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EXPERIMENTAL ARTICLES

Alterations in the Carbohydrate Composition of the Cytosol of Fungal Spores Caused by Temperature Variations and the Storage Process

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Abstract—Differences in the carbohydrate composition were revealed among spores of fungi belonging to *Zygomycetes, Ascomycota, Basidiomycota,* and *Oomycota*, part of the novel kingdom *Chromista.* It was shown for the first time that *Phytophthora infestans* contains arabitol in addition to glucose and trehalose. Sucrose was detected in *Pleurotus ostreatus* basidiospores. It was established that *Blakeslea trispora* stylospores contain inositol. The dependence of the spore carbohydrate composition on the temperature of the habitat of the corresponding species is discussed. It was shown that the cytosol of the conidia is dominated by trehalose and inositol under hypothermic conditions and by mannitol and glucose under hyperthermic conditions. *Neomycota* and *Eomycota* were shown to differ in their responses to stress (starvation), which correlated with the differences in the carbohydrate composition of the spore cytosols. Assuming that cytosol carbohydrates perform a protective function, we explain the higher viability of conidia compared to stylo- and sporangiospores.

Key words: fungi, spores, cytosol carbohydrates.

Recently, extensive data have been obtained which suggest that fungal spores related to different systematic groups differ in their chemical composition. Of special interest in this respect are spore cytosol carbohydrates, whose chemical composition is regarded as a systematic criterion [1–3].

Particularly significant differences in the carbohydrate composition of the spores were revealed between fungi (the kingdom *Fungi*) belonging to the divisions *Ascomycota* and *Basidiomycota* and those belonging to the class *Zygomycetes* [4–6]. In terms of the new systematics [7], oomycetes are referred to the kingdom *Chromista*, not *Fungi.* Oomycetes constitute the division *Oomycota*, the "pseudofungi." Based on an earlier suggestion, the differences in the carbohydrate composition are due to the fact that *Oomycota* fail to synthesize acyclic sugar alcohols [6], which are present in large quantities in *Ascomycota* and *Basidiomycota.* For example, the conidia of ascomycetous fungi contain mannitol, arabitol, meso-erythritol, and glycerol, in addition to trehalose [4, 5]. A comparison of *Fungi* with *Chromista* is of interest, because it can provide additional information concerning the legitimacy of classifying *Oomycota* into the novel kingdom *Chromista.* Cavalier-Smith [8] considers the new kingdom sufficiently closely related to the kingdom *Plantae.* The authors of the present paper adopt the system presented in [8], which includes the subkingdoms *Eomycota* (with the class *Zygomycetes*) and *Neomycota* (with the divisions *Ascomycota* and *Basidiomycota*).

Based on the available data on the protective role of cytosol carbohydrates under stress [9] and the novel concept that a spore is a "stress-adapted" cell, it was of considerable interest to investigate (i) the influence of the temperature of the species-specific habitat on the spore carbohydrate composition and (ii) the changes in the carbohydrate composition of various spore types during the storage process. These data are of importance in terms of fungal taxonomy and evolution, because they can help us establish unambiguous systematic links in the kingdom *Fungi.* The data are also of practical value, since information on the changes in the chemical composition of spores and the criteria of determining their viability are of paramount importance in biotechnological terms.

MATERIALS AND METHODS

This study was conducted with higher fungi belonging to *Ascomycota* (*Aspergillus japonicus* VKM F-2145, *A. niger* VKM F-33, *A. flavipesi* (from I.P. Bab'eva), *Penicillium kapustinskii, P. nigricans* (isolated by Bab'eva from the chernozem soils of the Southern Urals), and *Myceliophthora thermophila* (from the collection of the Institute of Microbiology, Russian Academy of Sciences)); *Zygomycetes* (*Blakeslea trispora* T(+) and T(–), *Cunninghamella japonica*

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VKM F-1204 (–), and *Absidia coerulea* VKM F-859 (–) and VKM F-858 (+)); *Basidiomycota* (*Stropharia rugoso-annulata, Agaricus bisporus, Lentinus edodes*, and *Pleurotus ostreatus* (from the collection of the fungus-breeding Joint-Stock Company Zarech'e)); and *Oomycota* (*Phytophthora infestans* from O.L. Ozeretskoskaya's collection).

Obtaining spores. To obtain spores, fungi and pseudofungi were grown on wort agar (*Ascomycetes*), potato–carrot agar (*Zygomycetes*), or oat agar (*Oomycetes*). Sporangiospores (SS spores) of *Blakeslea trispora* were obtained at a temperature of 24–26°C. The two types of conditions used for growing stylospores (ST spores) are given in Table 1 and in the text. The spore maturity was evaluated using the following criteria [10]: the color (dark in mature spores) and the capacity to germinate. Based on these criteria, the fungal spores used in this work were considered mature on the 4th–5th day of cultivation (*Aspergillus japonicus*), the 3rd–4th day (*A. niger* and *A. flavipesi*), the 5th–6th day (*Absidia coerulea*), the 6th–7th day (*Cunninghamella japonica*), the 7th day (*B. trispora*), the 5th–6th day (*Myceliophthora thermophila*), or the 7th–8th day (*Penicillium kapustinskii* and *P. nigricans*). Basidiospores were obtained from the sporophores of basidiomycetous fungi by preparing spore replicas of their caps on Petri dishes. Sporangia with zoospores were obtained by washing the surface of an 11-day-old *P. infestans* culture with distilled water and separating the sporangium fraction by filtration through a nylon sieve. All of the spores obtained were lyophilized.

Determining the carbohydrate composition of spores. Sugars were extracted with boiling water for 20 min; the procedure was repeated four times. Proteins were removed from the resulting extract [11]. The carbohydrate extract was further purified using a combined column with Dowex-1 (the acetate form) and Dowex 50W (the H^+ form). The quantitative sugar composition was determined by gas–liquid chromatography using trimethylsilyl sugar derivatives obtained from the lyophilized extract. Arabitol or α-methyl-D-mannoside served as an internal standard. Chromatography was performed on a Model 3700 gas–liquid chromatograph equipped with a flame-ionization detector and a 2-m-long glass column with 5% SE-30 on Chromaton (70–90 mesh). The temperature was increased from 170 to 270°C at a rate of 5–6 degrees per minute. Glucose, mannitol, arabitol, inositol, trehalose, and α-methyl-D-mannoside (Merck) were used as standards [11].

RESULTS AND DISCUSSION

This study was conducted with five types of spores: (1) conidia of higher fungi (*Ascomycota*); (2) sporangiospores (SS spores) and (3) stylospores (ST spores) of *Zygomycetes*; (4) basidiospores (*Basidiomycota*); and (5) sporangia with zoospores (*Oomycota*) (the systematics is according to [7]).

From the data in Table 1, it is evident that the tested mature spores significantly differed in terms of their carbohydrate composition. However, they all contained glucose and trehalose. These two carbohydrates were the major ones in *Mucorales* representatives, e.g., *C. japonica* and *A. coerulea*. Ascomycetous (*Eurotiales*) and basidiomycetous fungi also contained sugar alcohols. Curiously enough, we detected no glycerol in ascomycetous fungi, in contrast to the data obtained with *A. niger* [12]. However, mannitol was the predominant polyol in basidiomycetous fungi, which is consistent with the data available in the literature [3]. This polyol occurred in the spores of both ascomycetous and basidiomycetous fungi, in agreement with the work by Tan and Moore [13]. However, basidiospores, particularly those of *A. bisporus*, contained greater amounts of this sugar alcohol. Of particular interest is the high content of sucrose (10% of total carbohydrates) in the *P. ostreatus* spores (Table 1).

We investigated for the first time the carbohydrate composition of the cytosol of sporangia (with zoospores) of *P. infestans*, a representative of *Oomycota* (the kingdom *Chromista*). The carbohydrate composition established by us significantly differs from that characteristic of the *Fungi* kingdom: glucose is the predominant carbohydrate (over 80% of total carbohydrates), and only minimum amounts of trehalose occur. We also detected the sugar alcohol arabitol, which was earlier considered unusual for *Oomycota* [3].

From the results presented in Table 1, one can conclude that the habitat temperature influences the sugar composition and particularly the ratio between individual sugars. This fact is in some cases disregarded while establishing systematic links based on the carbohydrate composition. For instance, spores predominantly contain trehalose in *M. thermophila*, a thermophile with a temperature optimum of 42–43°C. An analogous pattern is characteristic of the conidia of *A. flavipesi* (a thermotolerant species). In contrast, *P. kapustinskii* belongs to psychrophiles, and its spores contain minimum amounts of trehalose, much mannitol, and no inositol. The conidia of *P. nigricans*, a mesophile, contain comparatively little mannitol, much trehalose, and some inositol.

The dependence of the ratio between the carbohydrates of the spore cytosol on the temperature of the species habitat manifests itself not only in *Ascomycota* but also in *Zygomycetes*. From the data in Table 1, it follows that the trehalose content is high in the ST spores of the (+) and (–) strains of *Blakeslea trispora* that form at 29–30°C, i.e., at higher temperatures than SS spores (whose optimum is 24–26°C). Accordingly, there is a clear-cut dependence of the carbohydrate ratio of the fungal spore cytosol on the optimum temperature. Sporulation occurring at elevated temperatures results in trehalose and inositol accumulation in the spores of both *Neomycota* and *Eomycota*. On the con-

Table 1. Sugar contents of the cytosol of fungal spores (% of total sugars)

Microorganism	Spore type	Glycerol	Erythritol	Arabitol	Glucose	Mannose and galactose	Mannitol	Inositol	Trehalose	Sucrose
A. coerulea	Sporangiospores	–	–	–	5.6	TA	–	–	94.4	–
*B. trispora**	ST(+)	–	–	–	41.3	–	–	TA	57.8	–
B. trispora	ST(–)	–	–	–	44.1	–	–	TA	56.9	–
B. trispora	SS(+)	–	–	–	57.6	–	–	TA	42.4	–
B. trispora	SS(–)	–	–	–	58.2	–	–	TA	41.8	–
C. japonica	Conidia	–	–	–	5.5	TA	–	–	94.5	–
M. thermophyla	Conidia	–	TA	TA	9.0	–	13.0	3.0	75.0	–
A. flavipesi	Conidia	–	TA	TA	12.0	–	12.6	5.4	70.0	–
A. japonicus	Conidia	–	TA	TA	12.2	–	21.5	2.8	59.3	–
P. kapustinskii	Conidia	–	TA	TA	13.0	–	46.0	TA	31.0	–
P. nigricans	Conidia	–	2.8	5.7	4.2	6.6	30.2	2.1	48.4	–
S. rugosa-annulata	Basidiospores	–	–	22.8	7.0	–	32.5	TA	37.7	–
A. bisporus	Basidiospores	–	–	TA	7.4	–	62.0	TA	26.7	2.9
L. edodes	Basidiospores	–	–	28.1	5.3	–	39.9	–	26.8	–
P. ostreatus	Basidiospores	–	–	5.1	19.9	–	30.1	6.9	27.3	10.6
P. infestans	Sporangia with zoospores	–	–	6.3	88.0	–	–	–	5.7	–

Note: "–" means that the sugar is lacking; TA denotes trace amounts (less than 1% of the total sugars).

*The ST spores of *B. trispora* were obtained by method 1, i.e., at 30°C for three days with subsequent spore maturation at 28°C for seven days.

trary, the spores of psychrophilic fungi (*P. kapustinskii*) contain more mannitol, less trehalose, and no inositol.

Temperature-dependent responses are different to some extent in different systematic groups of fungi. The *Mucorales* representatives tested, *C. japonicus* and *A. coerulea* (except *B. trispora*, see below), change only the trehalose contents upon changing the cultivation temperature, whereas *Ascomycota* and *Basidiomycota* also change the sugar alcohol levels (Table 1). Hence, the adaptation mechanisms are more diverse in *Neomycota* than in *Eomycota*. This conclusion is also supported by the data (see below) on the cytosol carbohydrate composition during the storage of sporangiospores and conidia.

A comparative study of the changes occurring during the storage of spore-containing materials was of considerable interest. It could also help us determine more accurately the differences in the carbohydrate composition of the spore cytosol of different fungi. We focused our attention on the fact that *B. trispora* spores lose their viability after 30–40 days of storage on nutrient agar slants. However, almost 100% of *A. japonicus* and *A. niger* conidia can germinate after 1 year (or even more) of storage.

The data presented in Fig. 1 indicate that the SS spores and ST spores of the (+) and (–) strains of *B. trispora* display the same pattern when stored on agar slants: the glucose content increases and the trehalose content decreases, although the opposite trend in the levels of individual sugars may occasionally occur at the initial stage of the experiment. A peculiarity of the (+) SS spores is that they contain more trehalose than glucose toward the end of the storage period, despite the decrease in the trehalose content during storage. The (–) SS and ST spores of *B. trispora* are particularly active in accumulating trehalose and depleting the trehalose pool. This seems to be an additional difference between the heterothallic strains of this species.

ST spores were obtained under different conditions in the experiments shown in Fig. 1: *B. trispora* was cultivated for 10 days at a constant temperature of 29–29.5°C (method 2) [11]. It is under these conditions that inositol accumulates in ST spores. The inositol content is especially high in the ST spores of the (+) strain of *B. trispora* (up to 5–6% of the dry biomass, Fig. 1d).

Another pattern occurred with *A. japonicus* conidia stored under the same conditions (on an agar layer, Table 2). Mature spores form on the 4th–5th day in this system. Their storage results in a decrease in the mannitol content and an increase in the trehalose content. The difference in the mannitol and trehalose contents is particularly manifest on the 20th–25th day of storage:

the trehalose content increases from 13–15% (in 4-day-old spores) to 30–33%, and the mannitol content drops from 52–54 to 46–48% of the total cytosol sugars (Table 2).

The differences in the carbohydrate composition between the spores of mucorous and ascomycetous fungi also manifest themselves during germination. The trehalose content decreases and the glucose content increases upon the swelling of mucorous spores. The trehalose content also decreases in ascomycete spores, but their mannitol content increases. However, the mannitol content drastically decreases and the glucose content increases upon the transition to the developmental stage characterized by the formation of the germ tube.

The following conclusions can be drawn from a comparison of the data obtained in this work with the available facts concerning the physiological function of cytosol carbohydrates [11, 13], particularly with the fact that resistance to environmental factors depends on a sufficiently high trehalose (and possibly sugar alcohol) content.

Like the stylosporangiospores of *Mucorales*, sporangiospores are not characterized by a dormant state during the storage period, in contrast to ascomycete conidia. The trehalose content increases during the storage period in ascomycete spores. This is characteristic of dormant spores. Probably, this is the reason why the conidia of ascomycetous fungi do not lose the capacity to germinate upon long-term storage on slants. Interestingly, the trehalose content of *A. niger* conidia increases in the course of slant desiccation.

In contrast to ascomycete spores, the spores of the mucorous zygomycetes studied resided in the active state. This follows from the gradual decrease in the content of trehalose, the "dormancy" carbohydrate, and the increase in the content of glucose, a metabolite that is characteristic of an active lifestyle. This may account for the observation that the spores of *C. japonica*, *A. coerulea*, and *B. trispora* cannot retain their viability during long-term storage on slants (in contrast to ascomycete spores). Importantly, the ST and SS spores of the (+) strain of *B. trispora*, which do not display such a drastic increase in the trehalose content, remain viable for a somewhat longer time than the spores of the (–) strain of *B. trispora*.

The above differences in the composition of the spores of ascomycetous and zygomycetous fungi apparently reflect the dependence of the carbohydrate composition on the sporulation type. In *Zygomycetes*, there is a continuous transition from the sporangial to the conidial sporulation type. *B. trispora* forms stylosporangia (sporangia with columns containing 200–300 stylospores) and sporangioles. Based on the sporulation type, *B. trispora* assumes, therefore, an intermediate position between purely sporangial (e.g., *Mucoraceae*) and conidial (*Cunninghamellaceae*) mucorous fungi. The data obtained suggest that conidia represent a more

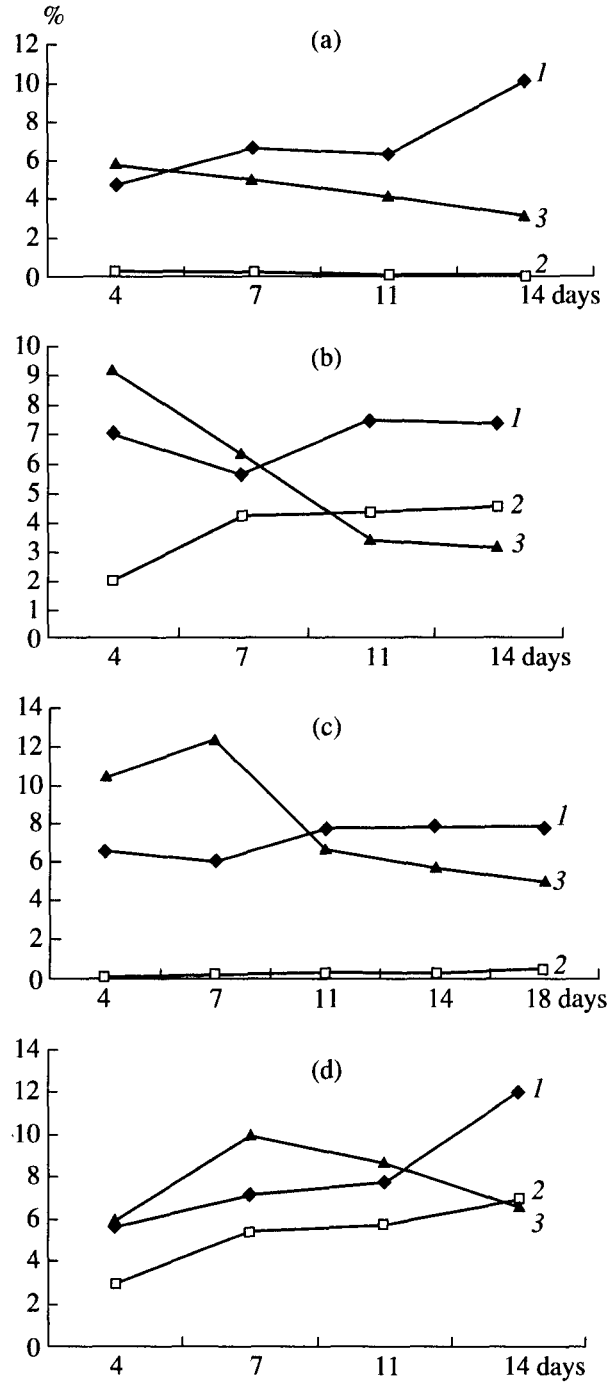

Fig. 1. Cytosol carbohydrate composition during the storage of stylo- and sporangiospores of the (+) and (–)T strains of *B. trispora* on solid medium. (a) Sporangiospores of the (–)T strain; (b) stylospores of the (–)T strain; (c) sporangiospores of the (+)T strain; (d) stylospores of the (+)T strain. (*1*) Glucose; (*2*) inositol; (*3*) trehalose.

advanced sporulation type and that they reach the dormant state sooner (which they retain for a long time); the spores representing the endogenous sporangial type have a longer maturation period.

In this work, sucrose was detected in the basidiospores of some *Basidiomycota*; the content of this disaccharide reached 10% of the total sugars in

Table 2. Dynamics of the carbohydrate composition (% of total sugars) of the cytosol during the storage of *A. japonicus* conidia

Age of conidia, days	Erythritol	Arabitol	Mannitol	Inositol	Glucose	Trehalose
4	6.1	12.0	52.0	0.9	14.0	13.0
23	3.5	9.3	46.0	0.1	12.0	30.0
52	2.5	2.0	28.0	–	7.0	60.5

P. ostreatus. This fact is of considerable interest, because sucrose is characteristic of plants and serves as a "dormancy sugar" in them (like trehalose in fungi, prokaryotes, and some animals). Interestingly, *Basidiomycota* spores contain two "dormancy" carbohydrates, trehalose and sucrose.

The occurrence of sucrose in *Basidiomycota* can be due to the fact that these fungi are especially closely related to plants in biological and evolutionary terms. Presumably, the coordinate evolution of fungi and plants is more ancient than the animal–plant coevolution. The *Plantae–Fungi* relationships seem to have been the most evolutionarily progressive among the relationships between different kingdoms of life [14]. The presence of sucrose in *Basidiomycota* seems to point to particularly close relations between this fungal group and plants and to the development of common biosynthetic pathways in the process of coevolution.

Another important fact established in this work is the presence of inositol in mucorous fungi. In terms of the current classification (based on the carbohydrate composition of the cytosol), mucorous fungi (*Zygomycetes*) belong to the P_1 type [6]: the representatives of this group do not form mannitol, but the cytosol of their mycelium contains glycogen, ribitol, and arabitol. Among the tested *Mucorales* representatives, *C. japonica* and *A. coerulea* spores contain no polyols, and *B. trispora* spores form only one sugar alcohol (inositol) under certain cultivation conditions. This polyol has also been identified in some *Ascomycota* spores, including ascomycete conidia. Based on the lipid composition of *B. trispora*, it was earlier suggested [15] that this fungus represents a transitional form between *Zygomycetes* and *Ascomycota*. The data on the presence of inositol, a typical ascomycete polyol, in *B. trispora* seem to support this suggestion. These data are evidence that the above systematic groups of fungi are interrelated.

The data obtained enable us to suggest a hypothesis concerning the role of inositol. The predominant accumulation of this polyol in *B. trispora* stylosporangia, which form at high temperatures, may bear witness to an involvement of stylospore formation in the *B. trispora* response to stress. Trehalose is partly replaced by inositol, which probably serves as a mandatory additional thermoprotector. Interestingly, an analogous mechanism was shown earlier to operate in *Ascomycota* [16].

The data on the carbohydrate composition of *P. infestans* sporangia militate against the generally accepted concepts. It has been assumed that oomycetes form no polyols and belong to the P_0 type [6]. The available literature contains no data on the presence of trehalose in these organisms. The data obtained by us indicate that glucose dominates the cytosol carbohydrates in *P. infestans* (about 90% of the total sugars). Apart from this sugar, we also detected trehalose (about 6%) and arabitol.

Oomycetes represent a group of organisms whose status is still controversial. Many researchers classified them into algae (not fungi) or regarded then as a self-contained phylum *Vaucheriophyta* or as the class *Oophyceae*, a peculiar evolutionary branch of the phylum *Vaucheriophyta* [17]. *Oomycota* were earlier placed in the kingdom *Fungi* as a separate phylum of this kingdom [18]; this was largely due to the morphological similarity of their reproductive organs (e.g., sporangia) to those of *Fungi*. Based on the recent data [8], they belong to the kingdom *Chromista*. The data obtained by us indicate that oomycete sporangia have nothing in common (in terms of the carbohydrate composition) with the fungal cells that share the same name (sporangia) but represent dormant stages in the *Fungi* life cycle. A high trehalose ("dormancy carbohydrate") and polyol content and a low glucose level characterize these fungal cells. Accordingly, the function of the so-called *Oomycete* "sporangia" with zoospores is clearly distinct from that of the fungal sporangia, and the morphological similarity is a result of convergent evolution. These facts can be regarded as additional evidence for the legitimacy of separating *Oomycota* from the kingdom *Fungi* and classifying them into plants [8], which is consistent with their polyol composition.

We demonstrated for the first time that the storage of conidia results in a decrease in the mannitol content and a concomitant increase in the trehalose content. Mannitol is considered a reserve polyol, which disappears shortly after spore germination [19]. This resembles to some extent the behavior of trehalose that also disappears in zygomycetes upon spore germination, because it is converted to glucose. Of interest in this context is the fact that stored spores are characterized by a different pattern of trehalose–mannitol relationships. Mannitol is degraded, and the trehalose level is increased. These data suggest different functional roles of the two reserve carbon sources, taking into account the fact that trehalose, apart from the reserve function, is also involved in the stabilization of membrane lipids [20]. The data in Table 2 indicate that an increase in the trehalose content at the expense of a decrease in the mannitol content is of foremost importance for retaining the spore viability.

Our data on the difference between *Ascomycota* and *Zygomycetes* in the cytosol sugar composition during the response to stress (starvation) significantly contribute to our knowledge of the mechanisms of dormancy and the involvement of mannitol therein. These data are also of practical value with respect to the storage of spore-containing materials. Importantly, the tested strains of *B. trispora* and *A. japonicus* are used in the industrial production of carotenoids and lytic enzymes [21].

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