained the highest SOD specific activity and more than 94% of total cell SOD activity detected (Table 1). In contrast, 95% of the total SOD activity detected in aerobically cultured *Escherichia coli* B cells was in the cytoplasmic fraction (Table 1). These results corroborate those of Britton and Fridovich (1977) for *E. coli* B while revealing the unusual periplasmic location of SOD in *A. magnetotacticum*.

Periplasm from *A. magnetotacticum* MS-1 cells cultured at 1% DOT with either NO_3^- (previously shown to be O_2-respiring and denitrifying) or with NH_4^+ (O_2-respiring), contained 6 electrophoretically distinct bands of SOD activity (Fig. 1a, lane b). Each band may represent a different enzyme or isozymes of a particular form. Because their activity was not affected by CN^- (Fig. 1b, lane b) all 6 appeared to be of the Mn- or Fe-type. Five were inhibited by H_2O_2 (Fig. 1c, lane b) as expected of FeSOD. One band, unaffected by CN^- or H_2O_2, was assumed to be a MnSOD. No differences between the cell periplasm and periplasm + cytoplasm fractions were evident with respect to the number or distribution of SOD bands present. Cells cultured at a DOT of 10%, expressed 9 bands of SOD activity (Fig. 1a, lane c) of which 4 were FeSODs and 5 were MnSODs (Fig. 1b,c, lane c). We estimated, from densitometric scans

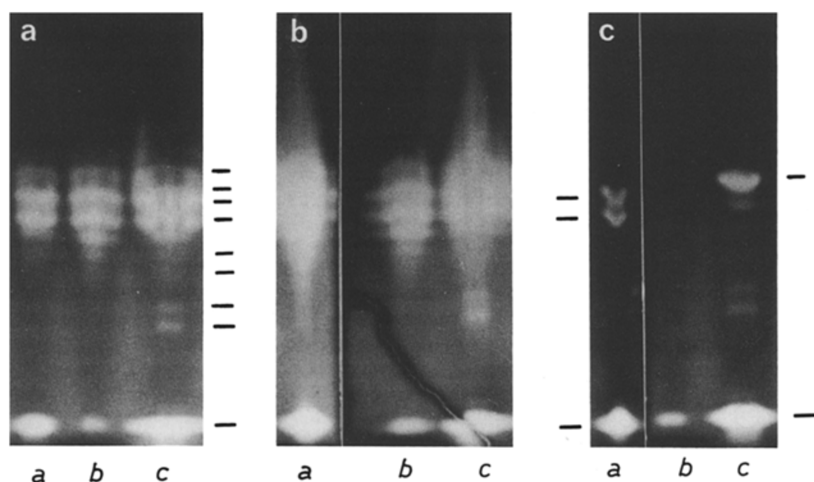

Fig. 1a–c
Non-reducing PAGE of *Aquaspirillum magnetotacticum* periplasmic fractions. 10 U SOD were applied per lane. **a** Control gel was soaked in buffer and stained for SOD activity according to the method of Beauchamp and Fridovich (1971), **b** soaked in 1.0 mM CN^- for 1 h prior to staining; **c** soaked in 5.0 mM H_2O_2 for 1 h prior to staining. Lanes: *a*: strain NM-$1A_A$, 1% DOT; *b*: strain MS-1, 1% DOT; *c*: strain MS-1, 10% DOT

Table 1. Distribution of SOD in *Aquaspirillum magnetotacticum* strain MS-1 and *Escherichia coli* B

Organism and cell fraction (obtained by)	SOD	
	Sp. act.[a]	Total act.[b] (%)
A. magnetotacticum MS-1		
OMP (Schnaitman 1981)	0.3	1
CMP (Schnaitman 1981)	0.3	5
CP + PP (Schnaitman 1981)	2.8	94
Periplasm (obtained by):		
Chloroform (Ames et al. 1984)	5.0	98
EDTA-lysozyme (Neu and Heppel 1965)	5.8	94
Freeze/thaw (Paoletti et al. 1987)	12.0	97
E. coli B		
Cytoplasm	101.5	95
Periplasm		
EDTA-lysozyme (Neu and Heppel 1965)	6.3	5

[a] Sp. act. = U × mg protein^{-1}
[b] Total activity (%) = activity in fraction × sum of activity in all fractions^{-1}

of non-reducing tube gels (data not shown), that FeSOD accounted for 88% and MnSOD for 12% of total SOD activity detected in cells cultured at a DOT of 1%. At a DOT of 10%, the activity of the MnSOD appeared to increase relative to that of the FeSOD (data not shown). We detected patterns of SOD activity similar to those of the wild-type strain in cells of the aerotolerant mutant NM-$1A_A$ cultured at a DOT of 1% (Fig. 1a–c, lane a). Cells of this mutant strain possessed 2 MnSODs not present in wild-type cells grown at 1% DOT (Fig. 1c, lanes a and b). No evidence of a CuZn SOD in any cell fraction of *A. magnetotacticum* was obtained, even though we did detect CuZn SOD activity in bovine erythrocytes and this was inhibited completely by incubation in the presence of 1 mM CN^- prior to staining. In addition, the presence of 1 mM CN^- in the reaction mixture used for kinetic spectrophotometric quantitation of SOD did not decrease SOD activity measured in a periplasmic fraction.

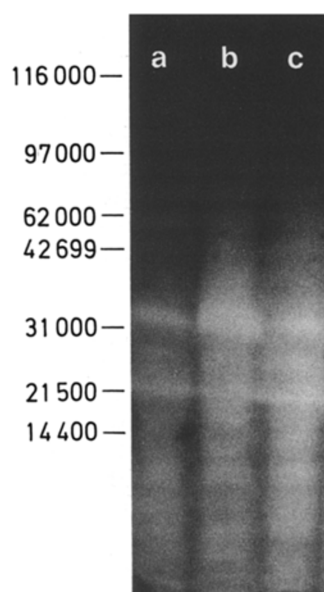

Fig. 2a–c. Reducing SDS PAGE of periplasmic fractions from cells of *Aquaspirillum magnetotacticum*. Approximately 10 U SOD were applied per lane. Sample were mixed with SDS, but not heated. The gel was washed for 8 h in 50 mM phosphate buffer (pH 6.8) and stained for SOD activity. Achromatic zones were removed from the gel, sliced into fragments and assayed for iron using the ferrozine assay (Stookey 1970). Iron was detected only in the lower achromatic band. *a*: Strain MS-1 cultured at 1% DOT; *b*: strain MS-1 cultured at 10% DOT; *c*: strain NM-$1A_A$ cultured at 10% DOT

Periplasmic fractions from *A. magnetotacticum* MS-1 cells cultured at a DOT of 1% (Fig. 2, lane a) or 10% (Fig. 2, lane b) were applied to reducing SDS-PAGE and subsequently assayed for SOD activity. A 23 Kdal band and a 35 Kdal band showed activity. Each band was cut from the gel, and finely diced prior to assay for Fe with ferrozine. The sample containing the 23 Kdal band gave a positive (Fe present) ferrozine reaction, whereas that containing the 35 Kdal band did not. On this basis we assigned apparent molecular weight values of 23 Kdal to the FeSOD and 35 Kdal to the MnSOD. It should be noted that the relative positions of FeSOD and MnSOD are reversed when

Table 2. Effect of N source and culture DOT on periplasmic SOD and soluble peroxidase and catalase activities of *A. magnetotacticum*

Strain	SOD[a]	Peroxidase[b]	Catalase[c]
MS-1			
NO_3^-, 1% DOT	11	4.8×10^{-2}	ND[d]
NO_3^-, 10% DOT	14	7.8×10^{-2}	ND
NH_4^+, 1% DOT	10	1.1×10^{-1}	ND
NH_4^+, 10% DOT	15	4.8×10^{-2}	ND
NM-1A_A			
NO_3^-, 1% DOT	22	2.1×10^{-2}	ND
NO_3^-, 10% DOT	110	1.0×10^{-3}	17
NH_4^+, 1% DOT	55	3.8×10^{-2}	ND
NH_4^+, 10% DOT	93	–[e]	–

[a] SOD values are U × mg protein^{-1} detected in periplasm obtained by F/T
[b] Peroxidase values are change in $A_{482} \times min^{-1} \times$ mg protein^{-1} from cell PP + CP fraction (see Methods)
[c] Catalase values are U × mg protein^{-1} from cell PP + CP fraction (see methods)
[d] ND = not detected
[e] Not determined

comparing reducing and non-reducing gels. A periplasmic fraction from cells of NM-1A_A cultured at a DOT of 10% displayed in SDS PAGE, two bands of activity which migrated identically (Fig. 2, lane c) to those from periplasm of strain MS-1 cells.

Variations in culture nitrogen supply, in mode of respiration (O_2 or NO_3^-) or in iron supply over the range 20 to 300 μM (as ferric quinate) were without significant effect on the types or amounts of periplasmic SOD detected in microaerobically grown cells (Table 2). However, cells cultured at a DOT of 10% contained roughly two-thirds more MnSOD activity than did cells cultured microaerobically (DOT of 1%). We observed no difference in MnSOD activity with variation in Mn concentration. At a trace or 30 μM Mn, MnSOD activity was the same as that of cells cultured with 15 μM Mn. This was true whether cells were cultured at 1% or at 10% DOT. Thus, Mn supplied at 15 μM in the usual culture medium (MSGM), was not at a limiting concentration for expression of MnSOD, as has been reported for other cultured bacteria (Meier et al. 1982; Martin et al. 1984).

The microaerophilic nature of wild-type *A. magnetotacticum* MS-1 cells may relate to their inability to express measurable levels of catalase (Table 2) and to their relatively low level of SOD. In our study, MS-1 cells expressed approximately 20% of the total cell SOD activity detected within aerobically cultured cells of *E. coli* (data not shown). We observed no correlation between *A. magnetotacticum* peroxidase activity and culture DOT (Table 2). The probable importance of catalase in O_2 protection in this organism is suggested by observations that cells of the wild-type strain MS-1 can be protected from O_2 by exogenously supplied catalase. It is also significant that the aerotolerant mutant strain NM-1A_A (having increased SOD and catalase activity at higher DOT; Table 2) can be cultured in an Erlenmeyer flask in free exchange with air. The increase in SOD activity in response to increased culture DOT was not unusual and has been observed with *Azotobacter vinelandii* (Dingler and Oelze 1987), *Campylobacter sputorum* (Niekus et al. 1978), and *Azospirillum brasielense* (Clara and Knowles 1984). However, the expression of catalase by cells of strain NM-1A_A cultured at elevated DOT was unexpected. We presume that cells of the wild type magnetic strain produce catalase but in undetectable quantity.

A periplasmic location for SOD activity could be important in wild-type cells of this obligately microaerophilic bacterium. Univalent O_2 reduction at membrane respiration sites could otherwise result in toxic OH^- formation from O_2^- via Haber-Weiss and Fenton reactions (McCord and Day 1978; Minotti and Aust 1987) given that these cells have such high intracellular iron concentrations at sites proximal to their membranes (Gorby and Blakemore 1988).

Our results comprise the first report of SOD activity (of either the Fe- or Mn-type) in a magnetic bacterium and the first report of periplasmic SODs in a Gram-negative bacterium.

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