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EXPERIMENTAL ARTICLES

Effect of the Medium Composition and Cultivation Conditions on Sporulation in Chemolithotrophic Bacteria

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Abstract—The possibility of regulating endospore formation by changing cultivation conditions was for the first time shown in acidophilic chemolithotrophic bacteria *Sulfobacillus thermosulfidooxidans* type strain 1269 and the thermotolerant strain K1 formerly described as "*S. thermosulfidooxidans* subsp. *thermotolerans*". Suppression of sporulation occurred when these strains were cultured in Manning's liquid medium with yeast extract. This medium was optimized by gradually reducing the concentrations of ferrous iron salts (the source of energy), phosphorous, nitrogen, and yeast extract and simultaneously increasing the concentrations of calcium, magnesium, and manganese (the elements important for sporogenesis) to attain higher yields of endospores by strains 1269 and K1. As a result, a new medium A was proposed, in which, under aeration, the life cycle of the strains studied culminated in sporulation at a level of 45 and 60%, respectively, of the total cell number. In a series of additional tests, the growth temperature and medium pH were adjusted to obtain the maximum yield of endospores. The optimal ranges found were 40–50°C and pH 1.8–2.2 for strain 1269 and 35–40°C and pH 2.5–2.7 for strain K1. An even higher yield of endospores, amounting to 55 and 75% for strains 1269 and K1, respectively, was obtained when the above growth conditions were combined (growth on medium A at optimal temperatures and pH under static conditions). Our results suggest a new approach to optimizing sporulation by acidophilic chemolithotrophs, which consists in limiting the energy and nutrient sources and using temperature and pH values within the tolerance bounds of these cultures but outside their growth optimum ranges.

Key words: chemolithotrophs, *Sulfobacillus thermosulfidooxidans*, medium optimization, sporulation.

It is well known that, in response to nutrient depletion in the stationary growth phase, spore-forming bacteria can produce endospores in the course of cellular differentiation. Endospores are specialized dormant forms that remain viable for a long time, exhibit no experimentally detected metabolic activity, and show increased resistance to adverse effects. The capacity to form endospores is found in a relatively small number of gram-positive and gram-negative bacteria, including *Bacillus*, *Sporolactobacillus*, *Thermoactinomyces*, *Alicyclobacillus*, *Sulfobacillus*, *Clostridium*, *Desulfotomaculum*, *Sporomusa*, and *Sporohalobacter* [1], which are all well studied by microbiologists, and a few others. Chemolithotrophic acidophilic spore-forming bacteria of the genus *Sulfobacillus* are currently represented by the three species *S. thermosulfidooxidans*, *S. acidophilus*, and *S. disulfidooxidans* [2–5], which, from the viewpoint of microbial biogeochemistry and environmental studies, are of particular interest in view of their capacity to oxidize ferrous iron, sulfide minerals, and elemental sulfur. Working with collection strains of *S. thermosulfidooxidans*, we noted that, under standard growth conditions [2], their endospore forming activity was extremely low or totally missing.

The starting point in our analysis of this effect was literature data that the life cycle of sporulating bacteria does not necessarily lead to the formation of endospores. For example, when initiated spores of *Bacillus cereus* strain T were transferred to a medium for sporulation with a limited source of nitrogen (to retard vegetative growth) and then to a glucose-rich (10^{-2} M) medium to suppress the formation of spores, the cell development microcycle ended in the formation of semirefractile cells. They exhibited hypometabolic activity and an altered ultrastructure but rapidly lost viability when stored [6]. When nutrient medium and cultivation conditions are modified [7] to suppress sporulation and increase biosynthesis of d_1 factors (anabiosis autoinducers), the life cycle of *Bacillus cereus* strain VKM 504 ends up in the formation of refractive cystlike forms that preserve viability over long periods of time and have all the necessary features of resting forms.

It could not be ruled out that the sporulation of acidophilic chemolithotrophic bacteria cultured under standard conditions [2] was suppressed because of a nonoptimal medium composition or cultivation conditions.

The purpose of this work was to elucidate the conditions optimal for the sporulation of the chemolithotrophic bacteria *Sulfobacillus thermosulfidooxidans* strain 1269 and strain K1.

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MATERIALS AND METHODS

The moderately thermophilic chemolithotrophic acidophilic bacterium *Sulfobacillus thermosulfidooxidans* strain 1269 [2] and the chemolithotrophic thermotolerant strain K1 (formerly, *"S. thermosulfidooxidans* subsp. *thermotolerans"* [3, 8]) were studied.

Bacteria were cultured under mixotrophic conditions in Manning's medium composed of (g/l) $(NH_4)_2SO_4$, 6; KCl, 0.2; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 1; and $Ca(NO_3)_2$, 0.02. $FeSO_4 \cdot 7H_2O$ (33.4 g/l) was used as a source of energy [9]. The solution of Fe^{2+} was separately autoclaved and added to the medium after its sterilization. The medium was supplemented with yeast extract (0.2 g/l; Difco, United States). The pH of the medium was adjusted to 1.8 or 2.7 (for strains 1269 and K1, respectively) by the addition of 0.1 N H_2SO_4. Modifications of the liquid medium will be described in the RESULTS section (Table 1). In several tests, cells were cultured on solid medium with 0.5% agarose.

Bacteria were also grown on a medium previously employed for cultivation of the heterotrophic *Bacillus acidocaldarius* [10] but with a decreased organic carbon content. This medium was composed of (g/l) $(NH_4)_2SO_4$, 2.5; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4 – 0.3; $CaCl_2 \cdot 6H_2O$, 0.25; soluble starch, 0.3; yeast extract, 0.3; and peptone, 0.3. The organic components were autoclaved separately and added after sterilization; the medium pH was adjusted to 1.8 and 2.7 by the addition of 0.1 N H_2SO_4.

The inoculum was introduced in a dose of 10 vol % to produce an initial cell density of 10^6 cells/ml. The cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of the medium for 2–10 days, depending on the aeration mode (shaker, 180 rpm or stationary conditions), at temperatures optimal for their growth (48°C for *S. thermosulfidooxidans* 1269 and 37°C for strain K1). Several modifications of cultivation conditions that we employed will be outlined below.

Microscopic examinations were performed using a Reichert microscope (Austria) equipped with a phase-contrast device. The total number of cells was determined in a Goryaev chamber by examining 100 squares and calculating the average number of cells in 1 ml of the suspension. The results were assumed reliable when the estimated standard deviation was under 5%.

RESULTS

When the acidophilic chemolithotrophic bacteria *S. thermosulfidooxidans* strain 1269 and strain K1 were cultured in the original Manning's medium with yeast extract, known to provide for good growth and biomass yield (10^8–10^9 cells/ml), virtually no sporulation in the stationary growth phase occurred, as demonstrated by microscopic examinations (Figs. 1a and 1b). However, small colonies containing endospores developed when solid agarized medium was inoculated with the obtained cell suspensions.

Table 1. Composition of media used to study sporulation in chemolithotrophic bacteria

Components	Concentration (g/l)		
	Manning's medium [9] with yeast extract	intermediate medium	new medium A
$(NH_4)_2SO_4$	6.0	3.0	1.5
KCl	0.2	0.15	0.1
K_2HPO_4	0.2	0.1	0.05
$MgSO_4 \cdot 7H_2O$	1.0	1.5	2.0
$Ca(NO_3)_2$	0.02	0.1	0.2
$FeSO_4 \cdot 7H_2O$	33.4	15.0	7.5
Yeast extract	0.2	0.15	0.15
$MnSO_4 \cdot 5H_2O$	–*	0.02	0.05

* Component was not used.

The bacteria under study failed to grow on a medium with high concentrations of peptone, yeast extract, and soluble starch (in total, 15 g/l), which is commonly used to cultivate the heterotrophic bacterium *Bacillus acidocaldarius* [10], later assigned to the genus *Alicyclobacillus* [11]. On a medium having the same mineral composition but a low organic matter content (totaling 0.9 g/l), the type strain *S. thermosulfidooxidans* 1269 exhibited poor growth and formed no spores. Meanwhile, strain K1 was able to grow on this medium over three to four culture transfers and formed endospores, which, in the stationary growth phase, constituted about 15% of the total number of cells (Table 2).

Our modifications of Manning's medium consisted in decreasing the concentrations of ferrous iron, nitrogen, phosphorous, and yeast extract and simultaneously increasing the concentrations of calcium, magnesium, and manganese salts as elements stimulating sporulation. The yields of endospores by *S. thermosulfidooxidans* 1269 and strain K1 on the "intermediate" medium (Table 1) were well above controls and amounted to 35 and 50% (Table 2), respectively. The composition of this medium was further optimized by trying other variants to obtain an even higher yield of endospores. On the medium A (Table 1), the endospore yield under aeration amounted to 45 and 60% for *S. thermosulfidooxidans* 1269 and strain K1, respectively (Table 2). In these experiments, bacteria were cultured at pH values (1.8–2.0 and 2.5–2.7) and temperatures (48 and 37°C) optimal for the growth of strains 1269 and K1, respectively. The obtained medium A, low in nutrients and rich in calcium, magnesium, and manganese, provided for the maximum sporulation at pH and temperature

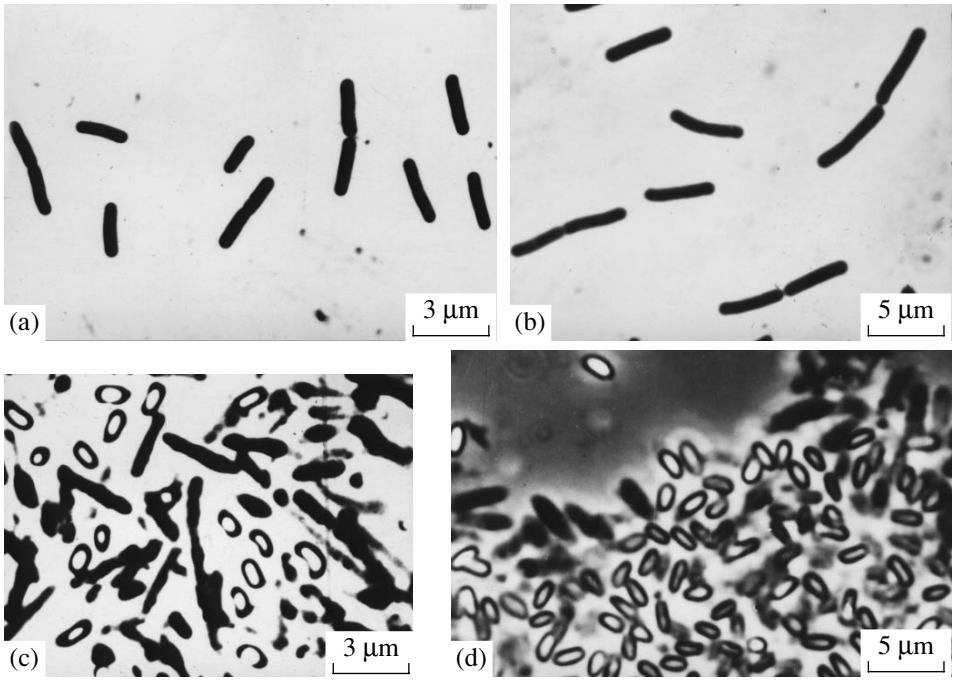

Fig. 1 Micrographs of stationary-phase cells of (a, c) *Sulfobacillus thermosulfidooxidans* 1269 and (b, d) strain K1 grown on (a, b) Manning's medium and (c, d) medium A at the temperature and medium pH optimal for sporulation. Phase-contrast microscopy.

values optimal for growth; this medium was used in subsequent experiments.

In the next series of tests, the influence of physicochemical factors (pH and temperature) on the sporulation of sulfobacilli grown on medium A was studied. The temperatures were varied from 30 to 55°C for *S. thermosulfidooxidans* strain 1269 and from 28 to 50°C for strain K1 to determine the maximum yield of endospores under static conditions at reduced aeration (the medium pH was fixed at values optimal for growth, i.e., 1.8–2.0 and 2.4–2.7, respectively). The highest number of spores was observed in *S. thermosulfidooxidans* 1269 cultured at 40–52°C and strain K1 cultured at 35–40°C (Fig. 2). It should be noted that both strains studied showed a high yield of endospores at temperatures nonoptimal for growth (Fig. 2). Thus, despite weak growth at 30°C, spores formed by *S. thermosulfidooxidans* 1269 constituted 25% of the total number of cells (Fig. 2). This was also true of strain K1 incubated at 50°C. Its cells were observed to form long filaments, apparently, in response to adverse growth conditions [3]; and, yet, the fraction of endospores in the culture was as high as 30%.

Further experiments on the influence of pH on the development of acidophilic chemolithotrophic bacteria on medium A at temperatures optimal for spore formation by each strain made it possible to identify the pH ranges ensuring the maximum production of endospores. In type strain 1269, their highest number, determined by phase-contrast microscopy (Figs. 1c and 1d), was observed at pH 1.8 to 2.2 and amounted to about 55%. In strain K1, the maximum yield of

Table 2. Yield of endospores formed by *S. thermosulfidooxidans* 1269 and strain K1 cultured on different media at the pH and temperature optimal for growth

Strains	Yield of endospores (% of total cell number)				
	Manning's medium [9] with yeast extract	intermediate medium	medium A	medium for *B. acidocaldarius* (15 g/l of organic compounds) [10]	medium for *B. acidocaldarius* with a low organic matter content (0.9 g/l)
Type strain *S. thermosulfidooxidans* 1269	0	35	45	–	Singular cells, no spores
K1	0	50	60	–	15 (growth over 3–4 transfers)

Note: "–" means that no growth observed.

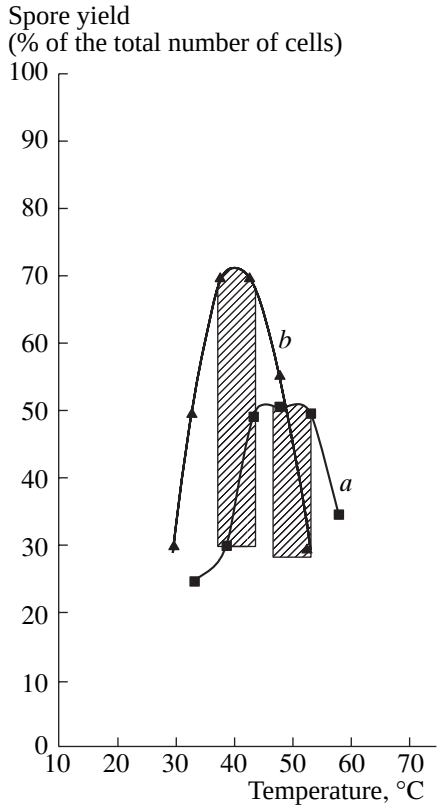

Fig. 2. Effect of temperature on sporulation in (*a*) *Sulfobacillus thermosulfidooxidans* 1269 and (*b*) strain K1 grown on medium A. Dashed regions are temperature optima for growth on the original Manning's medium.

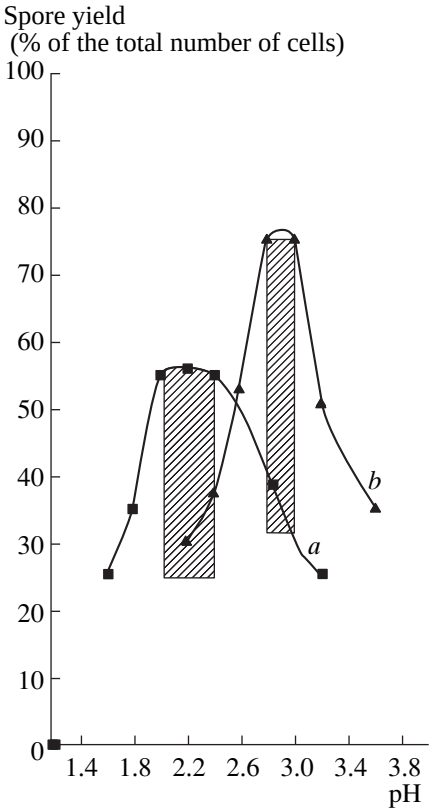

Fig. 3. Effect of pH on sporulation in (*a*) *Sulfobacillus thermosulfidooxidans* 1269 and (*b*) strain K1 grown on medium A. Dashed regions are pH optima for growth on the original Manning's medium.

endospores (75%) was noted in the pH range 2.5–2.7 under static conditions (Fig. 3). At pH 1.3 and 1.5 (values nonoptimal for the growth of *S. thermosulfidooxidans* 1269), endospores constituted, respectively, 30 and 35% of the total number of cells (Fig. 3). Despite the fact that strain K1 showed weak growth at pH values of 2.0 and 3.5, 30 and 35%, respectively, of the population of cells in the stationary growth phase was constituted by endospores (Fig. 3).

It is worth noting that the sporulation in the sulfobacilli under study is markedly affected by the aeration conditions. When cells were cultured on medium A at optimal pH and temperature but under O_2 deficiency created by static cultivation, the number of endospores was higher (55 and 75% for strains 1269 and K1, respectively) then when high oxygen content of the medium was maintained by shaking at 160–180 rpm (35 and 45%, respectively). This fact is in agreement with the recently reported data on the effect of oxygen shortage on the yield of another type of resting forms—cystlike refractive cells of *Bacillus cereus* (under conditions of sporulation repression) and *Micrococcus luteus* [7]. Based on our experiments with medium composition and cultivation conditions, a combination of these factors was determined that gave rise to the maximum yield of endospores (Table 3).

DISCUSSION

The sporulation of the moderately thermophilic strain *Sulfobacillus thermosulfidooxidans* and the thermotolerant strain K1 is known to be suppressed or even blocked when these organisms are cultured on Manning's standard medium with yeast extract and Fe^{2+} as the energy source. It is, therefore, important for maintaining collection cultures and industrial strains to optimize cultivation conditions in order to obtain a greater yield of endospores. Cells suspensions of sulfobacilli are known to produce single colonies that contain endospores when plated on agarized Manning's media. Based on this fact, it was supposed that the observed inhibition of sporulation in liquid cultures was not caused by a change in the genotype but resulted from nonoptimal cultivation conditions (composition of the medium and physical and chemical factors).

A commonly employed method to induce sporulation is to transfer a culture in the phase of growth deceleration to a mineral medium poor in nutrients. The approach we used was different and consisted in culturing chemolithotrophic spore-forming bacteria on a modified growth medium poor in the source of energy (ferrous iron salts) and nutrients (nitrogen, phosphorous, and yeast extract) and rich in elements stimulating

Table 3. Combination of conditions optimal for sporulation in chemolithotrophic bacteria

Cultivation conditions	*S. thermosulfidooxidans* 1269	Strain K1
Medium	Medium A (Table 1)	Medium A (Table 1)
Temperature	40–50°C	35–40°C
Medium pH	1.8–2.2	2.5–2.7
Aeration regime	Static	Static
Yield of endospores	55%	75%

sporulation, such as calcium, magnesium, and manganese. By modifying composition of the medium in this way, we were able to substantially increase the formation of endospores. The obtained increase in the spore production appears to be most of all due to the use of the new medium A and, secondly, to the optimal choice of temperature and medium pH, which was confirmed statistically.

We believe that increased sporulation by chemolithotrophic bacteria can be attained by their culturing under physical and chemical conditions (temperature and pH) outside the ranges optimal for their growth but still within their tolerance limits. It is worth noting that, even under unfavorable growth conditions (temperature and pH), the life cycle of bacilli cultured on medium A ended in sporulation, which can be treated as a reaction of cells to stress conditions.

The value of the proposed approach consists in the possibility of obtaining spore suspensions that can be stored for a long time and would preserve viability and be resistant to a wide spectrum of adverse factors. Modifying nutrient media and cultivation conditions can be effective when working with newly isolated chemolithotrophic bacteria, which rarely form spores when grown on standard media and under standard conditions. In addition, to confirm that the organism under study can indeed form spores is of taxonomic importance. That strain K1 can form spores in large amounts, as shown in this study, is also important in connection with the recent proposals to revise its taxonomic position. Thus, comparative 16S rRNA sequence analysis of the type strain *S. thermosulfidooxidans* 1269 and strain K1 and the results of DNA–DNA hybridization showed a remote phylogenetic affinity of these bacteria [12]. These strains were also found to differ significantly in their capacity to oxidize sulfide minerals and utilize organic compounds [12, 13]. In the present work, we also showed different growth characteristics of these two strains of sulfobacilli and different sporulation on a medium with a low carbon content (0.9 g/l). It follows that our approach to increasing sporulation by applying special conditions nonoptimal for growth can also be used as an additional test to clarify the taxonomic position of bacteria.

It should be borne in mind that the observed suppression of sporulation in *S. thermosulfidooxidans* 1269 and strain K1 might also be connected with the phenotypic variability of sulfobacilli and caused by the predominant development of a clone (variant) present in the studied population of chemolithotrophs and characterized by a low spore-forming ability under the given specific conditions. Clarifying whether this is indeed the case with the strains in hand requires additional investigation. At the same time, the heterogeneity of the microbial population caused by the presence of several phenotypes with different growth and physiological characteristics was found in many bacteria, including streptomycetes [14] and bacilli [15].

The observed distinctions in the capacity of chemolithotrophic bacteria to form spores during growth in liquid Manning's medium (no endospores detected) and during colonial growth on the same medium with agarose suggest a role of sporulation-inducing extracellular metabolites, the concentration of which in colonies with a large number of cells per unit volume is higher than in a liquid culture. This hypothesis is based on the previous evidence of the occurrence of an endogenous factor (*sporogen*) with a sporulation induction function in *Bacillus subtilis* [16]. In addition, microorganisms of different taxonomic groups (including spore-forming bacteria) were shown to possess a specialized autoregulation system controlling their development; this system involves extracellular low-molecular metabolites acting as anabiosis autoinducers (d_1 factors). Increasing their concentration in the cell suspension was shown to induce the formation of cystlike refractive cells in *B. cereus* (under the conditions of sporulation repression), *Pseudomonas carboxydoflava*, *Micrococcus luteus*, *Saccharomyces cerevisiae* and other microorganisms. The obtained cystlike refractive cells exhibited the typical features of resting cells: viability preserved over long periods of time, no experimentally detectable metabolic activities, increased resistance to adverse effects, and modified cell structure [7, 17–20]. It should be noted that refractive cells were occasionally observed in cultures of *S. thermosulfidooxidans* strain 1269 and strain K1 grown on Manning's medium with, e.g., arsenopyrite as the energy source, but their formation was in no way systematic. The identification of conditions propitious for the formation of refractive cells and verification of their belonging to the class of resting forms require further investigation.

The possibility of strategy choice (among sustaining vegetative growth, endospores formation, and formation of refractive cells) in response to a particular combination of trophic, physical, and chemical factors makes possible flexible adaptation of acidophilic chemolithotrophic bacteria to changes of environmental conditions in natural habitats.

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