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EXPERIMENTAL ARTICLES

The Role of Oxygen in the Regulation of the Metabolism of Aerotolerant Spirochetes, a Major Component of *"Thiodendron"* Bacterial Sulfur Mats

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Abstract—Two spirochete strains isolated earlier from *"Thiodendron"* bacterial sulfur mats grew better under microaerobic (0.3–0.5 mg O_2/l) than under anaerobic conditions. The microaerobic growth of these strains was accompanied by a twofold increase in the cell yield and the efficiency of glucose utilization, despite the fact that an additional amount of ATP (and, hence, glucose) was spent in this case for the synthesis of exopolysaccharides. Glucose metabolism under microaerobic conditions gave rise to more oxidized products (acetate and carbon dioxide) than under anaerobic conditions (formate, ethanol, pyruvate, and hydrogen). The paper considers two putative mechanisms implemented by aerotolerant spirochetes: adaptive (the use of a more efficient pathway of glucose catabolism) and protective (an enhanced synthesis of exopolysaccharides and the reduction of hydrogen peroxide by the reduced sulfur compounds thiosulfate and sulfide, yielding elemental sulfur). The formation of *"Thiodendron"* bacterial sulfur mats in saltwater environments is also discussed.

Key words: aerotolerant spirochetes, *"Thiodendron,"* metabolism regulation by oxygen, enzymes and products of glucose metabolism, hydrogen peroxide, hydrogen sulfide oxidation, bacterial sulfur mats.

"Thiodendron" bacterial sulfur mats, which were first found in mineral sulfide springs [1], are also widespread in marine and oceanic environments. These mats occupy vast areas of bottom sediments in high-productivity regions with an active inflow of biogenic or volcanic hydrogen sulfide and in regions of high hydrothermal activity [2]. Fermentative anaerobic spirochetes, which accumulate elemental sulfur intracellularly, are the major component of sulfur mats [3]. Unlike other known sulfurettes, which are mainly formed by chemoautotrophic sulfur-oxidizing bacteria (such as *Beggiatoa* and *Thioploca*), *"Thiodendron"* bacterial sulfur mats are chiefly made up of heterotrophic microorganisms [3, 4]. These mats occur on the surface of bottom sediments, for instance, in the sublittoral zone of marine ecosystems, where the concentration of dissolved oxygen varies greatly. Earlier, we observed the de novo formation of *"Thiodendron"* bacterial sulfur mats in the littoral and sublittoral zones of the White Sea during falling and rising tides, when the concentration of dissolved oxygen near the mats varied from 0.01 to 7 mg/l [2]. The reason for the preferential development of anaerobic spirochetes under oxidative conditions remained unknown.

This paper deals with the study of the effect of oxygen on the growth and carbon metabolism of two spirochete strains isolated earlier from two different sulfur mats. The adaptive and protective mechanisms implemented by these anaerobic spirochetes under microaerobic conditions and the functional role of reduced sulfur compounds in their metabolism are also discussed.

MATERIALS AND METHODS

Strains and cultivation conditions. The spirochete strains P and BM used in this study were isolated, respectively, from the *"Thiodendron"* bacterial sulfur mat of a saline mineral spring at the resort Staraya Russa (Novgorod oblast) and from such a mat in the sublittoral zone of the Gulf of Kandalaksha of the White Sea (Murmansk oblast) [3]. The strains are stored in DSMZ and the culture collection at the Institute of Microbiology.

The strains were cultivated in a medium containing (g/l) NaCl, 20.0; NH_4Cl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $MgCl_2 \cdot 6H_2O$, 3.0; glucose, 1.0; peptone, 1.0; Difco yeast extract, 0.5; and phosphates, 0.5 (pH 7.6). The medium was supplemented with vitamins, trace elements [3], and rifampicin (10 mg/l). Immediately before inoculation, the medium was boiled and supplemented with a 10% solution of $Na_2S \cdot 9H_2O$ (0.5 ml/l). In some experiments, the medium was reduced by adding Ti(III) citrate at a concentration of 50 mM.

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Cultivation was performed in 250-ml flasks containing 25 ml of the medium and sealed with rubber stoppers. In the case of anaerobic cultivation, the flasks were filled with nitrogen after inoculation. Microaerobic conditions were produced by admitting air into the flasks to give different oxygen concentrations in the gas phase (0.5, 1.4, 3, 5, or 10%). In the flasks that initially contained little oxygen, its concentration was measured by gas chromatography and, when required, was adjusted to the initial level. When the gas phase contained 0.5 or 1.4% oxygen, the concentration of dissolved oxygen in the sulfide-containing liquid phase was determined by the Winkler method [5]. Actually, the optimal concentration of oxygen in the gas phase turned out to be 1.4%, which corresponded to a concentration of dissolved oxygen in the liquid phase equal to 0.4–0.5 mg O_2/l.

Preparation of cell suspensions and cell extracts. Cells were harvested by centrifugation at 5000 *g* for 30 min, washed twice with 0.1 M phosphate buffer (pH 7.5) containing 2% NaCl and 50 μM sodium sulfide, and suspended in this buffer under the same gas phase that was used for cell cultivation. Then cells were disrupted by sonication with the aid of a UZDN-2T ultrasonic disintegrator (22 kHz, 3 min, 0°C). The cell homogenate was centrifuged at 9000 *g* for 30 min at 4°C, and the supernatant was used as a cell-free extract.

Physiological, biochemical, and analytical methods. The cell yield was determined by measuring the concentration of protein in exponential-phase (2 days of growth) cultures by the Lowry method. The concentration of glucose in the culture liquid was measured with phenol [6]. In this case, cells were preliminarily removed by centrifugation at 9000 *g* [6]. The amount of exopolysaccharides in their precipitate was also determined by the phenol method. The precipitate was preliminarily washed twice with the buffer.

The products of glucose metabolism (ethanol and acetate) were analyzed by gas chromatography on a Chrom-5 chromatograph (Czech Republic). The sample volume was 5 μl. The injector was kept at 200°C. The carrier gas was argon at a flow rate of 40 ml/min. Other gases were determined by using an LKhM-80 chromatograph with a thermal conductivity detector and argon as the carrier gas at a flow rate of 40 ml/min. The electric current through the detector was 30 mA. The column was kept at room temperature. Formate, pyruvate, and hydrogen sulfide were assayed spectrophotometrically at 515 nm [7], 505 nm [8], and 670 nm [9], respectively.

The production of hydrogen peroxide by the washed exponential-phase cells was determined in 50 mM phosphate buffer (pH 7.4) with luminol [10]. Catalase (2 μg/ml) and thiosulfate (1 mg/ml) were added to the medium immediately before measurements. The luminescence of luminol was measured with the aid of an LB-3PA luminometer (Klimbi, Russia).

The production of ATP in the cell suspensions was measured by the luciferin–luciferase method with the same luminometer [11].

The concentration of cytochromes in cell-free extracts was measured from the redox difference absorption spectra of dithionite-reduced minus air-oxidized preparations, which were recorded with a Pye-Unicam SP 1800 spectrophotometer (United Kingdom).

The activities of NADH oxidase, NADH peroxidase (EC 1.11.1.1), and acetate kinase (EC 2.7.2.1) were determined as described by Niimura *et al.* [12]. Alcohol dehydrogenase (EC 1.1.1.1) was assayed by the method of Schater and Schonheit [13]. The activities of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), hexokinase (EC 2.7.1.1), glucose-6-phosphate isomerase (EC 5.3.1.9), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), and phosphoglucomutase (EC 2.7.5.1) were measured at 340 nm [14]. Phosphate acetyltransferase (EC 2.3.1.8) and hydrogenase were assayed at 233 and 600 nm, respectively [15].

All the experiments were performed no fewer than two times. The results presented in the paper are the means of three independent measurements.

The morphology of spirochete cells was studied by using an NU-2 phase-contrast microscope (Karl Zeiss, Germany). Elemental sulfur inclusions in cells were observed with a polarization microscope.

RESULTS

The effect of oxygen on the growth of the spirochetes was studied under anaerobic and microaerobic (at different concentrations of oxygen in the gas phase) conditions without adding the reducing agents sodium sulfide or Ti(III) citrate to the medium. These experiments showed that neither of the two spirochetes could grow in the absence of the reducing agents, even if the gas phase was pure nitrogen. The addition of Ti(III) citrate to the medium induced the growth of the spirochetes under anaerobic but not under microaerobic (0.5 and 1.4% oxygen) conditions. Sodium sulfide stimulated the growth of the spirochetes when the concentration of oxygen in the gas phase varied from 0.2 to 1.4%, the accumulated biomass ranging from 27 to 41 mg protein/l. When the concentration of oxygen was raised to 2%, growth was very poor (6–8 mg protein/l) and the cultures contained a great number of rapidly lysing spheroplasts, which underwent complete lysis within 2 days. The maximum biomass (36–43 mg protein/l) was observed when the gas phase contained 1.4% oxygen, which corresponded to a concentration of dissolved oxygen in the liquid phase equal to 0.4–0.5 mg/l. In this case, the spirochete cells grew as long straight nonmotile filaments (sometimes with elemental sulfur inclusions), which formed a slimy fouling on the flask bottom. When the gas phase contained 0.5% oxygen, the spirochete cells were spiral and motile, like those grown anaerobically.

Table 1. The effect of oxygen on the cell yield of spirochetes, the synthesis of exopolysaccharides, and the efficiency of glucose utilization

Cultivation conditions	Strain	Cultivation time, h	Glucose consumption, mmol/l	Cell protein, mg/l	EPS		Cell yield, mg protein/mmol glucose
					mg/l	C_{EPS}/mg	
Microaerobic	P	24	1.1	20.3	ND	ND	18.4
Anaerobic	P	24	1.0	9.4	0	0	9.4
		48	1.7	15.0	0	0	8.3
Microaerobic	BM	48	2.0	29.8	53.2	15.2	14.9 (17.5)*
Anaerobic	BM	48	1.96	14.5	0	0	7.4

Note: ND stands for "not determined." The asterisk * marks the cell yield calculated with allowance for the consumption of glucose for the synthesis of EPSs.

Further experiments were carried out with spirochetes grown either anaerobically in the nitrogen atmosphere or microaerobically in the presence of 1.4% oxygen in the gas phase. These experiments showed that the spirochetes were tolerant to oxygen only if the medium contained sodium sulfide, even in small amounts. As for cytochromes, we failed to detect them in spirochete cells grown under any of the cultivation conditions (anaerobic or microaerobic). This indicates that the spirochetes do not contain the electron transport chain and are incapable of oxidative phosphorylation.

Table 1 shows the effect of oxygen on the growth of the spirochetes, the growth efficiency, and the synthesis of exopolysaccharides (EPSs). The growth efficiency with respect to the substrate consumed was twofold higher under microaerobic than under anaerobic conditions. The microscopic examination of the spirochete cells grown microaerobically showed that they were embedded in a slimy EPS matrix (Fig. 1). The proportion between the carbon contents of EPS and cells varied from 1.5 to 3. With allowance made for this circumstance, the efficiency of substrate utilization by the spirochetes should be increased by 18–25%.

The effect of oxygen on the glucose metabolism of the spirochetes was studied by measuring the end products (Fig. 2) and by assaying the enzymes of carbon metabolism. Under anaerobic conditions, the culture liquid contained formate, ethanol, hydrogen, pyruvate, acetate, and carbon dioxide, the first two products being dominant. Under microaerobic conditions, the yield of the oxidized products acetate and carbon dioxide increased by 3–5 times, whereas the production of formate, ethanol, and hydrogen was very low if it occurred at all. Moreover, the possibility cannot be excluded that these reduced products were produced under local anaerobic conditions. The specific yield of the major products acetate and CO_2 per millimole of glucose consumed was twofold higher under microaerobic than under anaerobic conditions.

The effect of oxygen on the enzyme activity of the spirochetes. As is evident from the results presented in Table 2, the spirochete cells did not contain glucose-6-phosphate dehydrogenase (the key enzyme of the oxidative pentose phosphate and the Entner–Doudoroff pathways) but did contain the major glycolytic enzymes, irrespective of the growth conditions.

The presence of oxygen substantially enhanced the activity of the enzymes involved in the oxidative conversion of glucose to acetate (Table 2; Fig. 3) but suppressed the enzymatic conversion of pyruvate to relatively reduced products (this is evident from the low activity of alcohol dehydrogenase and other enzymes involved in the anaerobic metabolism of glucose). Noteworthy is the high activity of NADH oxidase and NADH peroxidase, which are involved in the processes of NAD^+ regeneration and hydrogen peroxide conversion.

The results of the enzymatic assay agree well with the results of determination of the end products of glucose metabolism. The relevant differences between the two spirochete strains studied were insignificant.

It should be noted that the activity of phosphoglucomutase, which is involved in EPS synthesis, was higher in the cells grown microaerobically. This is in agreement with the high production of EPSs by the spirochete cells grown under microaerobic conditions.

The effect of oxygen on ATP formation. The specific rate of ATP formation in the suspension of the spirochete cells grown microaerobically was twofold higher than in the case of the anaerobic cells (Table 3). The addition of arsenate (an uncoupler of oxidative phosphorylation) suppressed the formation of ATP by both types of spirochete cells.

The effect of oxygen on the production of H_2O_2 and the oxidation of sulfur compounds. As was mentioned above, the activity of NADH oxidase in the spirochete cells grown microaerobically is higher than in the anaerobic cells (Table 2). Consequently, the microaerobically grown spirochete cells must produce hydrogen peroxide in greater amounts. Measurements showed that the spirochete cells grown anaerobically did not produce H_2O_2, whereas the microaerobically grown cells produced it at a rate of up to 5 μg/(min mg protein). The addition of catalase or thiosulfate to the incubation medium completely suppressed the formation of hydro-

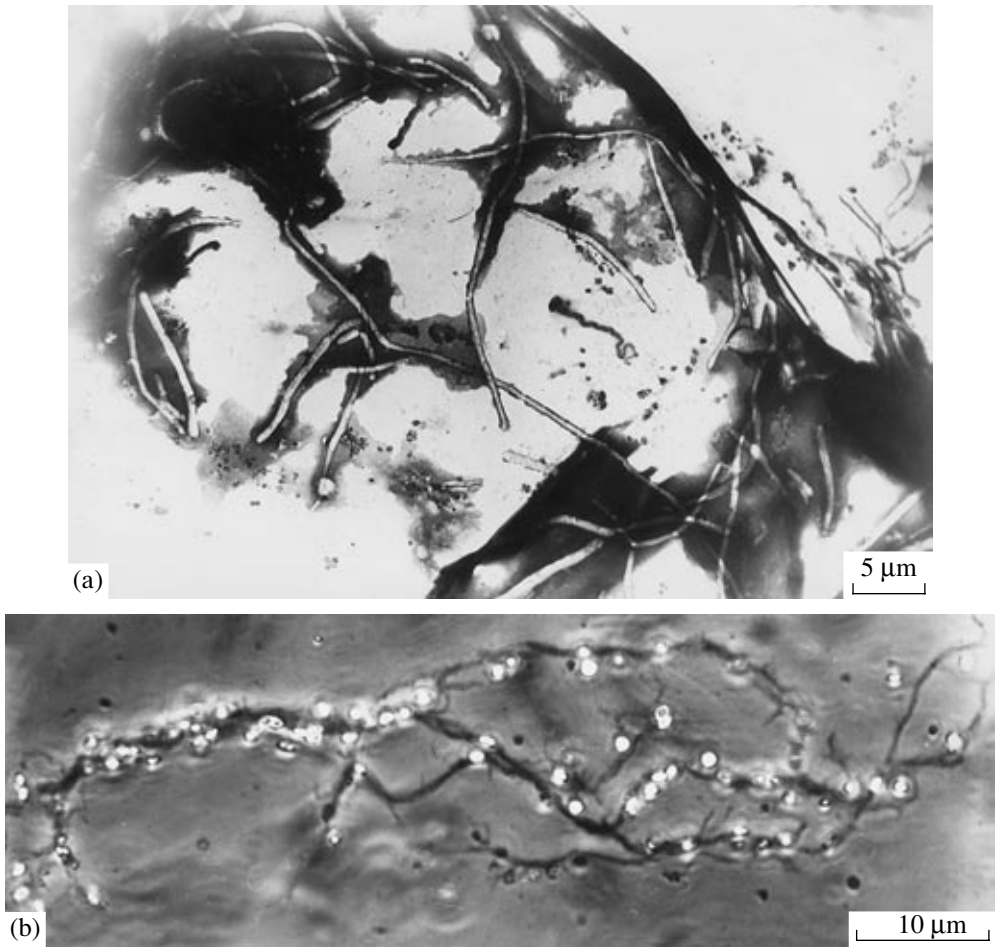

Fig. 1. Cells of the spirochete strain P grown under microaerobic conditions. (a) An electron microscopic image of spirochete filaments in the EPS matrix stained with ruthenium red. (b) A phase-contrast microscopic image of elemental sulfur granules occurring in spirochete cells.

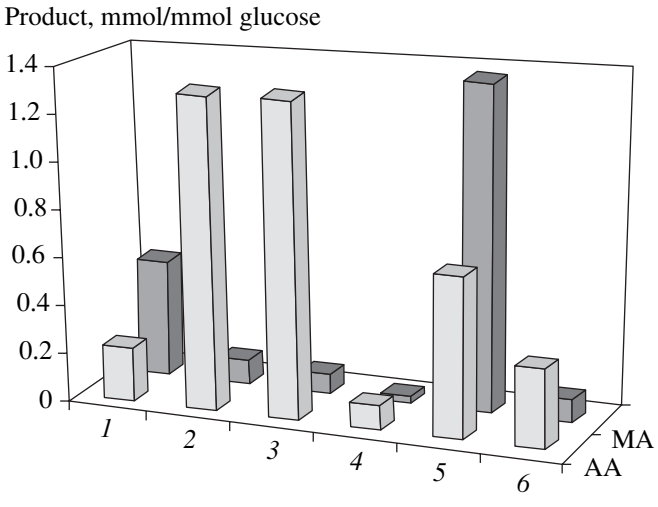

Fig. 2. The relative production of (*1*) acetate, (*2*) formate, (*3*) ethanol, (*4*) pyruvate, (*5*) carbon dioxide, and (*6*) hydrogen in spirochete strain P cells grown under anaerobic (AA) and microaerobic (MA) conditions.

gen peroxide. Consequently, like catalase, thiosulfate may remove H_2O_2 and thus prevent a cytotoxic effect of this compound. The addition of $HgNO_3$ to the spirochete culture incubated microaerobically in the presence of thiosulfate led to the formation of a precipitate composed of small yellow crystals of $Hg_2S_2O_4$, which is the product of thiosulfate oxidation by hydrogen peroxide. On the other hand, the addition of sodium sulfide to the cultivation medium of the spirochetes led to the formation of elemental sulfur inclusions in the spirochete cells (Fig. 1b). It was difficult to estimate the intensity of this process since the redox state of the cultivation medium was unstable.

DISCUSSION

All species of the genus *Spirochaeta* can ferment carbohydrates anaerobically by the glycolytic Embden–Meyerhof–Parnas pathway, the major end products being ethanol; acetate; hydrogen; carbon dioxide; and, in some species, formate or lactate [15, 16]. Two facultatively anaerobic spirochete species were found

Table 2. The effect of cultivation conditions on the activity of enzymes involved in the glucose metabolism of the two spirochete strains

Enzyme	Strain					
	P			BM		
	AA	MA	MA/AA, %	AA	MA	MA/AA, %
Hexokinase	64	64	100	33	33	100
Pyruvate kinase	13	13	100	33	33	100
Glucose-6-phosphate dehydrogenase	0	0	ND	0	0	ND
Glucose-6-phosphate isomerase	48	48	100	82	82	100
Fructose-1,6-bisphosphate aldolase	48	48	100	10	10	100
Phosphate acetyltransferase	1064	605	57	2545	2195	86
Acetate kinase	160	305	191	57	155	272
Alcohol dehydrogenase	50	15	30	136	40	29
Lactate dehydrogenase	ND	ND	ND	126	70	56
NADH oxidase	12	65	542	10	53	530
NADH peroxidase	27	59	219	17	80	267
Hydrogenase	46	18	39	ND	ND	ND
Phosphoglucomutase	30	100	333	30	180	600
Pyruvate dehydrogenase	129	ND	ND	ND	ND	ND

Note: Enzymatic activities are expressed in nmol/(min mg protein). AA, MA, and ND stand for "anaerobic," "microaerobic," and "not determined," respectively.

Table 3. The effect of cultivation conditions on the ATP formation rate (nmol ATP/(min mg protein)) in a cell suspension of the spirochete strain P

Experimental variant	Anaerobic conditions (AA)		Microaerobic conditions (MA)		MA/AA
	ATP	%	ATP	%	
Cell suspension	0.62	100	1.14	100	1.8
Cell suspension + glucose	0.92	148	2.02	177	2.2
Cell suspension + arsenate	0.07	11	0.09	8	ND
Cell suspension + glucose + arsenate	0.10	16	0.18	16	ND

Note: Glucose and arsenate were added to the medium at concentrations of 1 mg/ml and 10^{-7} M, respectively. The data are the means of three independent measurements. The confidence interval is 95%.

Table 4. The effect of cultivation conditions on the rate of hydrogen peroxide formation and the oxidation of thiosulfate in a cell suspension of the spirochete strain P

Cultivation conditions	H_2O_2, μg/(min mg protein)			$S/S_2O_3^{2-}$, μg/(min mg protein)
	Experimental variant			
	without additives	with glucose	with glucose and catalase	with glucose and thiosulfate
Microaerobic	2.8	3.9	0	ND
	3.7	4.9	0	6.5
Anaerobic	0	0	0	0

Note: Glucose, catalase, and thiosulfate were added to the medium at concentrations of 1 mg/ml, 1 μg/ml, and 4 mg/l, respectively. ND stands for "not determined."

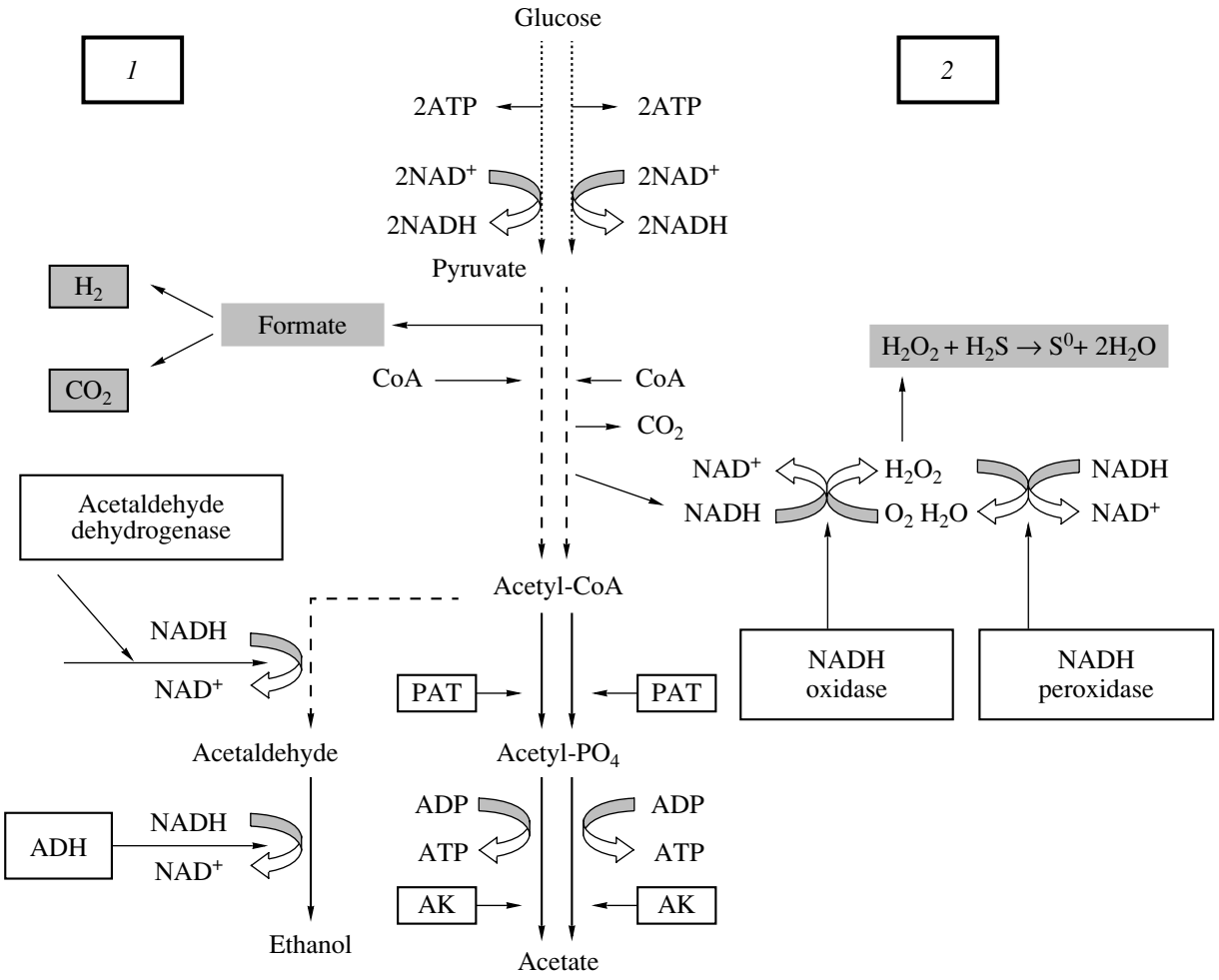

Fig. 3. A putative scheme showing the metabolism of glucose in the aerotolerant spirochete strains P and BM grown under anaerobic (*1*) and microaerobic (*2*) conditions. The solid, dotted, and dashed arrows show, respectively, the enzymatic reactions that were assayed completely, partially, and not at all. The end fermentation products are given in frames. AK is acetate kinase; PAT is phosphate acetyltransferase; and ADH is alcohol dehydrogenase.

to be able to grow aerobically by oxidizing glucose to pyruvate and the products of its oxidative decarboxylation, acetate and CO_2. In these spirochetes, ATP was produced due to oxidative phosphorylation driven by a reduced electron transport chain that contained only cytochromes *b* and *o* [16].

The spirochete strains P and BM under study do not contain cytochromes. The analysis of the end fermentation products of these strains and relevant enzymatic activities showed that they implement different biochemical pathways of glucose metabolism under anaerobic and microaerobic cultivation conditions (Fig. 3).

Under anaerobic conditions, the spirochetes metabolize glucose by a pathway similar to that of other representatives of this genus (Fig. 3, left). In this case, the major fermentation products are relatively reduced compounds (ethanol, formate, hydrogen, and pyruvate). The accumulation of the central intermediate of glycolysis, pyruvate, is likely to be due to the inhibitory action of hydrogen on pyruvate oxidation. The production of hydrogen and CO_2 in equimolar amounts suggests that they are produced from formate with the involvement of pyruvate dehydrogenase. The regeneration of NAD^+, which is necessary for the normal functioning of glycolysis, is provided by two reductive reactions at the final steps of ethanol formation.

Aerobic cultivation conditions provide for a balanced and almost complete utilization of pyruvate. The activation of pyruvate metabolism, as well as the involvement of oxygen in oxidative reactions other than oxidative phosphorylation, provides an advantage over the anaerobic metabolism of aerotolerant strains. The regeneration of NAD^+ necessary for glycolysis is provided by two enzymes, NADH oxidase and NADH peroxidase. NADH is produced not only by glycolysis but also by the pyruvate decarboxylation reaction (pyruvate $\longrightarrow$ acetyl-CoA + NADH + CO_2). The activation of acetate kinase, which catalyzes the production of ATP and acetate at the final stages of fermentation, enhances the

Fig. 4. *"Thiodendron"* bacterial sulfur mats against a background of gray laminaria mats at a depth of 17 m in the region of hydrothermal vents in Kraternaya Bay, Yankich Island, the Kuril Islands, the Sea of Japan. Submarine picture taken by V.G. Tarasov.

energy efficiency of glucose catabolism in the aerotolerant spirochetes (Fig. 2).

Thus, the microaerobic utilization of glucose has three advantages: (1) the enhanced metabolism of pyruvate and, hence, glucose; (2) the enhanced regeneration of NAD^+, necessary for glycolysis; and (3) the activation of the enzymatic production of acetate. In the final analysis, this augments the efficiencies of substrate utilization, ATP formation, and cell growth as a whole.

A disadvantage of the aerobic metabolism of aerotolerant spirochetes is the excessive accumulation of hydrogen peroxide because of unbalanced processes of its formation and decomposition (in particular, aerotolerant spirochetes have a higher activity of the H_2O_2-generating enzyme NADH oxidase as compared to the H_2O_2-decomposing enzyme NADH peroxidase). Similar mechanisms of metabolic activation were revealed in some other aerotolerant bacteria [12, 17].

The extensive production of hydrogen peroxide explains the necessity of adding reducing agents, such as thiosulfate or sodium sulfide, to the artificial cultivation media of aerotolerant spirochetes, as well as their restriction in nature to bodies of water with an intense intake of hydrogen sulfide. Moreover, the close association of spirochetes with sulfidogenic bacteria of the genera *Desulfobacter* and *Dethiosulfovibrio* in *"Thiodendron"* bacterial sulfur mats [2, 3, 18] favors the development of the spirochetes in the bottom water, with rapidly varying oxic conditions.

The detoxification of hydrogen peroxide with hydrogen sulfide is accompanied by the accumulation of elemental sulfur in cells. In marine environments, this process may occur on a global scale (Fig. 4), as is evident from the high rates of sulfide oxidation and organic matter degradation in *"Thiodendron"* bacterial sulfur mats [2].

The stressful effect of oxygen on aerotolerant spirochetes is also mitigated by another adaptive mechanism, namely, the synthesis of EPSs, which are an important component of the dense slimy matrix of *"Thiodendron"* bacterial sulfur mats. It is known that one of the functions of EPSs is the regulation of the diffusion rate of oxygen to the cell surface [19]. This phenomenon may be responsible for the wide distribution of *"Thiodendron"* bacterial sulfur mats in natural habitats with diverse oxygen concentrations. Due to the presence of the dense EPS matrix, the actual concentrations of oxygen near the cell surface of the component spirochetes may be considerably lower than near the surface of a *"Thiodendron"* bacterial sulfur mat. In other words, EPS synthesis may represent an important adaptive mechanism that helps bacteria to counteract the impact of environmental oxygen.

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