Microbiology, Vol. 72, No. 5, 2003, pp. 534–541. Translated from Mikrobiologiya, Vol. 72, No. 5, 2003, pp. 600–608.

EXPERIMENTAL ARTICLES

Oxidative Stress and Antioxidant Cell Protection Systems in the Microaerophilic Bacterium *Spirillum winogradskii*

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Received November 20, 2002; in final form, June 16, 2003

Abstract—The influence of oxygen availability during cultivation on the biosynthetic processes and enzymatic activities in the microaerophilic bacterium *Spirillum winogradskii* D-427 was studied, and the roles played by different systems of the defense against oxidation stress were determined. The metabolic adjustments caused by transition from microaerobic (2% O_2) aerobic conditions (21% O_2 of the gas phase) were found to slow down constructive metabolism and increase synthesis of exopolysaccharides as a means of external protection of cells from excess oxygen. This resulted in a twofold decline of the growth yield coefficient. Even though the low activity of catalase is compensated for by a multifold increase in the activities of other cytoplasmic enzymes that defend against toxic forms of O_2—peroxidase and enzymes of the redox system of glutathione (glutathione peroxidase and glutathione reductase)—massive lysis of cells starts in the midexponential phase and leads to culture death in the stationary phase because of H_2O_2 accumulation in the periplasm (up to 10 μg/mg protein). The absence in cells of cytochrome-*c*-peroxidase, a periplasmic enzyme eliminating H_2O_2, was shown. It follows that the major cause of oxidative stress in cells is that active antioxidant defenses are located in the cytoplasm, whereas H_2O_2 accumulates in the periplasm due to the lack of cytochrome-*c*-peroxidase. The addition to the medium of thiosulfate promotes elimination of H_2O_2, stops cell lysis under aerobic conditions, lends stability to cultures, and results in a threefold increase in the growth yield.

Key words: microaerophily, cytochrome-*c*-peroxidase, catalase, SOD, glutathione redox system, exopolysaccharides, thiosulfate, *Spirillum winogradskii.*

Many microorganisms under aerobic conditions are exposed to highly reactive forms of oxygen, with detrimental or lethal consequences for cells. Some aerobic microorganisms, called microaerophiles, are able to grow only in low-oxygen natural or laboratory media because of their high sensitivity to products of incomplete reduction of oxygen. Their high sensitivity can arise from a number of endogenous and exogenous factors. The most important one is the low activity of the enzymatic systems implicated in detoxication of reactive forms of oxygen, primarily of catalase, superoxide dismutase (SOD), and peroxidase. In natural environments, microaerophily can be connected with the inhibiting action of exometabolites on the enzymes involved in antioxidant cell defenses in microbial communities [1]. It can also be connected with the presence of reactive forms of oxygen produced in photochemical of chemical reactions or as a result of fluctuations in the oxygen content of aquatic environments [2]. There, apparently, can be significantly different causes for microaerophily in different representatives of this group of microorganisms.

The sources of microaerophily and the mechanisms of antioxidant defenses in bacterial cells are not yet well understood. For example, there are several catalase-positive bacteria that fail to show stable growth under aerobic conditions [3]. It was established that low-molecular-weight thiols (glutathione) constitute important components of antioxidant defenses against H_2O_2 in animal cells. However, the available data on the role of the redox system of glutathione in the protection of bacterial cells are scarce and inconsistent [4, 5]. The evidence concerning the functional role of another enzyme—cytochrome-*c*-peroxidase—in the processes connected with the protection of cells from the action of toxic forms of oxygen is also very limited.

In this work, microaerophily of *Spirillum winogradskii*, a new representative of organotrophic sulfur-oxidizing spirilla, was studied. Our goal was to determine the influence of different oxygen concentrations on biosynthetic processes and enzymatic activities in this bacterium and to study enzymatic and nonenzymatic systems of antioxidant cell defenses.

MATERIALS AND METHODS

Bacteria and culture conditions. The microaerophilic *Spirillum winogradskii* sp. nov., strain D-427, DSMZ 12756, from the culture collection of the Laboratory of Ecology and Geochemical Activities of Microorganisms, Institute of Microbiology, Russian Academy of Sciences, was used. The organism was cul-

0026-2617/03/7205-0534$25.00

tured in a modified MPSS nutrient medium [6] with succinate (1 g/l) as the growth substrate; in several experiments, succinate was replaced with acetate (1 g/l). Microaerobic conditions were established as described elsewhere [8] by replacing air with argon in bottles (0.5 l) with a freshly boiled medium (50 ml) and then aseptically injecting air with a syringe in amounts of 50, 25, 10, or 5%, which corresponded to the concentrations of dissolved oxygen in the liquid medium of 2.9, 1.5, 0.6, and 0.3 mg/l, respectively. Under aerobic growth conditions, the oxygen content of the medium was 8.5 mg/l. To sterilize the injected gas, nozzles equipped with ultrafilters (pore size, 0.2 μm) were used. Experiments to study the effect of O_2 on bacterial growth were carried out using the above-specified medium free of peptone.

Analytical methods. Bacterial cell mass was determined from the suspension optical density measured at 500 nm with a Specol-10 spectrophotometer (Czech Republic). Cells were precipitated by culture centrifugation at 5000 g and 4°C for 30 min and then washed in a 0.1 M Tris–HCl buffer (pH 7.5). The cell homogenate was obtained by disrupting cells in an ice bath using a UZDN-2T ultrasonic disintegrator operating at 500 W and 22 kHz for 2 min. The supernatant of cell extracts was obtained by centrifugation of the homogenate at 9000 g and 4°C for 30 min. Protein in whole cells, obtained by culture centrifugation at 9000 g for 30 min, and in the supernatant (dissolved protein formed as a result of cell autolysis) was determined by the Lowry method. The absence of cells in the supernatant was verified by microscopic examination. Oxygen in the gas phase was determined on an LCM-80 chromatograph equipped with a katharometer (carrier gas, argon flown at 40 ml/min; filament current, 80 mA; column temperature, ambient). Acetate in the medium was determined by the method of gas adsorption chromatography using a Chrome-5 chromatograph (Czech Republic) with a flame ionization detector. The overall content of carbohydrates was determined by the phenol method on an SF-26 spectrophotometer at $\lambda = 488$ nm. Hydrogen peroxide in cell suspensions was determined by the chemiluminescence technique with luminol on an LB-3PA luminometer (Klimbi, Russia). The rate of oxygen consumption was measured on a PU-1 polarograph [8].

Enzymatic activity assays. Enzymatic activities were determined using cells of 2-days-old cultures at the end of exponential growth. The activities of the following enzymes in the culture supernatant were determined on an SF-26 spectrophotometer by conventional procedures. Catalase (EC 1.11.1.6), superoxide dismutase (SOD), and peroxidase (EC 1.11.1.7) were assayed as described elsewhere [9]; cytochrome *c* peroxidase (EC 1.11.1.5) was determined according to [10] with preliminary reduction of cytochrome *c* with dithionite [11]; phosphoenolpyruvate carboxykinase (EC 4.1.1.38), phosphoenolpyruvate carboxylase (EC 4.1.1.31), pyruvate kinase (EC 2.7.1.40), pyruvate carboxylase (EC 6.4.1.1), NADH-dependent malate dehydrogenase (MDH) (EC 1.1.1.37), phosphoenolpyruvate synthase, phosphoglucoisomerase (EC 5.3.1.9), fructose-bisphosphatase (EC 3.1.3.11), phosphoglucomutase (EC 2.7.5.1), NADP-dependent glutamate dehydrogenase (EC 1.4.1.2), alanine dehydrogenase (EC 1.4.1.1), NADP-dependent MDH (EC 1.1.1.82), NADP-dependent malic enzyme (ME) (EC 1.1.1.40), and NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) were assayed according to [11]; and the activities of aminotransferases—aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2)—were determined using the Lahema Diagnostica biotest. The enzymes of the glutathione redox system were assayed spectrophotometrically: glutathione peroxidase (EC 1.11.1.9) was determined from a decrease in the concentration of reduced glutathione in the incubation medium measured at $\lambda = 412$ nm and glutathione reductase (EC 1.6.4.2) was determined from a decrease in the NADPH concentration in the incubation medium measured at $\lambda = 340$ nm [4].

All test were replicated two to five times; the figures display results of representative tests. The data in the tables are averages of three measurements, and their spread was within 10–15%.

RESULTS

Influence of Oxygen on Growth Processes and Biomass Accumulation

The results of experiments performed to measure the effect of different concentrations of oxygen in the gas phase on the accumulation of cell mass in the medium are shown in Fig. 1. The accumulated biomass was maximal when the concentration of O_2 in the gas phase was 2%; it exceeded by 1.8-fold the biomass accumulated in an air atmosphere.

Microscopic examinations revealed massive lysis of cells in the course of culture growth in an air atmosphere. Therefore, in subsequent experiments, the effect of the oxygen concentration on biomass accumulation and cell lysis was studied in more detail during the growth of spirilla both under aerobic and microaerobic conditions (Fig. 2). It can be seen from Fig. 2 that, in the midexponential phase, the protein content of intact whole cells in the culture developing under microaerobic conditions was higher than in cells grown under aerobic conditions by a factor of 2–2.5. However, under aerobic conditions, the content of dissolved protein of lysed cells in the culture liquid was higher than that in the microaerobic culture by a factor of 2.1–2.3. The net contents of cellular and extracellular protein in both test variants were roughly equal. The stable growth and long-preserved viability of cells in microaerobic cultures were in marked contrast with the growth instability and fast death of cells exposed to air.

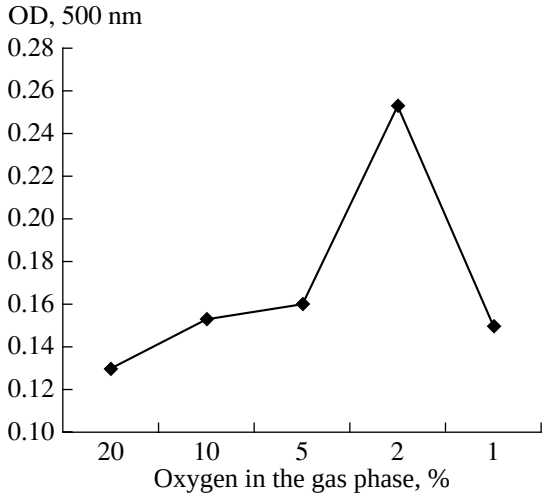

Fig. 1. Effect of oxygen content of the gas phase on biomass production in *S. winogradskii*. The experiment duration was 48 h (end of exponential growth); the low cell yield at the gas phase oxygen content of 1% can presumably be explained by growth limitation by the shortage of oxygen.

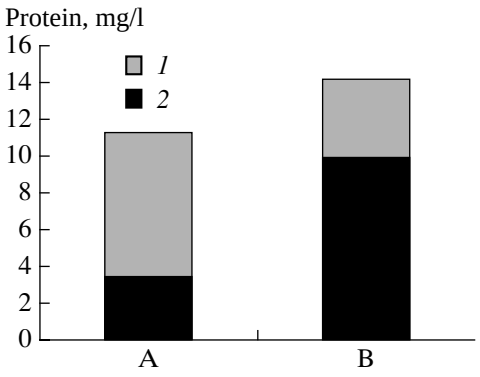

Fig. 2. Effect of oxygen concentration in the gas phase (A, 21%; B, 2%) on the growth and accumulation biomass in a culture of *S. winogradskii*. (*1*) Lysed cells and (*2*) intact cells; the experiment duration was 48 h (end of exponential growth).

The massive lysis of spirilla under aerobic conditions could be due to the formation of hydrogen peroxide in cells or in the medium, as was shown to be the case with many aerobic microorganisms sensitive to oxygen [10, 12, 13]. Therefore, the conditions giving rise to the accumulation of H_2O_2 in cells and the mechanisms of antioxidant defenses against the action of toxic forms of oxygen, operating at the cellular and subcellular levels, were studied in more detail.

Influence of Oxygen Content during Cultivation on the Accumulation of H_2O_2 in Cells

As seen from Table 1, the rate of H_2O_2 formation in cells was directly connected with the oxygen regime of cultivation and attained a maximum of 1.2 nmol/(min mg protein) for growth in air. In the stationary phase, the H_2O_2 content of cells could be as high as 10.4 μg per 1 mg of cell protein. Under microaerobic conditions, the rate of hydrogen peroxide accumulation dropped sevenfold.

No accumulation of H_2O_2 in the culture liquid free of cells was detected in assays employing the chemiluminescence method. The entire hydrogen peroxide was associated with the bacterial cell mass and, probably, could also be present in exopolysaccharides pelleted together with cells. The specificity of the luminol reaction with H_2O_2 was demonstrated by the lack of luminescence in test variants where the cell suspension was first treated with pyruvate or catalase.

The polarographic method showed 30% inhibition of H_2O_2 production in the presence of antimycin A, known to inhibit the cytochrome *b* cytochrome *c* segment of the respiratory chain, indicating the interrelation of this process with the operation of the electron transport chain (ETC).

Enzymatic Defenses against Toxic Oxygen Species

In order to compare the effectiveness of different enzymatic systems protecting spirilla cells against toxic oxygen species in cells grown under aerobic and anaerobic conditions, the activities of enzymes involved in detoxication of H_2O_2 and $O_2^{\cdot}$ were measured.

The results are presented in Table 2. Under aerobic growth conditions, the activities of most enzymes, except for catalase, were notably higher than under microaerobic ones. The catalase activity in cells did not depend on the availability of oxygen and was extremely low, one to two orders of magnitude lower than that in many aerobic heterotrophic bacteria [3, 13]. Likewise, the activity of SOD did not depend on the presence of oxygen in the growth medium; however, its level was comparable to that observed in many aerobic bacteria.

The activities of enzymes of the glutathione redox system under aerobic growth conditions were several times higher than under microaerobic ones. Specifically, the activity of glutathione peroxidase, directly participating in the reduction of H_2O_2, was four times higher, and that of glutathione reductase, sustaining the pool of reduced glutathione in the cytoplasm, was higher by a factor of 3.4. Meanwhile, the activity of peroxidase, which is not implicated in the glutathione redox system, remained quite low compared to other aerobic organisms, even despite its severalfold increase in cells grown in an air atmosphere. Our assays show that, in *S. winogradskii*, the activity of this enzyme is as low as 0.45 nmol/(min mg protein), whereas in *E. coli*, it is 3.0 nmol/(min mg protein).

Activity assays of cytochrome *c* peroxidase, another enzyme interacting with H_2O_2, showed its absence in cells irrespective of the culture conditions.

Table 1. Effect of the oxygen regime during cultivation on the H_2O_2 production rate and accumulation in cell suspension of *S. winogradskii*

Test variant 1* (O_2 content of the gas phase, %)	Rate of H_2O_2 production, nmol/(min mg protein)	Accumulation of H_2O_2 in cells, μg/mg protein***
Succinate (21)	1.2	10.4
Succinate (21) + catalase (2 μg/ml)	0.0	0.0
Succinate (21) + pyruvate (1 μg/ml)	0.0	0.0
Succinate (5)	0.4	2.7
Succinate (2)	0.3	1.5
Supernatant	0.0	0.0

Test variant 2** (O_2 content of the gas phase was 21%)	Rate of O_2 uptake, nmol/(min mg protein)	Rate of O_2 uptake upon addition of catalase, nmol/(min mg protein)	Rate of H_2O_2 production, nmol/(min mg protein)	Inhibition, %
Succinate	45.7	27.4	35.3	
+ rotenone, 1×10^{-3} M	45.7	27.4	35.3	0.0
+ antimycin, 1×10^{-7} M	32.0	19.2	24.7	30

Note: H_2O_2 was determined * by the chemiluminescence method; ** by the polarographic method; *** in the stationary phase of growth.

A multifold increase, under aerobic culture conditions, in the activities of enzymes of the glutathione redox system, and, above all, in the activity of glutathione peroxidase, is direct evidence of the participation of this system in the H_2O_2 decomposition in cells. However, as can be inferred from Figs. 1 and 2 and data in Table 3, the increased activities of enzymes with antioxidant action fail to prevent the lytic damage inflicted to cells by H_2O_2, formed in the course of respiration.

Nonenzymatic Defenses against Toxic Oxygen Species

Formation of polysaccharides. Previous light microscopic and, particularly, electron microscopic studies of *S. winogradskii* revealed the formation of large amounts of extracellular exopolysaccharides and capsules of a polysaccharide origin [6]. Shown in Table 3 are the results of the experiments to study the effect of oxygen on the accumulation of cellular protein and exopolysaccharides and on the efficiency of growth substrate utilization under different culture conditions. The data obtained show that, when acetate is the growth substrate, the overall cell yield, with the protein of lysed cell taken into account, is 1.5 times greater under microaerobic conditions than in aerobic growth. In the latter case, extracellular polysaccharides and mucous capsules form in the course of growth. According to our calculations, their synthesis takes up about 50% of the acetate carbon utilized by cells. With succinate used as the growth substrate, the amount of produced polysaccharides increased five- to sevenfold, in addition to the overall cell yield increase (data not reported). The growth yield coefficient *K* (measuring the utilization efficiency of a given substrate) calculated from data in Table 3 was two times higher for the culture grown under microaerobic conditions. This increase apparently reflects the reduced burden on the energy metabolism due to the decreased use of acetate for exopolysaccharide synthesis.

The formation of polysaccharides in microorganisms is known to start with the synthesis of their precursor—phosphoenolpyruvate (PEP)—which not only participates in the early stages of gluconeogenesis but also serves as a substrate for a large number of biosyn-

Table 2. Effect of the oxygen regime during cultivation on the activities of enzymes involved in the removal of toxic oxygen species in *S. winogradskii*

Enzymes	A	B	A/B
	aerobic conditions (21% O_2)	microaerobic conditions, (2% O_2)	
Catalase, μmol/(min mg protein)	0.4	0.3	1.3
SOD, units/mg protein	9.8	8.4	1.2
Peroxidase, nmol/(min mg protein)	0.45	0.1	4.5
Glutathione peroxidase, nmol/(min mg protein)	215.0	50.7	4.2
Glutathione reductase, nmol/(min mg protein)	57.0	16.3	3.5
Cytochrome *c* peroxidase, nmol/(min mg protein)	0.0	0.0	

Note: Cells from a two-day-old culture (end of exponential growth).

Table 3. Effect of the oxygen regime during cultivation on utilization of acetate, polysaccharide synthesis, and the growth yield coefficient in *S. winogradskii*

Cultivation conditions	Acetate utilization		Polysaccharide synthesis		Protein synthesis**		*K*
	mmol/l	C, mg/l	mg/l	C, mg/l*	mg/l	C of protein, mg/l***	
Aerobic	0.68	16.3	5.6	2.2	2.5	3.0	3.7
Microaerobic	0.51	12.2	2.8	1.1	3.8	4.6	7.5

Note: Experiment duration, 24 h; *K* is the growth yield coefficient, mg protein/mmol acetate.
* Carbon of polysaccharides estimated as glucose carbon.
** Protein of cells (whole and lysed).
*** Cell carbon calculated according to [8].

thetic reactions and for the TCA cycle (Fig. 3). The activities of PEP carboxykinase, PEP carboxylase, and PEP synthase in spirilla cells are listed in Table 4.

Under aerobic growth conditions, the activities of PEP carboxykinase and PEP carboxylase, responsible for the conversion of oxalacetic acid to PEP, increased only insignificantly. Under the same conditions, the activity of phosphoenolpyruvate synthase, implicated in the conversion of pyruvic acid to PEP, exceeded by an order of magnitude the activities of the two above-mentioned enzymes. It should be noted that the activity of pyruvate kinase, catalyzing the reverse conversion of PEP to pyruvic acid, increased markedly under microaerobic conditions, with a corresponding increase in the rate of constructive metabolism.

The synthesis of carbohydrates is known to be preceded by a number of successive enzymatic reactions of gluconeogenesis (Fig. 3). The activities of several enzymes, such as fructose-bis-phosphatase, phosphoglucoisomerase, and phosphoglucomutase, implicated in gluconeogenesis in spirilla cells, were 2.5–5 times higher under aerobic conditions than under microaerobic conditions (Table 4), which is indicative of the increased activities of biosynthetic processes directed toward the production of polysaccharides.

Effect of thiosulfate on growth. Like other heterotrophic sulfur bacteria, strain D-427 can oxidize several reduced sulfur compounds: thiosulfate to tetrathionate and sulfide to elemental sulfur, which is accumulated in its cells [7, 15]. The effect of thiosulfate on the growth processes was determined for different regimes of aeration. With 1 g/l of thiosulfate in the growth medium, no cell lysis was noted under aerobic conditions and the cell yield increased threefold over the controls grown in a thiosulfate-free medium (data not shown). As shown by further assays, the biomass increase observed in these experiments was not due to thiosulfate utilization in the energy metabolism, and the absence of H_2O_2 accumulation in cells could be explained by its chemical interaction with thiosulfate (unpublished data).

Table 4. Activities of enzymes responsible for the synthesis of PEP and carbohydrates in *S. winogradskii* grown under different oxygen regimes (nmol/(min mg protein))

Enzymes	Cultivation conditions		
	aerobic (A)	microaerobic (B)	A/B
PEP synthase	400.0	300.0	1.3
PEP carboxykinase	14.4	12.8	1.1
PEP carboxylase	30.2	21.3	1.4
Pyruvate kinase	28.0	37.0	0.76
Fructose-bis-phosphatase	48.9	11.0	4.4
Phosphoglucoisomerase	48.9	13.0	3.7
Phosphoglucomutase	31.1	14.0	2.2

Activities of the Constructive Metabolism Enzymes

As seen from Table 3, virtually no synthesis of extracellular polysaccharides proceeds in microaerobically grown *S. winogradskii*. Although similar quantities of total protein (protein of intact and lysed cells) are synthesized in cultures grown under aerobic and microaerobic conditions, the yield coefficient in the second case is 1.6–2 times higher. To identify the reasons for the reduction in the efficiency of substrate utilization during aerobic growth, the activities of enzymes participating, both directly and indirectly, in the processes of cellular protein biosynthesis were determined.

It follows from Table 5 that the activities of different enzymes of the TCA cycle showed a different dependence on the medium aeration regime. During aerobic growth, the activities of all enzymes of the TCA cycle were lower than during microaerobic growth. The most marked (two- to fivefold) decline in activity was exhibited by enzymes involved in the supply of reducing equivalents (malic-enzyme) and amino acids (glutamate dehydrogenase and aminotransferases) for a constructive metabolism.

The data on the effect of H_2O_2 on different enzymes of the TCA cycle are presented in Table 6. The addition of H_2O_2 to the supernatant of cell homogenate in physiological concentrations (1 mg/l) was found to cause a notable activity reduction of NADH-dependent malate dehydrogenase. The enzyme coming from microaero-

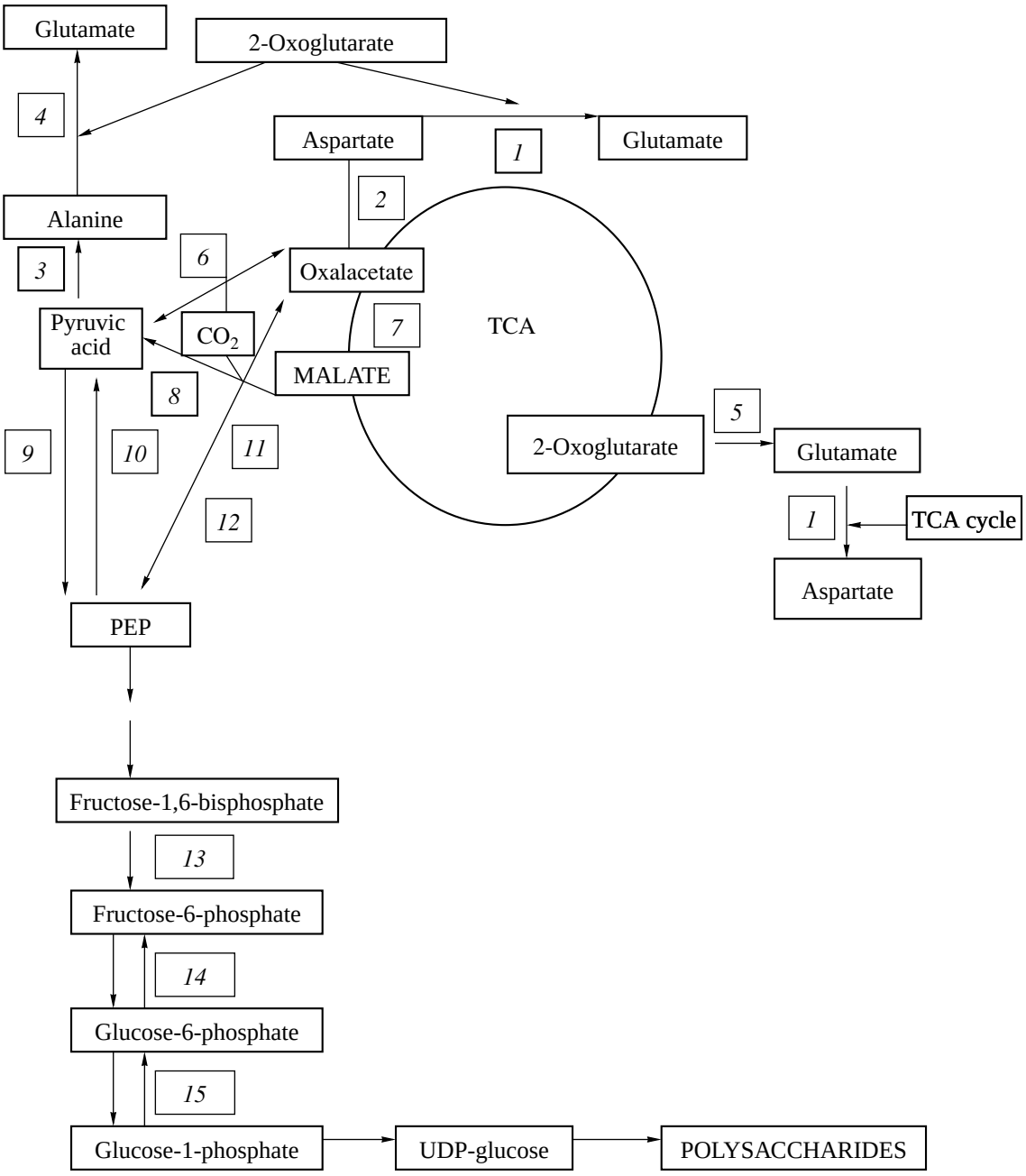

Fig. 3. Major pathways of gluconeogenesis and constructive metabolism in *S. winogradskii*. The enzymes the activities of which were determined are designated by numbers as follows: *1*, aspartate aminotransferase; *2*, aspartate dehydrogenase; *3*, alanine dehydrogenase; *4*, alanine aminotransferase; *5*, glutamate dehydrogenase; *6*, pyruvate carboxylase; *7*, NADP-dependent MDH; *8*, NADP-dependent malic enzyme; *9*, phosphoenolpyruvate synthase; *10*, pyruvate kinase; *11*, phosphoenolpyruvate carboxykinase; *12*, phosphoenolpyruvate carboxylase; *13*, fructose-bis-phosphatase; *14*, phosphoglucoisomerase; and *15*, phosphoglucomutase.

bically grown cells proved to be particularly sensitive to the presence of H_2O_2. At the same time, the activity of NADP-dependent isocitrate dehydrogenase was not affected by the presence of H_2O_2. These facts can apparently be explained by the presence in the active center of malate dehydrogenase of an oxidant-sensitive SH group, which does not occur in the other enzyme studied. Different sensitivities to toxic forms of O_2 not only cause inhibition of growth processes but also inevitably disrupt the coordinated operation of the TCA cycle and the ETC and interfere with processes of constructive and energy metabolism.

DISCUSSION

The results of our investigation into the effect of the oxygen regime of cultivation on the biosynthetic processes and the activities of several enzymatic antioxi-

Table 5. Effect of oxygen content of the growth medium on the activities of the TCA cycle enzymes implicated in constructive metabolism (nmol/(min mg protein))

Enzymes	Cultivation conditions		
	Aerobic (A)	Microaerobic (B)	B/A
MDH (NADP-dependent)	80.6	120.8	1.5
ME (NADP-dependent)	32.2	153.0	4.8
MDH (NADH-dependent)	17.8	26.8	1.5
Pyruvate kinase	27.8	36.8	1.3
Pyruvate carboxylase	11.4	28.8	2.5
Glutamate dehydrogenase (NADP-dependent)	13.8	67.2	4.9
Aspartate dehydrogenase (NADP-dependent)	7.7	27.2	3.5
Alanine dehydrogenase (NADP-dependent)	6.9	8.6	1.2
Aspartate aminotransferase	28.0	79.0	2.8
Alanine aminotransferas	17.0	45.0	2.6

Table 6. Effect of hydrogen peroxide on the activities of some enzymes of the TCA cycle in *S. winogradskii* (nmol/(min mg protein))

Enzyme	Supernatant		Supernatant + H_2O_2		A/C	B/D
	A	B	C	D		
Malate dehydrogenase (NADH)	14.6	22.3	7.2	4.2	2.0	5.3
Isocitrate dehydrogenase (NADP)	80.6	80.6	67.8	74.5	1.2	1.07

Note: Enzymatic activities were determined in the supernatant of the homogenate of cells grown under aerobic (A and C) and microaerobic (B and D) conditions. The concentration of H_2O_2 in the supernatant was 2 μg/ml.

dant defense systems in *S. winogradskii* show that the growth with readily available oxygen gives rise to accumulation of significant amounts of intracellular H_2O_2. Our assays show that, despite a marked rise in activity of the glutathione redox system and peroxidase, the concentration of H_2O_2 in cells of a two-day-old culture can reach 10 μg per mg protein. In *S. winogradskii*, H_2O_2 formed in the ETC during respiration accumulates mostly in the periplasmic space, as earlier shown to be the case with several microaerophilic bacteria [14, 15]. In the absence in cells of *S. winogradskii* of an effective means to remove H_2O_2, this process under aerobic growth conditions leads to cell lysis, starting in the midexponential phase, and, eventually, to culture death in the stationary phase of growth.

Studies of the known enzymatic cell defenses against reactive oxygen species, and first and foremost H_2O_2, show that, when active catalase is absent, other antioxidant enzymes situated in the cytoplasm can play a major role in the decomposition of H_2O_2. These enzymes are represented by peroxidase systems, including glutathione peroxidase, an enzyme of the glutathione redox system. Indeed, under aerobic conditions, the activities of peroxidase and glutathione peroxidase in cells increased three- to fivefold as compared to microaerobic growth. The increased activities of these enzymes are unable to completely counterbalance the toxic action of H_2O_2, and the lysis of cells takes place not only under aerobic but also under microaerobic conditions. However, this increased activity during aerobic growth may have a positive effect on the state of other enzymes and cell components. For example, glutathione peroxidase is known to protect other enzymes, e.g., glutamine synthase, from inactivation under oxidative stress [16].

The role played by cytochrome *c* peroxidase, a periplasmic enzyme, is not yet well understood. In addition to participating in the ETC reactions [15], this enzyme, in several microorganisms, has a most important function in eliminating H_2O_2 that accumulates in the periplasm and is not subject to the activities of cytoplasmic enzymes [10].

In our view, the lysis of cells in *S. winogradskii* during aerobic growth in the absence of cytochrome *c* peroxidase takes place because H_2O_2 accumulates predominantly in the periplasm and, to some extent, outside cells [15, 16], while the enzymatic antioxidant defenses are localized in the cell cytoplasm.

During growth in the presence of readily available oxygen, cells of spirilla experience substantial rearrangement of their metabolism. Specifically, the enzymes implicated in gluconeogenesis are activated, and the synthesis of mucous capsules and exopolysaccharides is stepped up as a means to protect cells from surplus oxygen by creating a physical and chemical barrier. The measured activities of enzymes participating in gluconeogenesis are in agreement with quantitative estimates of synthesized extracellular polysaccharides. An increase in gluconeogenesis under aerobic conditions causes a notable increase in the share of utilized substrate spent on biosynthetic activities. The production of extracellular polysaccharides is known to be an energy intensive process [17]. At the same time, bacterial growth under microaerobic conditions, with reduced gluconeogenesis, and therefore, reduced synthesis of exopolysaccharides, is accompanied by the increased activities of enzymes implicated in constructive metabolism. This point is demonstrated by a marked increase in the activity of pyruvate kinase, converting PEP into pyruvic acid, which is then used in the cell's constructive metabolism. By contrast, under aerobic conditions, an increase is observed in the activities of a number of enzymes contributing to the synthesis of PEP as the initial substrate for gluconeogenesis (Fig. 3). The change in enzymatic activities under microaerobic

conditions and the reduction of total expenditures on assimilatory purposes as compared to aerobic growth give rise to a twofold increase in the yield coefficient, which is a measure of the efficiency of substrate utilization.

Several exogenous chemical compounds of different origin and cell metabolites may participate in cell defenses against reactive forms of oxygen. Among reduced sulfur compounds, these are dithiothreitol, metabisulfite [18], sulfide, and thiosulfate [19]. Our study of *S. winogradskii* indicates that thiosulfate is one of the most efficient antioxidant protectants. The oxidation of thiosulfate to tetrathionate in spirilla cell suspensions does not involve additional expenditure of oxygen and proceeds without the participation of enzymes specific to sulfur metabolism. It is not connected, therefore, with lithoheterotrophy and is a means of removal of toxic O_2 products.

Comparing the results of our study of *S. winogradskii* with the data available for other microaerophilic microorganisms, it can be concluded that major components of antioxidant defenses may differ significantly in representatives of different groups of microorganisms. The evidence available in the literature [19] also suggests that, in many microaerobic bacteria developing organotrophically in sulfide-containing aquatic environments with an unstable oxygen regime, reactive forms of oxygen, as in *S. winogradskii*, are eliminated predominantly by means of nonenzymatic reactions involving reduced inorganic sulfur compounds present in the environment. This fact is largely responsible for the affinity of these organisms to such biotopes.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project nos. 02-04-18196, 02-04-49185, and 02-04-063369, and by the federal program integration, project no. 30282/1553.

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