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EXPERIMENTAL ARTICLES

Intracellular Polysaccharide of an Anaerobic Psychrophile *Clostridium algoriphilum*

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Abstract—Polysaccharide was revealed in the cytoplasm of spore-forming *Clostridium algoriphilum* 14D1 = VKM B-2271 = DSM 16153 grown on glucose at 5°C. This polymer was isolated, purified, and identified as a glycogen-like compound based on analysis of its hydrolysis products. The ratio of the polysaccharide to the dry biomass weight did not change in the course of culture growth. Limitation of *C. algoriphilum* 14D1 growth by nitrogen resulted in a doubling of the polysaccharide/dry biomass ratio. The transition of *C. algoriphilum* 14D1 cells from optimal conditions into carbon-free medium resulted in utilization of intracellular polysaccharide. The amount of polysaccharide was shown to depend on glucose concentration, type of the carbon substrate, and growth temperature. Future investigations of polysaccharide functions in *C. algoriphilum* 14D1 cells are discussed.

Keywords: extreme conditions, cryopeg, survival, *Clostridium algoriphilum* 14D1, intracellular polymers, glycogen-like compound, polysaccharide hydrolysis.

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Arctic cryopegs are nonfrozen soils and lenses of free water that occur within permafrost and are characterized by constant subzero temperatures [1]. They are of interest as models of the Solar System's planets of the cryogenic type. The study of microorganisms inhabiting cryopegs is aimed at understanding the survival strategy under extreme conditions in the geological past. These microorganisms are of interest as possible components of artificial communities capable of pollutant destruction in a frigid climate. Moreover, such microorganisms can be used as sources of the low-temperature-active enzymes for food industry, wastewater treatment, and molecular biology [2].

Microorganisms belonging to all three domains (*Bacteria, Archaea*, and *Eukarya*) were found in cryopegs. The cold-adapted microbial communities contained viable aerobic and anaerobic heterotrophic bacteria, sulfate reducers, acetogens, and methanogenic archaea [3, 4], as well as eukaryotic microorganisms, including mycelial fungi and yeasts [5].

The psychrophilic, anaerobic, spore-forming bacterium *Clostridium algoriphilum* sp. nov. was isolated from Yakutsk Lake, Kolyma Lowland [6]. This bacterium can be considered as a model organism capable of survival under simultaneous impact of subzero temperatures (below −10°C) and high salinity (170–300 g/l) [7].

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Formation of intracellular polymers (polysaccharides, lipids, and polyphosphates) is one of the most important properties that promote cell survival. These compounds can serve as sources of carbon and/or energy. Intracellular accumulation of polymers (polysaccharides and polyhydroxybutyrate) is typical of clostridia [8]. Clostridial polysaccharides are represented by glycogen- or starchlike polymers, which are composed of glucose units connected by α-(1-4) bonds and branched via α-(1-6) bonds [8]. These compounds fulfill mainly a reserve function; they can also serve as endogenous substrates for sporulation and as energy sources for cell differentiation [9]. The accumulation of intracellular polysaccharide (or granulose) in *C. acetobutylicum* was associated with the culture transition from the stage of acetate and butyrate accumulation to producing acetone, butanol, and ethanol [10]. There is information that impairment of glycogen metabolism in *Mycobacterium smegmatis* resulted in growth cessation [11]. An intracellular glycogen-like compound was involved in karyogenesis of *Streptococcus mutans* [12]. Glycogen synthesis in *Salmonella enteritidis* correlated with biofilm formation and culture virulence [13].

At present, data on polysaccharides of psychrophilic bacteria including clostridia are scarce or absent [14, 15].

The aim of this work was to study the nature and accumulation of the intracellular polymer in the psy-

chrophilic anaerobic, spore-forming bacterium *C. algoriphilum* 14D1.

MATERIALS AND METHODS

Microorganism. The study was carried out with the anaerobic bacterium *C. algoriphilum* 14D1 = VKM B-2271 = DSM 16153 obtained from the Culture Collection of the Laboratory of Anaerobic Bacteria, Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino).

Cultivation was performed in liquid medium containing the following (g/l): KH_2PO_4, 0.7; K_2HPO_4, 0.7; NH_4Cl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 5.0; sodium ascorbate, 1.0; and glucose, 2.0 in Hungate tubes or in bottles containing 10 or 80 ml of the medium, respectively, at 12, 5, 0, and −5°C.

To study the dynamics of polysaccharide accumulation in the course of the culture growth, samples were withdrawn at certain time intervals and stored at −20°C until analysis.

To study the effect of glucose concentration (0.1, 0.25, 1, 2, and 4 g/l) on the polysaccharide content, cells were grown in bottles up to the late exponential phase.

To study the effect of the carbon substrate type on the amount of intracellular polysaccharide, cells were grown in bottles in media with glucose, trehalose, or peptone (2.0 g/l each) up to the late exponential phase.

To study the effect of temperature and salinity on polysaccharide production, the late-exponential-phase cells were washed off from the medium, transferred into fresh media with glucose (2 g/l) and different NaCl concentrations (5, 50, 100, and 150 g/l), and incubated at an appropriate temperature for a month.

Optical density of the culture was measured at 600 nm on a Specol-221 spectrophotometer (Germany). The cell doubling time (t_d) was determined as the time of twofold increase in optical density. The growth rate was calculated using the formula: $\mu = \ln 2/t_d$. The biomass dry weight was determined by drying of filtered and washed cells in a vacuum at 80°C for one day.

Electron microscopic examinations were performed as described earlier [6].

To isolate the intracellular polysaccharide, the late-exponential-phase cells were collected by centrifugation, washed twice with 0.1 M sodium phosphate buffer (pH 7.0), and disrupted by using an MSE ultrasonic disintegrator (United Kingdom). Protein was removed from the obtained homogenate by addition of trichloroacetic acid to the final concentration of 1.5% with subsequent centrifugation on a K-24 centrifuge (Germany) at 10000 *g* for 20 min. The amount of polysaccharide in the supernatant was assayed by the anthrone method [16]. Pure polysaccharide was isolated from the deproteinized cell homogenate by the Stams method [17]. The polysaccharide was precipitated by adding ethanol to the final concentration of 50% and centrifuged at 10000 *g* for 20 min; the supernatant was removed, and the pellet was dried at 37°C.

Staining of the polysaccharide with Lugol's solution. Purified polysaccharide (10 mg) was dissolved in 2 ml of distilled water and hydrolyzed with amyloglucosidase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) at 37°C for 2 h. The sample (0.1 ml) was transferred into a cuvette and supplemented with 0.2 ml of Lugol's solution (0.2% iodine in 2% KI solution). The extinction of hydrolyzed and nonhydrolyzed preparations was measured at 495 nm; Lugol's solution diluted tenfold was used as control.

Hydrolysis and analysis of monomer composition. Enzymatic hydrolysis of the polysaccharide was carried out using amyloglucosidase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) and -amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2). Enzymes (1 mg/ml) were added into water solution of the polysaccharide (5 mg/ml), and the mixture was incubated at 37°C for 4 h. Acid hydrolysis was performed using 2.5 N sulfuric acid at 120°C for 30 min. Products of hydrolysis were identified by high-performance liquid chromatography (HPLC) on a Biotronic LC 2000 carbohydrate analyzer (Germany).

Analysis of elemental composition of the polysaccharide. The C, H, and N amounts were determined on a 1106 analyzer (Carlo Erba Strumentazione, Italy). Phosphates were assayed by the Novikov method [18].

Protein concentration was measured spectrophotometrically by the Bradford method [19]. Bovine serum albumin (BSA) was used as a standard.

Nucleic acid content of the pure polysaccharide preparation was determined by measuring extinction at 260 nm [19] on a UV-160 double-beam spectrophotometer (Shimadzu, Japan).

RESULTS

Electron microscopic examinations. The study of ultrathin sections of *C. algoriphilum* 14D1 cells grown at optimal or subzero temperatures revealed that the cytoplasm of the cells grown at optimal temperature (5°C) was completely filled with an electron-transparent substance, whereas, in the cells grown at −5°C, the amount of this substance was considerably lower (Fig. 1). It was assumed that accumulation of the electron-transparent substance was a growth-dependent process.

The nature of the intracellular polymer in *C. algoriphilum*. Since cell homogenates treated with Lugol's solution were dark brown in color, it was suggested that the cytoplasm contained a polysaccharide. Pure polysaccharide (134 mg) was obtained from native cells grown in a bottle with 80 ml of the medium with

the use of the Stams method [17]. It was found that the ratio of the polysaccharide (mg) to protein content (mg) within the cells was 36. In the preparation of the polymer, the level of C, H, and PO_4 was 41, 6.6, and 0.27%, respectively. Nitrogen, protein, and nucleic acids were not revealed in the polysaccharide preparation.

Identification of the polysaccharide was carried out with the use of acid and enzymatic hydrolysis with subsequent HPLC analysis of the products. Glucose was the only product of acid hydrolysis; it also reached 93% in the reaction mixture after enzymatic hydrolysis with amyloglucosidase. Only maltose was formed after polysaccharide hydrolysis with α-amylase.

Polysaccharide treated with Lugol's solution was brown-colored, indicating the formation of the iodine–glycogen complex. The ability of polysaccharide to bind iodine was lost after its hydrolysis with amyloglucosidase; the extinction of the complex was 0.489 at 495 nm. Thus, the cells of *C. algoriphilum* 14D1 grown at –5°C accumulated a glycogen-like compound composed of D-glucose residues connected mainly via α-(1-4) bonds.

Patterns of polysaccharide accumulation in *C. algoriphilum* cells. The study of polysaccharide accumulation in the course of *C. algoriphilum* growth in the medium with glucose concentration of 2 g/l (Fig. 2) revealed that the polysaccharide content in the biomass remained rather constant (25–28%).

As seen from Table 1, the polysaccharide content of the biomass increased with increasing glucose concentration in the medium. The polysaccharide amount in *C. algoriphilum* cells depended also on the type of the carbon source (Table 2). In the media with equal concentrations of glucose, trehalose, or peptone, polysaccharide accumulation was maximal on glucose (27% of dry cells) and minimal on peptone (no more than 5% of dry cells).

Under growth limitation by nitrogen (initial glucose concentration in the medium was 2 g/l), the polysaccharide content reached 50% of dry biomass. When the cells grown in the medium with 2 g/l glucose were transferred into carbon-free medium, the polysaccharide content of the biomass decreased from 27 to 17% over 7 days.

C. algoriphilum is an obligate psychrophile with the temperature optimum of 5°C [6]. Optical density of the culture grown at 12°C did not exceed 0.21 (Table 3), and polysaccharide content of the biomass was slightly lower than that at optimal temperature; at 0°C, polysaccharide accumulation increased by ~10%. At subzero temperature (–5°C), optical density reached 0.86 and the polysaccharide content of the biomass was 23%. The pattern of polysaccharide accumulation was the same at all temperatures: the ratio of the polysaccharide amount to the cell dry weight remained constant in the course of culture growth.

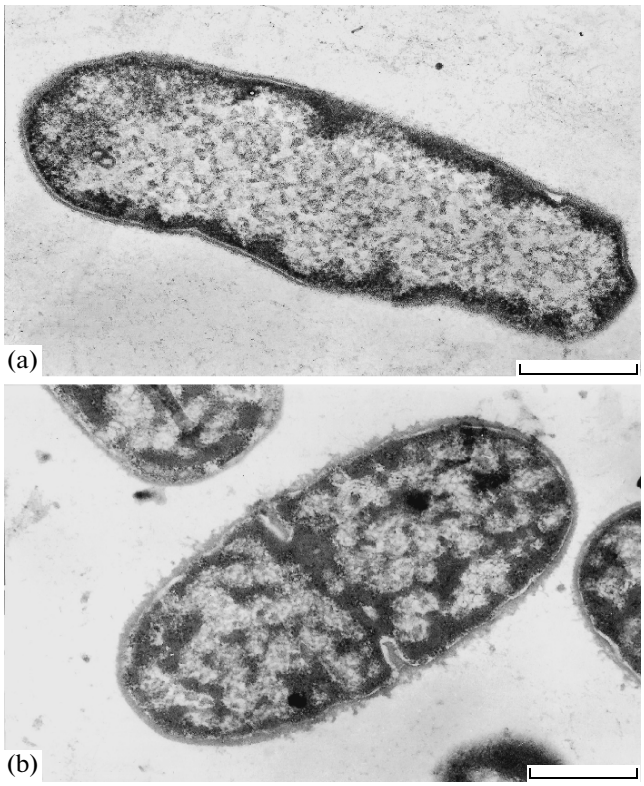

Fig. 1. Ultrathin sections of *C. algoriphilum* cells grown at optimal (5°C) (a) and subzero (–5°C) (b) temperatures. Scale bar, 0.5 μm.

The study of simultaneous impact of two factors (temperature and salinity) revealed that the maximum polysaccharide accumulation occurred at the highest salt concentration and the lowest temperature (Table 4). At 12°C and salt concentration of 5 g/l, polysaccharide content of the biomass decreased to 10% to the mass of dry cells (Table 5); no increase in the optical density of the culture was observed. Probably, under strongly nonoptimal conditions, glucose transport was impaired and cells survived due to consuming the endogenous substrate.

Table 1. Effect of glucose concentration on the intracellular polysaccharide content of *C. algoriphilum*

Glucose, g/l	Polysaccharide, mg glucose equiv./mg dry biomass
0.10	0.15
0.25	0.19
1.00	0.23
2.00	0.27
4.00	0.31

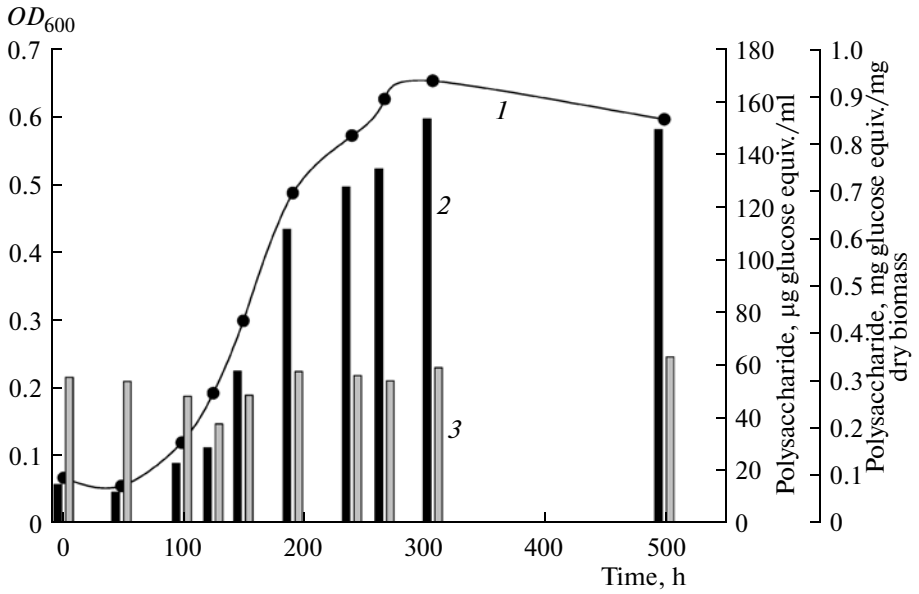

Fig. 2. Dynamics of the intracellular polysaccharide of *C. algoriphilum* in the course of growth: optical density (*1*); polysaccharide amount per unit of volume, mg glucose equiv./ml (*2*); and polysaccharide content of the cells, mg glucose equiv./mg dry biomass (*3*).

DISCUSSION

Glycogen-like Compound of the Bacterium C. algoriphilum

C. algoriphilum is an obligate anaerobic psychrophilic bacterium isolated from an extreme environment (cryopeg). It was reasonable to suggest that the intracellular polysaccharide of this bacterium differed in the composition and structure from those of mesophilic and thermophilic clostridia [20]. However, our preliminary data did not confirm this assumption. Probably, the role of the intracellular polysaccharide in adaptation of *C. algoriphilum* to cryopeg conditions is associated with the patterns of its accumulation rather than with the composition.

Effect of Stress Factors on Polysaccharide Accumulation

Stress factors (elevated temperature, salinity, and hydrogen peroxide) are known to induce the genes encoding glycogen synthesis in yeast *Saccharomyces cerevisiae* [21]. It can be assumed that polysaccharide accumulation in *'C. algoriphilum'* is a temperature-dependent process. However, according to our data (Tables 3 and 4), temperature and the combined action of temperature and salinity had no significant effect on the intracellular polysaccharide amount in *'C. algoriphilum'*. Nonoptimal temperature possibly induced both synthesis and degradation of the glycogen-like compound in an equal degree, as was observed in *S. cerevisiae* [21]. In this case, energy expenditure for opposite processes (biosynthesis and biodegradation of the polysaccharide) increased and the polysaccharide content of the biomass remained unchanged.

Table 2. Effect of the substrate type on the intracellular polysaccharide content of *C. algoriphilum*

Substrate	Polysaccharide, mg glucose equiv./mg dry biomass
Glucose	0.27
Trehalose	0.20
Peptone	0.05

Does the Intracellular Polysaccharide of C. algoriphilum Play the Role of a Reserve Substance?

The polysaccharide of *'C. algoriphilum'* might perform the function of a reserve substance. It should be noted that a compound plays the role of an energy reserve if it meets the following requirements: (i) it is accumulated when the energy inflow from exogenous sources exceeds cell requirements for growth; (ii) it is utilized when the energy inflow is insufficient for supporting cell growth, division, and survival; and (iii) it is degraded with release of energy in the form available for cells [22, 23]. Our results demonstrate that the intracellular polysaccharide of *C. algoriphilum* meets these requirements: (i) under growth limitation by nitrogen, polysaccharide content of the biomass almost doubled compared to the optimal conditions, and (ii) the polysaccharide was utilized when the cells were transferred into carbon-free medium.

Table 3. Accumulation of the polysaccharide in *C. algoriphilum* at different cultivation temperatures

Growth temperature, °C	OD_{600}max	Doubling time, h	Polysaccharide, mg glucose equiv./mg dry biomass
–5	0.86	432	0.23
0	0.80	67	0.36
5	0.69	20	0.27
12	0.21	76	0.22

Possible Involvement of C. algoriphilum Polysaccharide in Sporogenesis

When calculated per unit of volume, the polysaccharide amount increased with increasing optical density (Fig. 2); however, when calculated per milligram of dry cells, it remained constant.

It was shown that the glycogen-like polymer of *C. thermocellum* was degraded after growth cessation and presumably was involved in sporogenesis [20]. Spore-forming and nonspore-forming strains of *C. pasterianum* differed in the character of polysaccharide accumulation [24]. The spore-forming strain accumulated polysaccharide up to 60% of dry cells at the beginning of the stationary phase; the second strain produced polysaccharide (about 15%) during the whole period of growth, much like the bacterium *C. algoriphilum*, which was characterized by poor sporulation. Among the studied stress factors (nonoptimal temperatures, freezing, the absence of carbon substrate, and extreme values of pH and salinity), only transition into the cryopeg water promoted sporulation of *C. algoriphilum*. Thus, in its native environment, polysaccharide accumulation in *C. algoriphilum* possibly stimulates sporogenesis.

Does the Polysaccharide in C. algoriphilum Cells Create Favorable Conditions for Biochemical Reactions?

The cell cytoplasm is known to act as an environment for biochemical events. Probably, the polysaccharide of *C. algoriphilum* is not only involved directly in biochemical reactions but also promotes development of optimal cytoplasmic viscosity for the low-temperature biochemical reactions, thus ensuring survival of *C. algoriphilum* under cryopeg conditions.

Table 4. Effect of temperature and salinity on the intracellular polysaccharide content of *C. algoriphilum*

NaCl concentration, g/l	Final concentration of the polysaccharide (%) at different temperatures		
	12°C	5°C	–5°C
5	10	19	27
50	27	27	31.8
100	27	27	30.4
150	27	32	33.4

The results of our study of the intracellular polysaccharide of the anaerobic psychrophilic clostridium *C. algoriphilum* provide some insight into the inhabitants of extreme ecotopes. However, the obtained data don't confirm direct involvement of the intracellular polysaccharide in the adaptation of *C. algoriphilum* to cryopeg conditions. Further studies based on investigating the enzymes involved in the synthesis and degradation of the intracellular polysaccharide are required to elucidate the role of this polymer in *C. algoriphilum*. It is of interest to determine whether osmolyte–cryoprotectors (trehalose and/or sucrose) are produced in *C. algoriphilum* cells [21, 25, 26]. Of importance also is the study of the mutants that are defective in synthesis of intracellular polysaccharides.

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