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EXPERIMENTAL ARTICLES

Catalase and Superoxide Dismutase in the Cells of Strictly Anaerobic Microorganisms

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Abstract—Strictly anaerobic microorganisms relating to various physiological groups were screened for catalase and superoxide dismutase (SOD) activity. All of the investigated anaerobes possessed SOD activity, necessary for protection against toxic products of oxygen reduction. High specific activities of SOD were found in *Acetobacterium woodii* and *Acetobacterium wieringae*. Most of the investigated clostridia and acetogens were catalase-negative. A significant activity of catalase was found in *Thermohydrogenium kirishiense*, in representatives of the genus *Desulfotomaculum*, and in several methanogens. *Methanobrevibacter arboriphilus* had an exceptionally high catalase activity after growth in medium supplemented with hemin. Hemin also produced a strong positive effect on the catalase activity in many other anaerobic microorganisms. In methanogens, the activities of the enzymes of antioxidant defense varied in wide ranges depending on the stage of growth and the energy source.

Key words: catalase, superoxide dismutase, anaerobes.

It is well known that the products of oxygen reduction—hydrogen peroxide, superoxide radical, and hydroxyl radical—are highly toxic for cells and bring about damage to cell macromolecules [1]. Therefore, to relieve the toxic effect of oxygen, aerobic and facultatively anaerobic microorganisms possess catalase (EC 1.11.1.6), which uses hydrogen peroxide as an electron donor and catalyzes its two-electron reduction to water and oxygen, and superoxide dismutase (SOD, EC 1.15.1.1), which catalyzes the reaction of disproportionation of the superoxide radical.

At the early stage of investigation of the enzymes of antioxidant defense, a hypothesis was put forward that the inability of strict anaerobes to grow in the presence of oxygen is due to the lack of catalase, resulting in hydrogen peroxide accumulation in the cells during metabolic processes. However, later, catalase was found in various anaerobic microorganisms, in particular among many representatives of *Bacteroides* [2]. Fridovich failed to find SOD activity in some anaerobic bacteria and hypothesized that this enzyme is specific to aerobic and aerotolerant microorganisms and that cells that do not reduce oxygen do not normally encounter superoxide radicals and do not need SOD; therefore, they are very sensitive to the adverse effect of oxygen [1]. However, other researchers found that not only aerobic and facultatively anaerobic bacteria but also strict anaerobes, *Desulfovibrio* and *Clostridium* in particular, possess SOD activity [3, 4]. The occurrence of SOD in strict anaerobes (which is much more frequent than it was formerly supposed) raises a question about the physiological role of SOD and its origin in these microorganisms. The fact that strict anaerobes can grow only in oxygen-free environments makes the functions of SOD under such conditions disputable. Probably, only under unfavorable aerobic conditions (which may be encountered by strict anaerobes rather frequently, especially by pathogenic species of *Clostridium* and *Bacteroides*), does the synthesis of SOD induced by molecular oxygen provide the defense of an anaerobic organism against the lethal effect of $O_2^{-\bullet}$. It has been suggested that only microorganisms possessing SOD can survive the presence of oxygen; the presence of catalase is not so important [3, 4]. The studies of the resistance of microorganisms to oxygen revealed a clear-cut connection with SOD activity: the species with high SOD activity exhibit higher aerotolerance than species with low SOD activity or species lacking SOD [5].

We studied the catalase and SOD activities in cells of some strict anaerobes with the aim of finding microorganisms with the highest activities of enzymes of antioxidant defense.

MATERIALS AND METHODS

This work used the following bacteria: the clostridia *Clostridium acetobutylicum* 6, *C. acetobutylicum* 7, *C. acetobutylicum* 8, *C. butyricum* 19, *C. butyricum* 21, *C. butyricum* 22, and *C. butyricum* 23 (from the collection of Microbiology Department, Moscow State Uni-

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Catalase and SOD activity in strictly anaerobic microorganisms

Microorganism	Specific activity of catalase, U/mg protein		Specific activity of SOD, U/mg protein (– hemin)
	– hemin	+ hemin (30 μM)	
Fermenters			
Clostridium acetobutylicum 6	1	112	11
C. acetobutylicum 7	<0.01	<0.01	13
C. acetobutylicum 8	<0.01	1	15
C. butyricum 19	<0.01	<0.01	6
C. butyricum 21	1	1	3
C. butyricum 22	<0.01	1	1
C. butyricum 23	<0.01	1	4
C. symbiosum	<0.01	ND	ND
Acetogens			
C. formicoaceticum	<0.01	6	12
Sporomusa sphaeroides	0.7	1	11
Acetobacterium woodii	0.8	5	26
A. wieringae	9	9	38
A. poludosum Z-7390	0.3	9	3
Sulfidogens			
Desulfotomaculum nigrificans subsp. *salinus* 435	189	180	4
D. kuznetsovii 17	39	39	1
Thermoanaerobes			
Thermohydrogenium kirishiense 360	58	60	5
T. lactoethylicum 149	8	18	15
Methanogens			
Methanobrevibacter cuticularis RFM-1	54	ND	ND
M. arboriphilus SA	0.1	ND	ND
M. arboriphilus DH1	7	300	15
M. arboriphilus AZ	9	240	22
"*Methanolobus*" sp. NaT-1	0.2	ND	ND
Methanobacterium marburgensis	1	ND	ND
Methanopyrus kandleri	<0.01	ND	ND
Methanococcus voltae	<0.01	ND	ND
Methanosarcina barkeri Fusaro	40	42	31

Note: ND stands for "not determined."

versity) and *C. symbiosum* from DSMZ (Braunschweig, Germany); the acetogens *C. formicoaceticum*, *Sporomusa sphaeroides*, *Acetobacterium woodii*, *A. wieringae*, and *A. poludosum* Z-7390, obtained from the Laboratory of Relict Microbial Communities (Institute of Microbiology, Russian Academy of Sciences); the sulfate-reducing bacteria *Desulfotomaculum nigrificans* subsp. *salinus* 435 and *D. kuznetsovii* 17 from the Laboratory of Microbial Biogeochemistry (Institute of Microbiology, Russian Academy of Sciences); *Thermohydrogenium kirishiense* 360 and *T. lactoethylicum* 149 from the collection of the Microbiology Department, Moscow State University; and the methanogens *Methanobacterium marburgensis*, *Methanobrevibacter cuticularis* RFM-1, *Methanobrevibacter arboriphilus* SA, *M. arboriphilus* DH1, *M. arboriphilus* AZ, *Methanopyrus kandleri*, *Methanococcus voltae*, "*Methanolobus*" sp. NaT-1, and *Methanosarcina barkeri* Fusaro from DSMZ (Braunschweig, Germany).

For cultivation of anaerobes, we used corresponding optimized media described in the literature [6]. All cultures were grown until the end of the logarithmic phase

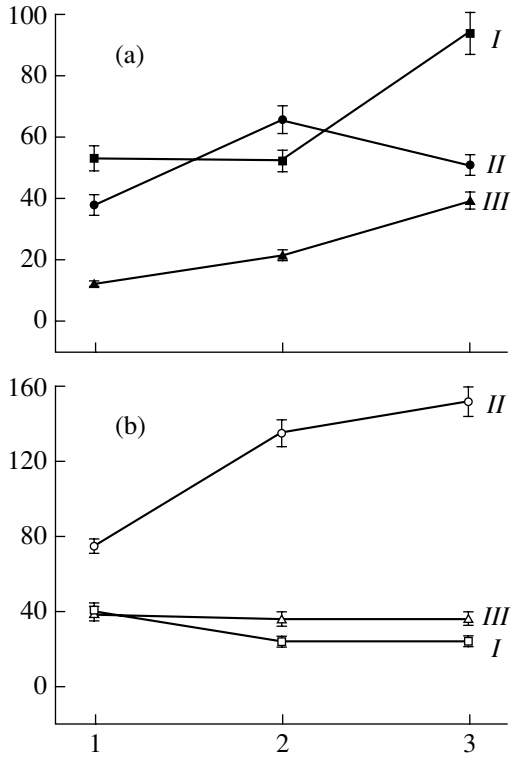

Fig. 1 Activities of (a) SOD and (b) catalase during the growth of *Methanosarcina barkeri* Fusaro on different substrates: (*I*) growth on medium with methanol; (*II*) growth on medium with acetate; (*III*) growth on medium with H_2 + CO_2. *Y* axis, specific activity, U/mg of protein; *X* axis, phases of growth: 1, mid-log phase; 2, late log phase; 3, stationary phase.

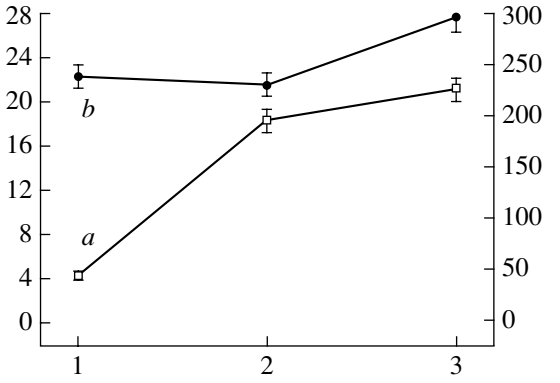

Fig. 2 Activities of (*a*) SOD and (*b*) catalase during the growth of *Methanobrevibacter arboriphilus* AZ on medium with H_2 + CO_2. Left *Y* axis, specific activity of SOD, U/mg of protein; right *Y* axis, specific activity of catalase, U/mg of protein; *X* axis, phases of growth: 1, mid-log phase; 2, late log phase; 3, stationary phase.

under anaerobic conditions at a cultivation temperature optimal for a given microorganism. A 30 mM sterile anaerobic hemin solution in 0.2 M KOH was added to the media (1 ml/l) when necessary.

To obtain cell extracts, cells of anaerobic microorganisms were washed by centrifugation (9000 *g*; 20 min; 4°C) in 50 mM K-phosphate buffer (pH 7.0). Then the biomass was resuspended in the same buffer and was ultrasonically disrupted (UZDN-2T; 22 kHz; 40 μA; six 45-s sessions with cooling in ice for 2 min between the sessions). Methanogens were disrupted in a Huge press in a frozen state at an excessive pressure of 3 t/cm^2. Cell fragments were removed by centrifugation (30 000 *g*; 20 min; 4°C).

The catalase activity was determined spectrophotometrically [7]. The spectrophotometric assays of SOD activity employed xanthine oxidase and cytochrome *c* [8]. The protein concentration in cell extracts was determined by the Bradford method [9], using bovine serum albumin as the standard. The data presented in this paper are statistically processed (using the Statistica software) results of at least three replicate experiments.

RESULTS AND DISCUSSION

According to our data, representatives of the genus *Clostridium* usually had no catalase activity; only two strains out of eight tested had a low activity of catalase (table). Indeed, the majority of known *Clostridium* species do not possess catalase, are peroxide-sensitive, and their growth is suppressed by very low concentrations of H_2O_2 (30–60 μM). However, some clostridia have been reported to possess catalase [10]. Apart from vegetative cells, a low activity of this enzyme was also discovered in spores of *Clostridium butyricum* 35/11 [10]. The addition of hemin (30 μM) to the medium resulted in the appearance of catalase activity in several clostridia investigated (table); in *C. acetobutylicum* 6, which had a low activity of the enzyme, catalase activity increased hundredfold after the addition of hemin. A similar significant effect was produced by hemin on the catalase activity in *Methanobrevibacter arboriphilus* (see below). Probably, microorganisms for which a positive effect of hemin was shown can synthesize the apoenzyme of catalase but cannot synthesize heme. It is necessary to note that hemin did not produce any significant effect on the growth of clostridia or other microorganisms used in our experiments.

The acetogenic bacteria investigated by us possessed a very low activity of catalase except for *Acetobacterium wieringae*, which exhibited a catalase activity of about 9 U/mg protein (table). Hemin (30 μM) produced a significant stimulatory effect on the catalase activity in most acetogens studied (table). For example, in the presence of hemin in the medium, catalase activity was found in *C. formicoaceticum*, and the catalase activity of *Acetobacterium woodii* and *Acetobacterium poludosum* increased 7- and 27-fold, respectively (table). It is known that *C. formicoaceticum* cells contain cytochromes and, thus, can synthesize heme. Hence, the effect of exogenous hemin added to the medium on the catalase activity of this bacterium is of

an uncommon nature. On the other hand, *A. wieringae* does not have cytochromes and cannot synthesize heme; nevertheless, we recorded no increase in the catalase activity of this bacterium in the presence of hemin. A possible explanation is the rather high concentration of yeast extract (which can contain heme) in the rich medium used for the cultivation of this acetogen. As already mentioned, the SOD activity of *A. wieringae* is relatively high as compared to other acetogens. We could not find any literature data on the catalase properties in acetogens, but we can suppose that, like clostridia, acetogenic bacteria are peroxide-sensitive and SOD is their first line of defense against the toxic effect of oxygen. An open question is the reason for the lack of catalase activity in some species: it may the absence of the corresponding gene or this gene may be expressed only under the conditions of oxidative stress.

Contrary to clostridia and acetogenic bacteria, the sulfate-reducing bacteria *Desulfotomaculum nigrificans* subsp. *salinus* and *D. kuznetsovii* exhibited a high catalase activity (table); in *D. nigrificans* subsp. *salinus*, the catalase activity (190 U/mg) was comparable with that characteristic of aerobic microorganisms. The addition of hemin (30 μM) to the media did not stimulate the catalase activity in these sulfate reducers (table). Representatives of *Desulfotomaculum* are the dominant sulfate reducers in temporarily flooded soils such as rice field soils; they occur in oxygen-free marine and freshwater sediments and in animal guts, i.e., in the environments in which oxygen can penetrate. It may be supposed that in these microorganisms catalase plays the main role in the protection of cells against the toxic products of oxygen reduction. It has been reported that some sulfate reducers, *Desulfovibrio desulfuricans* [11] and *Desulfovibrio gigas* [12] in particular, are capable of withstanding exposure to air for several days without loss of viability. As for catalase activity, it is known that some sulfate-reducing bacteria lack it; however, in *D. gigas* the catalase activity is high and reaches 53 U/mg of protein [12].

In the cells of anaerobic thermophilic bacterium *Thermohydrogenium kirishiense* 360 isolated from an industrial biocenosis, we also detected a high catalase activity (table). Hemin (30 μM) did not produce any positive effect on the catalase activity of this bacterium, in contrast to the catalase activity of *T. lactoethylicum* 149, which increased twofold after hemin addition.

High catalase activities, comparable to those of aerotolerant anaerobes, are exhibited by some methanogens, e.g., *Methanobrevibacter cuticularis* RFM-1 and *Methanosarcina barkeri* Fusaro (table; [13]). As can be seen from the table, only some methanogens have pronounced catalase activity, and *Methanopyrus kandleri* and *Methanococcus voltae* are catalase-negative. *Methanobrevibacter arboriphilus* DH1 and AZ, which cannot synthesize heme, exhibit a very high catalase activity (up to 300 U/mg of protein) in the presence of hemin in the medium [14]. It is well known that methanogens are strict anaerobes; however, data have been published [15] indicating certain aerotolerance of some of them: in particular, *Methanosarcina barkeri* can survive in drained rice fields and withstand the contact with oxygen for several hours. There are oxygenated zones in the termite gut, which is the main habitat for the representatives of *Methanobrevibacter.* It can be suggested that such methanogens need highly active enzymes of antioxidant defense for rapid elimination of toxic oxygen derivatives.

All of the representatives of *Clostridium* investigated by us possessed SOD activity. Strains of *C. butyricum* had 3- to 5-fold lower SOD activity than *C. acetobutylicum* strains (table). It is known that many clostridia possess SOD [4], which is in agreement with their ability to survive exposition to oxygen for eight and more hours.

We failed to find any published data about SOD of acetogenic bacteria. All five species of acetogens investigated by us had pronounced SOD activity (table); in *Acetobacterium woodii* and *Acetobacterium wieringae*, SOD activity was the highest (26 and 38 U/mg of protein, respectively); this is 2- to 22-fold higher than SOD activity in the clostridia studied by us. Moreover, such a level of SOD activity is close to the activity level exhibited by aerobic cells of *Escherishia coli* B. Taking into account the fact that all known acetogens are anaerobes with a high sensitivity to oxygen, it is difficult to explain the presence of highly active SOD in cells of these microorganisms. It can be supposed that some obligate anaerobes inhabiting environments that can be penetrated by oxygen (in particular, aquatic environments, as is the case with acetogens) possess high activities of enzymes of antioxidant defense for rapid minimization of the toxic effect of oxygen.

Sulfate-reducing bacteria of genus *Desulfotomaculum* used in our experiments also contained SOD, but its activity was low (table), lower than the SOD activity in most investigated clostridia and much lower than in acetogens. It is known that many sulfate reducers, e.g., *Desulfomicrobium norvegicum* [16] and *Desulfovibrio gigas* [12], possess SOD, but its activity in cell extracts of these bacteria is not high (1–4 U/mg of protein).

SOD is also present in thermophilic bacteria of the genus *Thermohydrogenium* (table); SOD activity in the cells of these bacteria is close to that in the cells of clostridia.

The activity of SOD in cells of the most strict anaerobes—the methanogenic archaea *Methanosarcina barkeri* and *Methanobrevibacter arboriphilus*—is close to SOD activity in such less strict anaerobes as strains of *C. acetobutylicum* (table; [17]). Previously, the presence of SOD with the a similar specific activity was shown for *Methanobacterium bryantii* [18] and *Methanobacterium thermoautotrophicum* [19].

SOD and catalase activities were found in cell extracts of bacteria grown anaerobically. This fact suggests that in the microorganisms studied, the enzymes

of antioxidant defense are expressed constitutively during the growth in the absence of oxygen. The expression of SOD and catalase under anaerobic conditions may protect cells in case of a sudden exposure to oxygen.

We also measured the activities of SOD and catalase in different phases of growth of the cultures of strict anaerobes *Methanosarcina barkeri* Fusaro and *Methanobrevibacter arboriphilus* AZ. In the case of *Methanosarcina barkeri*, we investigated cells grown on methanol, acetate, and $H_2 + CO_2$.

Catalase activity was much higher when *Methanosarcina barkeri* was cultivated on acetate than when it was grown on methanol or $H_2 + CO_2$ (Fig. 1). Interestingly, the catalase activity virtually did not change during the course of growth of *Methanosarcina barkeri* on $H_2 + CO_2$, whereas in a methanol-grown culture it peaked in the mid-log growth phase, and in an acetate-grown culture it exhibited a maximum in the stationary phase of growth (two times higher as compared with the beginning of log phase) (Fig. 1). For *Methanobrevibacter arboriphilus* AZ, it was also shown that catalase activity has a maximum in the stationary phase of growth (Fig. 2).

The lowest activity of SOD was characteristic of archaea grown on $H_2 + CO_2$; the specific activity of SOD in extracts of acetate- or methanol-grown cells was 2- to 3-fold higher (Fig. 1). Except for the case of acetate-grown cells, a pronounced increase in SOD activity (2- to 3-fold) occurred in the stationary phase of growth (Fig. 1). In the case of *Methanobrevibacter arboriphilus* AZ, the maximum of SOD activity (which was 5 times higher than in the mid-log phase) also occurred in the stationary phase of growth (Fig. 2). Probably, in the stationary phase of growth, when the rate of cell death and the possibility of superoxide radical formation are high, the cells need an intensification of antioxidant defense.

It may be supposed that in *Methanosarcina barkeri* and *Methanobrevibacter arboriphilus*, catalase and SOD are regulated enzymes whose activity depends on the energy source and/or culture growth phase.

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