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EXPERIMENTAL ARTICLES

Biochemical Mechanisms of Temperature Adaptation in the (+) and (–) Strains of *Blakeslea trispora*

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Abstract—Evidence obtained with industrial β-carotene-superproducing (+)T and (–)T strains, which fail to form zygotes, suggests that the lipids in the mycelium of the (–) strain of *Blakeslea trispora* lack linolenic acid. This circumstance apparently accounts for the fact that the (+) and (–) strains of *B. trispora* use different adaptive mechanisms to cope with an increase or decrease in cultivation temperature. In the (+) strain, temperature adaptation is based on changes in the ratio between linoleic and linolenic acyls and, also, involves shortening of acyl chains. In addition, the (+) strain contains a larger amount of protective carbohydrates, such as arabitol and trehalose. This strain is characterized by the presence of glycerol, a cryothermoprotector that protects fungal cells at low temperatures. The (–) strain lacks these biochemical mechanisms, but its neutral lipids contain a comparatively high amount of sterols and their esters. These facts enable us to interpret the enhanced thermotolerance of the (–) strain and its capacity to grow at high temperatures in terms of biochemical adaptation. In the light of the data obtained with wild-type and industrial strains, it is suggested that the lack of linolenic acid in the lipids should be considered an essential sex-specific property of the heterothallic strains of *Blakeslea trispora.*

Key words: heterothallism, *Blakeslea trispora*, lipids, linolenic acid, linoleic acid, trehalose.

The question of whether heterothallic strains of mycelial fungi display physiological and biochemical differences has been discussed in the literature since the beginning of the 20th century [1]. Recently, this issue has received special attention in light of new data suggesting that bisexuality manifests itself in higher eukaryotes in the biochemical features of these organisms as well as in their morphological properties. For example, overheating causes a more pronounced response (resulting in release of the adrenocorticosteroid hormone and hydrocortisone) in females than in males [2]. Attention should also be given to earlier findings that pointed to significant differences between the two sexes, for example, the Manoiloff test [3], in which female blood but not male blood is specifically stained by reagents.

With regard to the heterothallic strains of lower eukaryotes such as fungi, representatives of the order *Mucorales* have been studied in the greatest detail. In the late 1980s, significant differences were revealed with respect to the pattern of regulating the sexual process. A unique biochemical mechanism of synthesis of sex hormones (trisporic acids) by different-sex strains [4] was disclosed. Subsequently, differences between the (+) and (–) strains of *Blakeslea trispora* were also established relating to the synthesis of the isoprenoid compounds ubiquinone Q_9, ergosterol, and β-carotene [5].

Further research yielded the intriguing finding that the membrane lipids of the (–) strain of *Blakeslea trispora* lack linolenic acid; i.e., this heterothallic strain is either Δ^{12}-desaturase-deficient or exhibits low Δ^{12}-desaturase activity. This feature was found to occur in the wild-type VKM F-987 (–) strain at various ontogeny stages (in mycelium and in dormant cells, i.e., stylo- and sporangiospores). It was also established that (–) strain lipids contain more sterols and their esters. These biochemical properties, along with the comparatively high optimum growth temperature, indicate that the (–) strain is more thermotolerant than the (+) strain of *B. trispora* [6]. On the basis of these findings and taking into account recent data on the involvement of unsaturated fatty acids and protective carbohydrates in maintaining the dynamic state of biological membranes under temperature stress [7], we suggested that heterothallic strains may differ in terms of biochemical mechanisms of temperature adaptation.

The goal of this work was to investigate changes in the lipid and carbohydrate composition of the cytosol of the (+) and (–) strains of *B. trispora* that occur under hypo- and hyperthermy.

MATERIALS AND METHODS

This work used (+)T and (–)T strains of *B. trispora* from the Collection of the Winogradsky Institute of Microbiology (Russian Academy of Sciences). Our experiments were conducted using 2-day-old cultures

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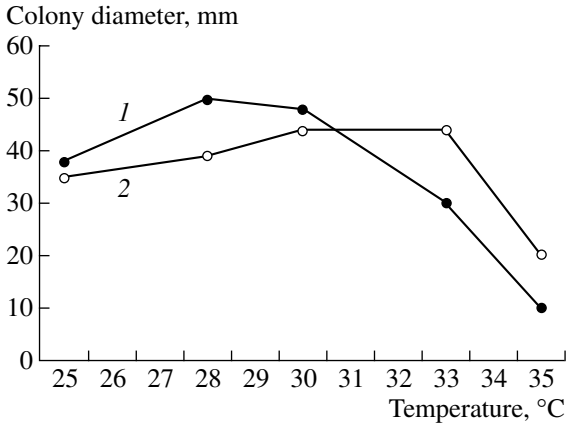
of (+) and (–) strains grown on hydrolyzed maize–soybean medium as an inoculum. 10% of the inoculum was added to 250-ml matrasses containing 30 ml of Goodwin medium [8]. Cultivation was carried out at 27–28°C for 66–68 h using a shaker (150 rev/min). Some of the matrasses were then incubated for 6 h at 20°C (cold stress (CS)) and 33–34°C (heat stress (HS)).

The lipids were extracted according to the Folch method: the samples were treated three times with a chloroform–ethanol mixture (2 : 1) for 1 h at room temperature using a magnetic stirrer. The samples were broken down by freezing–thawing them in liquid nitrogen and grinding them with quartz sand. Separation of the total lipid fraction into phospholipids (PLs), glycolipids (GLs), and neutral lipids (NLs) was carried out on a column with silica gel L (100/160 mesh, Chemapol, the Czech Republic) using solvents with varying polarity degrees [9]. The composition of the NLs and PLs was analyzed by ascending thin-layer chromatography on glass plates with KSKG silica gel (Laene Kalur, Estonia). The NLs were separated in a hexane–diethyl ether–acetic acid (85 : 15 : 1) solvent system. In order to separate the PLs, we consecutively used two solvent systems moving in the same direction: (i) hexane–diethyl ether–acetic acid (85 : 15 : 1) and (ii) chloroform–methanol–acetic acid–water (25 : 15 : 4 : 2). The lipids were applied to a plate in the amount of 50–100 μg. The chromatograms were sprayed with 5% sulfuric acid in ethanol and subsequently heated to 180°C to visualize the stains. The PLs were identified using individual markers and a phospholipid extract from porcine brain. Qualitative tests for amino groups (with ninhydrin) and choline-containing lipids (with the Dragendorff reagent) were carried out. The neutral lipids were identified with individual markers: mono-, di-, and triacylglycerols, free fatty acids, sterols (ergosterol), and hydrocarbons (Sigma). The chromatograms were quantitatively analyzed by scanning them with a Shimadzu CS-9000 dual-wavelength flying spot scanner.

The fatty-acid composition of the NLs, PLs, and GLs was determined using a Model 3700 gas–liquid chromatograph with a flame-ionization detector and a 2-m-long glass column filled with 17% diethylglycol succinate on the solid carrier Chromosorb W-AW ΔMGS-HP (80–100 mesh). The chromatography was carried out in an isothermal mode at a column temperature of 170°C. Lipid identification was performed using individual markers of methyl ethers of fatty acids (Sigma).

In order to determine the carbohydrate composition of the cytosol of fungal spores, sugars were extracted with boiling water for 20 min; this procedure was performed four times. Proteins [10] and charged compounds were removed from the obtained extract using a combined column with the ion-exchange resins Dowex-1 (acetate form) and Dowex 50W (H^+). Lyophilization was followed by preparation of the trimethylsilyl derivatives of the sugars, which were assessed by gas–liquid chromatography, with α-methyl-D-mannoside (Merck) used as the internal standard. The chromatography was carried out on a Model 3700 gas–liquid chromatograph with a flame-ionization detector and 2-m-long glass column with 5% SE-30 on a Chromaton 70–90 mesh. The temperature of this system was gradually increased from 130 to 270°C at a rate of 5–6°C/min. Glucose, mannitol, arabitol, inositol, and trehalose (Merck) were employed as markers.



Temperature dependence of the growth of the (*1*) (+) and (*2*) (–) strains of *B. trispora* in a surface culture.

The results obtained were statistically treated using the median (Me) method, with n values of 2–3 [12].

RESULTS AND DISCUSSION

The heterothallic (+)T and (–)T strains of *B. trispora* were obtained using mutagenesis. They differ from the original wild-type strains used in earlier studies in that they superproduce carotene and fail to form zygotes. Despite the malfunctioning sexual reproduction mechanism, the (–)T strain retains features that were previously revealed in the wild-type (–) strain [6]. It grows at high temperatures (Fig. 1), and its lipids lack linolenic acid (Table 1). These data are of special interest because the lack of $C_{18:3}$ in the lipids of the (–)T strain is a more stable trait than zygote formation.

This features of the lipids of the (–) strain suggests that heterothallic strains differ in terms of their mechanisms of biochemical adaptation to temperature changes. According to the data listed in Table 1, the phospholipid acyl chains of the (–) strain modulate the degree of desaturation (DD) of the lipid bilayer only by changing the linoleic acid content. This process does not involve the acyl chains of another unsaturated (oleic) acid. The linoleic acid content is significantly higher under CS than under HS, whereas the amount of $C_{16:0}$, a fatty acid with a shorter chain, remains virtually unchanged.

Nevertheless, the (+) strain increases its linolenic and linoleic acid contents under CS due to the influence of unfavorable temperatures. HS causes an increase in

Table 1. Alterations in the fatty-acid composition of the phospholipids (% of the total) of the (+) and (–) strains of *B. trispora* as a result of changes in the cultivation temperature

Fatty acids	(–)T			(+)T		
	Control	CS	HS	Control	CS	HS
$C_{16:0}$	15.4	13.9	15.6	15.2	15.3	22.1
$C_{16:1}$	6.6	3.6	3.9	3.3	3.3	3.6
$C_{16:2}$	–	–	–	–	–	–
$C_{17:0}$	6.0	1.3	3.3	3.1	0.8	3.1
$C_{17:1}$	–	–	0.8	–	–	–
$C_{18:0}$	3.8	–	1.2	1.0	1.1	1.6
$C_{18:1}$	13.7	13.3	13.1	18.5	17.5	13.8
$C_{18:2}$	54.5	67.9	47.1	26.4	31.5	31.7
$C_{18:3}$	–	–	–	20.0	22.2	13.4
$C_{20:0}$	–	–	–	–	–	–
X	–	–	15.0	12.5	8.3	10.7
DD	1.29	1.53	1.14	1.35	1.50	1.21

Note: *X* indicates an unidentified fatty acid; DD, the degree of desaturation; CS, cold stress; and HS, heat stress.

the palmitic acid level of phospholipids; i.e., it turns on the mechanism of shortening acyl chains and changes the $C_{18:3}$, $C_{18:2}$, and $C_{18:1}$ contents. This process is accompanied by a decrease in the DD of the lipid bilayer. The (–) strain lacks this mechanism of adaptation under analogous conditions. Importantly, both *B. trispora* strains are characterized by similar DD values under CS, while the DD of the (–) strain is lower under HS, which probably provides for the more intense growth of this strain under hyperthermy.

We also revealed differences in the phospholipid composition of the two strains. CS increases the neutral lipid content of the (–) strain and decreases that of the (+) strain. However, HS causes an increase in the neutral lipid content of both strains, which is more pronounced in the (–) strain (Table 2). An appreciable difference was also observed in the phospholipid composition, particularly in the phosphatidylcholine and phosphatidylethanolamine contents under the effect of HS and CS. The maximum phosphatidylethanolamine content in the (–) strain of *B. trispora* occurred under HS (it was twofold higher than in the control system). Only an insignificant increase in the phosphatidylethanolamine content was observed in the (+) strain under analogous conditions. A different pattern is characteristic of phosphatidylcholine, another bulk phospholipid located in the membranes. Under HS, its level is lower in the (–) strain and higher in the (+) strain than the control value. This effect is specific to the majority of mesophilic fungi at high cultivation temperatures. In addition, HS strongly influences the triacylglycerol content of the (–) strain, while the level of these neutral lipids (their percentage in the total fraction) slightly decreases in the (+) strain. It should be noted that the (+) strain is capable of forming saturated hydrocarbons, whose level significantly increases under CS (Table 2). Interestingly, the (–) strain forms more sterols and sterol esters than the (+) strain. Sterols prevail under CS, and their level in the (–) strain is twice as high as in the (+) strain. As we pointed out earlier [13], this is characteristic of thermophiles.

Heterothallic strains differ in terms of the intensity of lipid formation. Temperature stress stimulates lipid formation to a larger extent in the (–) strain, although the maximum lipid yield is attained under HS in both different-sex strains (Table 2).

The temperature protector function in a fungal cell is performed by "chemical chaperones" [14] that include noncyclic polyols and disaccharides. In this context, our research was aimed at investigating the effect of CS and HS on the carbohydrate composition of the cytosol of heterothallic strains of *B. trispora*. The data listed in Table 3 demonstrate the appreciable dif-

Table 2. Lipid composition of the (+) and (–) strains of *B. trispora* at various cultivation temperatures

Strain	Experimental variant	Lipids, % of the dry weight	Lipid fractions, % of total			Neutral lipids, % of total						Phospholipids, % of total			
			NLs	GLs	PLs	DGs	Sts	FFAs	TAGs	SEs	HCs	PC	PI	PS	PEA
(–)T	C	17.9	69.5	5.9	24.6	11.5	24.9	5.4	37.5	20.8	–	45.0	traces	16.4	38.6
(–)T	CS	16.1	72.7	5.2	22.1	11.2	22.5	7.2	37.8	21.2	–	35.9	–	18.5	45.7
(–)T	HS	27.4	82.7	6.2	11.1	12.0	29.4	traces	56.7	1.9	–	27.0	4.1	4.5	64.5
(+)T	C	18.3	83.3	2.6	16.1	3.5	12.7	traces	77.8	3.5	2.6	28.7	traces	9.5	61.8
(+)T	CS	16.5	73.2	8.0	18.8	traces	14.6	traces	76.5	traces	8.9	29.1	traces	7.5	63.4
(+)T	HS	23.0	85.4	4.6	10.0	8.3	12.5	traces	74.1	5.0	traces	32.4	traces	traces	67.6

Note: C stands for control variant; CS, cold stress; HS, heat stress; NLs, neutral lipids; GLs, glycolipids; DGs, diacylglycerols; Sts, sterols; FFAs, free fatty acids; TAGs, triacylglycerols; SEs, sterol esters; HCs, hydrocarbons; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PEA, phosphatidylethanolamine.

Table 3. Sugar composition of the mycelium in the (+) and (–) strains of *B. trispora* at various cultivation temperatures

Strain	Experimental variant	Sugars, % of the dry weight				Total sugars, % of the dry weight	Sugars, % of total			
		glycerol	arabitol	glucose	trehalose		glycerol	arabitol	glucose	trehalose
(–)	Control	traces	0.08	0.50	traces	0.58	traces	13.9	86.1	traces
(–)	CS	traces	0.11	0.49	0.15	0.75	traces	14.2	65.1	20.7
(–)	HS	traces	0.28	0.60	1.13	2.0	traces	13.9	30.0	56.1
(+)	Control	0.23	0.42	1.72	3.04	5.4	4.2	7.8	31.8	56.2
(+)	CS	0.27	0.62	1.93	3.62	6.4	4.2	9.6	30.0	56.2
(+)	HS	0.08	0.10	1.25	4.72	6.2	1.3	1.6	20.3	76.7

Note: CS stands for cold stress and HS, for heat stress.

ferences that exist between the (+) and (–) strain with respect to composition of protective carbohydrates and glycerol content. Importantly, arabitol accounts for up to 14% of the sugars contained in the (–) strain. The (+) strain is characterized by a higher arabitol content (up to 14% of the dry biomass), and it contains glycerol. Under CS, the two tested heterothallic strains undergo different changes in their carbohydrate composition. The trehalose content remains virtually unchanged in the (+) strain, butincreases in the (–) strain. However, the trehalose level increases under HS in both strains, and this effect is more pronounced in the (–) strain. The total sugar content also increases in both strains under HS (Table 3).

Hence, our study, conducted with the (+) and (–) strains of *B. trispora*, confirms the suggestion that all the lipid fractions lack linolenic acid in the (–) strain not only under optimum conditions but also under hypo- and hyperthermy. This finding was previously made in studies with other heterothallic fungi. Hence, it is also characteristic of the (–)T strain, which is of particular interest as this strain, unlike the wild-type strains studied earlier, does not form zygotes and supersynthesizes β-carotene in combination with the (+)T strain. This circumstance suggests that the lack of linolenic acid–synthesizing capacity and respective desaturase activity in the (–) strain is a sufficiently stable trait that determines the sexual characteristics of *B. trispora* strains.

In terms of modern concepts, the structural and dynamic properties of polyunsaturated acyl chains in lipid bilayers differ from those of saturated and monoene acyls [7]. Polyunsaturated acyl chains are relatively organized structures, and their presence in membranes promotes rapid lateral diffusion and ensures a high degree of local order and relatively limited molecular movements in the hydrophobic zone of the lipid bilayer. As far as polyunsaturated acids are concerned, *Mucorales* only contain linolenic acid, which provides for a substantial increase in the DD of the lipids [15]. This enables the system to change the fluidity of the lipid bilayer in a more dynamic fashion, particularly at low temperatures. The (+) strain, containing both linoleic and linolenic acid, uses a more efficient system of regulating the fluidity of the lipid bilayer, since the lipids of this strain can more rapidly modulate the DD of the lipid bilayer due to the presence of di- and triene acyls. The (–) strain also lacks the mechanism of regulating the fluidity of the lipid bilayer based on shortening acyl chains. In contrast, an increase in the palmitic acid content occurs in the phospholipid fraction of the (+) strain. During CS, adjusting the appropriate lipid DD in the (–) strain requires that the membrane contains twice the amount of linolenic acid found in the (+) strain. However, even such a membrane probably fails to ensure the optimum growth of this strain at 28°C. Nevertheless, the same temperature is optimum for the (+) strain, as can be seen from its growth curve (Fig. 1). A temperature of 31–32°C is optimum for the (–) strain, but this strain accumulates less biomass than the (+) strain even at this temperature.

Thus, we have established that a lack of linolenic acid in the fatty-acid fraction is a valid criterion enabling us to determine the sex of the heterothallic strains of *B. trispora*. We showed that it is inactive Δ^{12}-desaturase that is responsible for the observed differences in the mechanisms of temperature-dependent regulation and the DD values of lipid acyl chains. The fact that the optimum fluidity of the lipid bilayer cannot be attained due to a lack of linolenic acid accounts for the higher thermotolerance of the (–) strain as compared to the (+) strain, as well as for the lesser biomass accumulated by this strain (similarly to other thermophiles [13]).

In the light of these data concerning the greater adaptive potential of the (+) strain, the fact that it contains three times more protective carbohydrates, including arabitol and trehalose, than the (–) strain is of special interest. In addition, the (+) strain contains a wider variety of protective carbohydrates, e.g., glycerol. Apart from its osmoprotective function, glycerol is currently viewed as a natural antifreeze agent [16].

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