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Ubiquinone and carotene production in the *Mucorales* *Blakeslea* and *Phycomyces*

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Abstract The filamentous fungi *Phycomyces blakesleeanus* and *Blakeslea trispora* (*Zygomycota*, *Mucorales*) are actual or potential industrial sources of β-carotene and lycopene. These chemicals and the large terpenoid moiety of ubiquinone derive from geranylgeranyl pyrophosphate. We measured the ubiquinone and carotene contents of wild-type and genetically modified strains under various conditions. Light slightly increased the ubiquinone content of *Blakeslea* and had no effect on that of *Phycomyces*. Oxidative stress modified ubiquinone production in *Phycomyces* and carotene production in both fungi. Sexual interaction and mutations in both organisms made the carotene content vary from traces to 23 mg/g dry mass, while the ubiquinone content remained unchanged at 0.3 mg/g dry mass. We concluded that the biosyntheses of ubiquinone and carotene are not coregulated. The specific regulation for carotene biosynthesis does not affect even indirectly the production of ubiquinone, as would be expected if terpenoids were synthesized through a branched pathway that could divert precursor flows from one branch to another.

Introduction

Colored carotenes are demanded as strong antioxidants and as pigments in food, pharmaceuticals, and cosmetics (El Agamey et al. 2004; Stahl and Sies 2005); in addition, β-carotene is the main provitamin A. The filamentous *Zygomycetes Blakeslea trispora* and *Phycomyces blakesleeanus* have been converted into attractive sources of carotenes by protracted improvements of strains and culture conditions (Avalos and Cerdá-Olmedo 2004; Cerdá-Olmedo 2001; Ciegler 1965; Mehta et al. 2003). The same cultures could provide ubiquinone, ergosterol, organic acids, edible oil, and other products as well. Ubiquinone, an essential cellular component involved in electron transport in most biomembranes (Turunen et al. 2004), is an ingredient of cosmetics (Hoppe et al. 1999) and a common additive in health diets. Its medical applications include the treatment of cardiovascular diseases (Sarter 2002), hypertension (Wilburn et al. 2004), and neurodegenerative disorders such as Alzheimer's, Huntington's, and Parkinson's diseases and Friedreich's ataxia (Beal 2004; Cooper and Schapira 2003; Shults 2003).

Ubiquinone is composed of a quinone core with a solanesyl side chain. In both *Phycomyces* (Spiller et al. 1968) and *Blakeslea* (Bu'lock and Osagie 1973; Obolnikova et al. 1971), the side chain consists of nine isoprene units, i.e., 45 carbon atoms (C45), while the carotenes consist of eight isoprene units (C40). Fungal terpenoids are synthesized exclusively via the mevalonate pathway (Disch and Rohmer 1998). Dimethylallyl pyrophosphate (C5) is extended by repetitive condensation with isopentenyl pyrophosphate (C5) to produce compounds of various lengths, including geranylgeranyl pyrophosphate (C20) and solanesyl pyrophosphate (C45). Two molecules of geranylgeranyl pyrophosphate are joined to produce phytoene (C40). Successive dehydrogenations and cyclizations give rise to phytofluene, ζ-carotene, neurosporene, lycopene, γ-carotene, and β-carotene, a yellow compound that is the final product in the *Mucorales*. The close metabolic

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relationship of ubiquinone and β-carotene makes their coregulation an attractive research problem, which could have practical consequences for the optimal production of both compounds.

In a branched pathway, changes in the precursor flow into one branch usually modify the flows into other branches. Even when the flows are strictly controlled, mutational disruption of the control mechanisms may be expected to cause concomitant flow variations in the branches. Mutants with large differences in carotene concentration have been isolated in *Phycomyces* (Cerdá-Olmedo 1985, 2001) and *Blakeslea* (Mehta et al. 2003), allowing a direct test of this possibility. For the conversion of geranylgeranyl pyrophosphate into β-carotene, these fungi possess two structural genes (Arrach et al. 2001; Rodríguez-Sáiz et al. 2004; Torres-Martínez et al. 1980), whose mutations often affect the regulation of the pathway. Six additional genes are defined by regulatory mutants of *Phycomyces*. Gene assignments are not available for the regulatory mutants of *Blakeslea*.

The regulation of carotene biosynthesis has received considerable attention in *Phycomyces* (Bejarano et al. 1988; Govind and Cerdá-Olmedo 1986; Mehta and Cerdá-Olmedo 2001; Mehta et al. 1997; Murillo and Cerdá-Olmedo 1976) and to a lesser degree in *Blakeslea* (Mehta and Cerdá-Olmedo 1995; Mehta et al. 2003). These studies have led to vast gains in yield and have showed a number of differences in the responses of these fungi to various stimuli. Thus, blue light increases the accumulation of carotene in *Phycomyces* whether applied continuously or briefly (Bejarano et al. 1991). *Blakeslea* does not respond to continuous illumination (Sutter 1970), but shows a modest increase after illumination of dark-grown mycelia (Quiles-Rosillo et al. 2005).

The rising interest on the functions and practical applications of ubiquinone contrasts with the limited knowledge of its biosynthesis and regulation (Grünler and Dallner 2004; Meganathan 2001; Szkopinska 2000). In mammals, ubiquinone biosynthesis is regulated to allow limited variations. No universal inhibitor or activator of ubiquinone biosynthesis is known: compounds that are active in one organism may have no influence in others.

The purpose of this work was to establish whether the biosyntheses of ubiquinone and carotene are coregulated and to find conditions that lead to high contents of both.

Materials and methods

Strains

We used the strains of *Phycomyces blakesleeanus* Burgeff and *Blakeslea trispora* Thaxter previously described (Cerdá-Olmedo 1985, 2001; Cerdá-Olmedo and Lipson 1987; Mehta et al. 2003) and listed in Table 1 together with new analyses of their carotene contents (only the main carotene had been measured previously in most of them). The wild-type strains F921 of *Blakeslea* and NRRL1555 of *Phycomyces* were used when not otherwise stated. The strain names refer to the original collections: NRRL, *Phycomyces* wild types from the Northern Regional Research Laboratory (now National Center for Agricultural Utilization Research, Peoria, IL, USA); F, *Blakeslea* wild types from VKM (All-Russian Collection of Microorganisms, Moscow, Russia); C, *Phycomyces* mutants from Prof. Max Delbrück; S and SB, mutants from our own collections of *Phycomyces* and *Blakeslea*, respectively.

Culture conditions

Cultures were grown for 4 days on 25-ml standard minimal agar (Cerdá-Olmedo and Lipson 1987) in Petri dishes, 85 mm in diameter, in the dark at 22°C (*Phycomyces*) or 30°C (*Blakeslea*), unless otherwise stated. The carbon and nitrogen sources in the standard medium are 20 g/l D(+) glucose and 2 g/l L-asparagine and were replaced by other compounds as indicated. All media were adjusted to pH 5.4 ±0.1 after autoclaving, but the acetate media were left unadjusted (pH 6 to 7). L-Leucine was dissolved in 10 mol/l HCl, and the pH of the medium was adjusted to 4 before sterilization, to keep leucine dissolved, and to pH 5.4 afterward. Oligomycin A (Sigma Chem., St Louis, MO, USA) was dissolved in ethanol (10 g/l) and added to the sterile media at 50°–55°C for a final concentration of 0.1 mg/l established by former assays.

Each Petri dish was inoculated with 10^4 spores, randomly spread over the agar surface; *Phycomyces* spores were preactivated by heating at 48°C for 10 min. When indicated, the inoculum was a 4- to 7-days-old fragment of mycelium, about 2×2 mm, placed on the center of the agar surface. For light exposure, cultures were incubated on a white surface under a set of five fluorescent lamps (Philips TLD 36 W/54 CE) placed 50 cm away. The light fluence was measured with a calibrated photodiode (PIN-10DP/SB, United Detector Technology, Hawthorne, CA, USA) coupled with an amperimeter (model 485, Keithley Instruments, Cleveland, OH, USA).

Chemical analyses

Ubiquinone and carotene concentrations were measured by high-performance liquid chromatography in petroleum ether extracts of lyophilized mycelia (Kuzina and Cerdá-Olmedo 2006) and expressed as mass per dry mycelial mass. Extracts obtained by freezing the media (−20°C for at least 2 h), thawing the media (22°C for 1 h), and

Table 1 Strains used in this work, together with their relevant genotypes[a] and their carotene contents[b]

Strains	Genotype[a]	β-carotene	γ-carotene	Lycopene	Phytoene
Blakeslea					
F811	*(−)*	0.48	0.06	0.05	0.19
F812	*(+)*	0.21	0.07	0.06	0.04
F921	*(−)*	0.22	0.09	0.06	0.07
F986	*(+)*	0.08	0.04	0.02	0.05
SB34	*car-5 (−)*	0.11	0.18	0.23	0.06
SB39	*car-7 (+)*	0.27	0.09	0.06	0.07
SB51	*car-19 (+)*	0.20	0.19	0.14	0.09
SB53	*car-21 (−)*	0.43	0.11	0.08	0.15
SB63	*car-20 car-22 (−)*	1.33	0.60	0.48	0.30
SB65	*car-23 (−)*	0.40	0.20	0.16	0.17
F811 + F812		2.02			0.58
F921 + F986		3.90			0.41
SB34 + SB39		13.1	0.85	2.41	1.60
SB63 + SB51		19.3	0.49	2.49	0.35
Phycomyces					
NRRL1465	*(+)*	0.02			0.06
NRRL1554	*(+)*	0.13			0.05
NRRL1555	*(−)*	0.08			0.04
C5	*carB10 (−)*				1.61
C6	*carA12 carR27 (−)*			traces	
C9	*carR21 (−)*			2.04	0.32
C115	*carS42 (−)*	2.83			0.09
C2 ∗ C115	*carA5 (−) ∗ carS42 (−)*	2.90			0.06
C5 ∗ C115	*carB10 (−) ∗ carS42 (−)*	2.75			0.08
C9 ∗ C115	*carR21 (−) ∗ carS42 (−)*	1.47		7.29	0.59
S92	*carRA91 (−)*			traces	
S508	*carD174 (−)*	0.63			0.10
S555	*carS42 carF181 (+)*	4.83			0.30
S566	*carS42 carF181 (+)*	6.69			0.62
S568	*carS42 carF181 (−)*	6.16			0.42
S571	*carA5/carA+ carF181/carF+ (+)/(−)*	14.7			0.16
S566 + S568		17.1			0.09
NRRL1555 + NRRL1465		0.25			0.14
NRRL1555 + NRRL1554		0.36			0.27

[a] The symbols (+) and (−) designate the two sexes of the Mucorales. The *car* mutations modify carotene production. The symbol + between strain names and genotypes indicates that these are present in mixed cultures, a low-set asterisk, ∗, that they are the components of a heterokaryon, a virgule, /, that they are in heterozygosis in a diploid or a partial diploid.

[b] In milligrams per gram dry mass. Each value is the mean of two to eight independent determinations; the average standard error for the 86 values shown was 15.8%, but smaller for the larger values. Blanks indicate less than 0.01 mg/g dry mass.

centrifuging the liquid (1,000×g, 10 min, 22°C) were used for glucose determination as described (Kuzina and Cerdá-Olmedo 2006).

Rapid color tests for ubiquinone

We put aliquots (5, 10, 20, and 40 μl) of four solutions of ubiquinone-10 in ethanol (2, 40, 200, and 1000 mg/l) on filter paper, waited for them to dry, and examined them with a 302-nm ultraviolet lamp (UVM-57, UVP, San Gabriel, CA, USA) either directly or after covering them with one drop (about 10 μl) of a solution of rhodamine 6G (obtained from Merck, Darmstadt, Germany) in ethanol (5 g/l).

Results

Coregulation of ubiquinone and carotene contents

Sexual interaction and mutations in various genes cause enormous variations in the carotene content of *Blakeslea* and *Phycomyces*. The ubiquinone content remained unaltered while the carotene content varied from undetectable to

more than 100 times the wild-type level (Fig. 1 and Table 1). The average for ubiquinone in the 33 strains and mixtures of strains of opposite sex was 0.28 mg/g dry mass, with a standard deviation of 0.034; no strain deviated from the average more than it would be expected by random chance. The average of the wild types was 0.29 with a standard deviation of 0.044.

Time courses

Wild-type *Blakeslea* and *Phycomyces* exhibited similar patterns of growth and terpenoid production (Fig. 2). We used the standard strains F921 of *Blakeslea*, chosen from many wild types because of its carotene content, and NRRL1555 of *Phycomyces*, used in many laboratories for physiological, biochemical, and genetical research.

Ubiquinone concentrations increased with age, but this increase was less marked in *Phycomyces* cultures grown from spores. Ubiquinone became more abundant than β-carotene, and *Blakeslea* contained more β-carotene than *Phycomyces*. Carotene concentrations increased with age in *Blakeslea*, reaching their maxima in cultures grown for 3 to 4 days, but increased only slightly in *Phycomyces*.

Growth on the standard minimal medium is limited by the exhaustion of the carbon source, which occurs on the third or the fourth day of incubation in the case of *Blakeslea* and on the fifth day in the case of *Phycomyces*. *Blakeslea* grew and consumed glucose a little faster than *Phycomyces* in keeping with the incubation temperatures, 30°C for *Blakeslea* and 22°C for *Phycomyces*.

Inoculation of a small mycelial fragment in a Petri dish produced biomass more slowly than inoculation of about 10^4 spores, due essentially to a delayed start. The single large colony grown from a mycelial fragment was internally heterogeneous, probably more so than the small colonies grown from many spores. Mycelial samples taken from the center of large colonies after 4 days of incubation contained

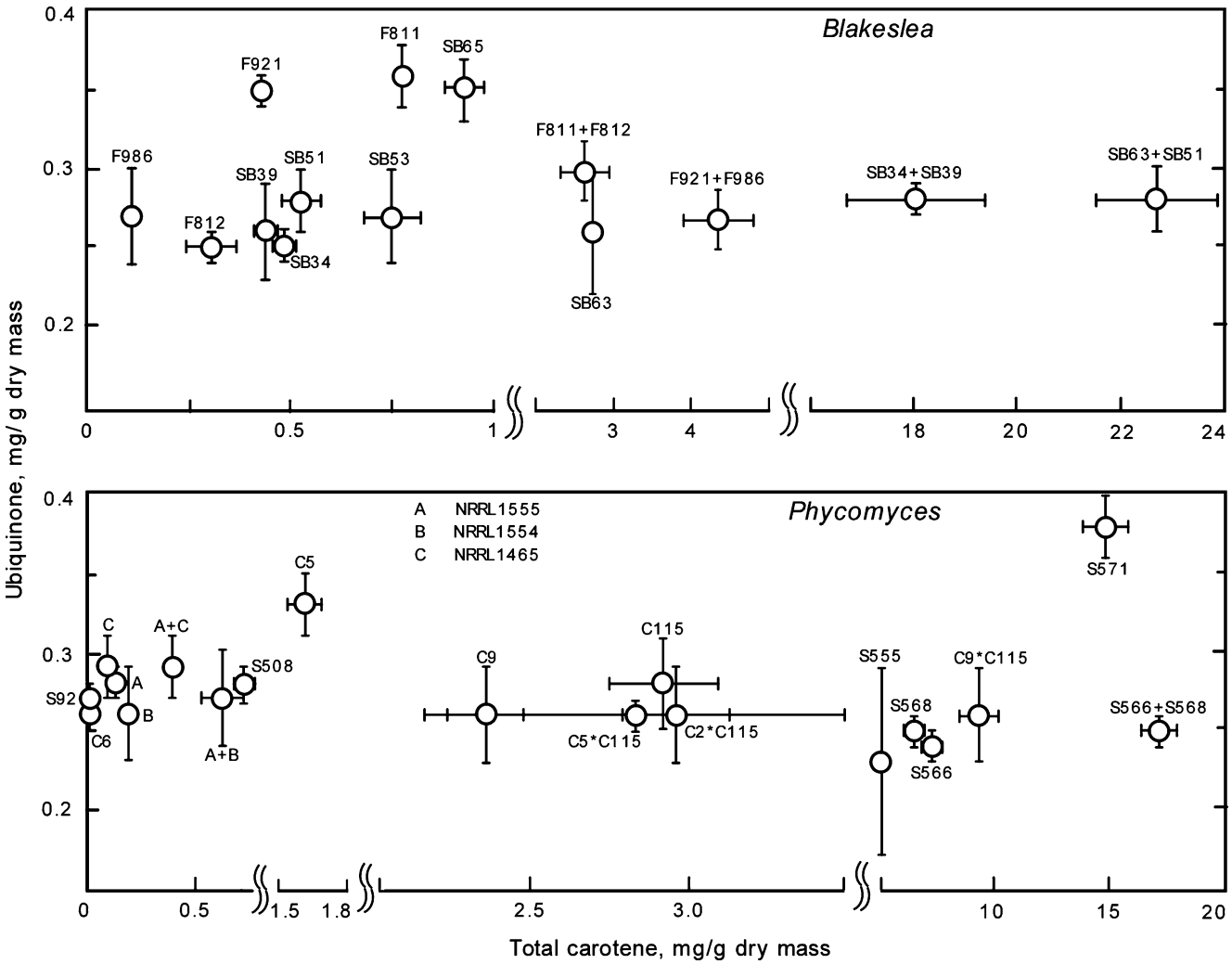

Fig. 1 Ubiquinone and carotene content (mean and its standard error, two to eight independent determinations) in 4-day-old mycelia of the strains shown in Table 1. The total carotene content is the sum of the contents of β-carotene, γ-carotene, lycopene, and phytoene

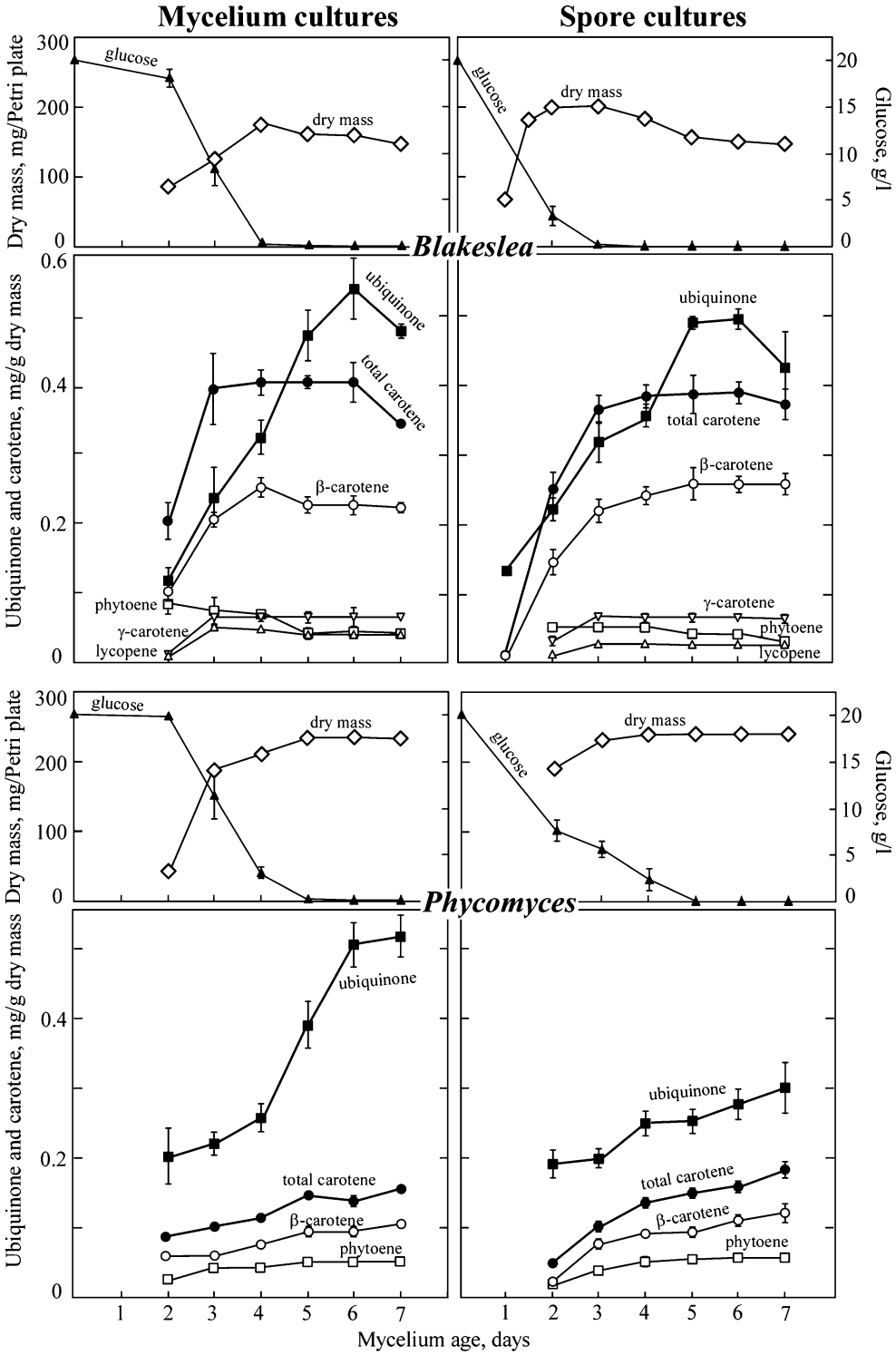

Fig. 2 Time course of ubiquinone and carotene mycelial contents, mycelial growth, and glucose content of the medium. Wild-type *Blakeslea* and *Phycomyces* mycelia were grown from spores and from mycelial fragments. The values are the mean and its standard error in two to seven independent experiments

23% more ubiquinone and 10% more carotene than samples from the edges.

Carbon and nitrogen sources

Mycelial growth and ubiquinone and carotene content varied considerably with the carbon and nitrogen sources (Fig. 3). Neither *Blakeslea* nor *Phycomyces* grew with leucine as the only carbon and nitrogen source or in acetate media with the final pH adjusted to 5.4.

Acetate, lactate, and leucine, which were used efficiently when mixed with glucose (media 3, 5, 7, 10, and 14), were poor carbon sources when used alone. The worst growth of *Blakeslea* (under one fifth of the dry mass on minimal agar)

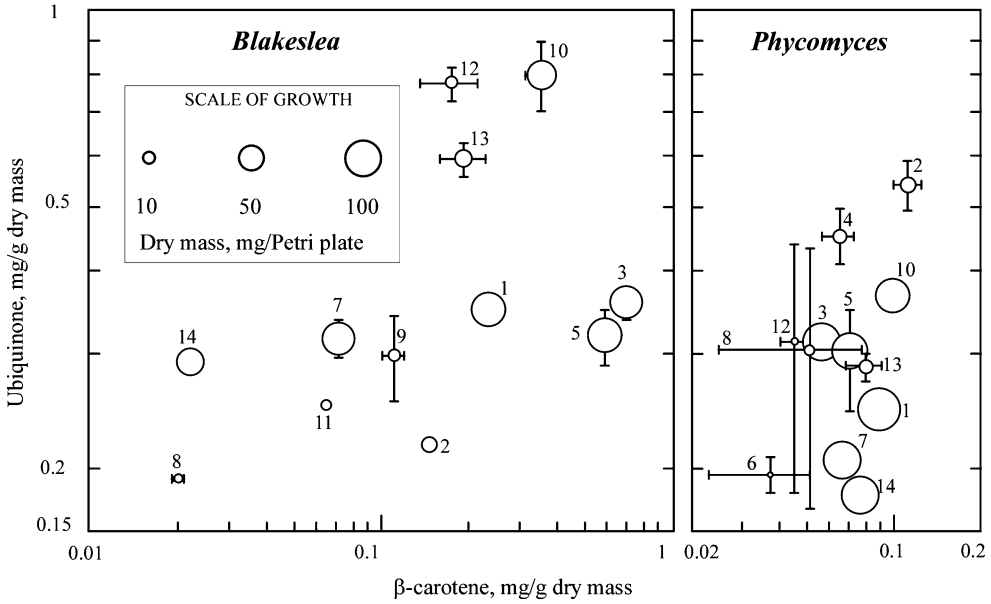

Fig. 3 Growth and ubiquinone and carotene production in 4-days old wild-type *Blakeslea* and *Phycomyces* cultures with different carbon and nitrogen sources. (*1*) Standard glucose-asparagine minimal medium. Glucose replacements ("full" denotes the concentration that provides 8-g/l carbon, the same as 20-g/l glucose; "half" denotes the concentration that provides 4-g/l carbon): (*2*) full potassium acetate, (*3*) half glucose and half potassium acetate; (*4*) full sodium acetate, (*5*) half glucose and half sodium acetate; (*6*) full D,L-lactate, (*7*) half glucose and half D,L-lactate, (*8*) half D,L-lactate; (*9*) full L-leucine, (*10*) half glucose and half L-leucine, (*11*) half L-leucine; (*12*) 6.56-g/l L-leucine and 0.82-g/l sodium acetate; (*13*) 1.31-g/l L-leucine and 4.1-g/l sodium acetate; (*14*) half glucose, half D,L-lactate and asparagine replaced by NH_4Cl at the same nitrogen concentration. The values are the means and their standard error in at least two independent determinations. The insert shows the biomass scale. The media that were not shown produced scarce biomass. The values are the mean and its standard error in two to seven independent experiments

was observed on media 4 (sodium acetate) and 6 (lactate) and of *Phycomyces* on media 9 and 11 (leucine). The results on these media are not shown in Fig. 3 because of the scarce biomass. The poor growth on media 12 and 13 is explained by their low-carbon content. Lactate alone was toxic to both fungi and, at the full concentration, blocked completely the growth of *Blakeslea* and brought that of *Phycomyces* down to 2% of the value with glucose. NH_4Cl can be used by both fungi as the only nitrogen source instead of asparagine, but not with better results (medium 14).

The ubiquinone content of *Blakeslea* was between 0.19 and 0.80 mg/g dry mass in the various conditions that allowed substantial growth. The highest ubiquinone concentration, which represents 2.3 times the average on minimal agar (0.36 mg/g dry mass), was found on medium 10 (mixture of leucine and glucose), on which *Blakeslea* maintained substantial growth, 72% of the average on the standard minimal agar. The average ubiquinone content in all cultures without leucine was 0.28 mg/g.

The maximum concentration of ubiquinone in *Phycomyces* (0.54 mg/g) was obtained with potassium acetate (medium 2), but the dry mass was only 15% of the average on the standard minimal agar. Medium 10 (mixture of leucine and glucose) was more convenient, because it led to an ubiquinone production 1.5 times the average on standard minimal agar (0.25 mg/g dry mass) while allowing substantial growth.

The β-carotene content of *Blakeslea* varied widely (from 0.02 to 0.68 mg/g dry mass) around the average of 0.24 mg/g on standard minimal agar. The highest value was obtained with medium 3 (mixture of glucose and potassium acetate). The carotene content varied less in *Phycomyces* cultures, with a maximum of only 0.11 mg/g on medium 2 (potassium acetate).

The stimulation of ubiquinone and carotene production by leucine led us to try adding it to the standard glucose–asparagine minimal agar. The ubiquinone and carotene contents were increased in both fungi to the same extent, 40% on the average, and the results were about the same with leucine concentrations from 0.6 to 4.5 g/l.

Effect of light

The two fungi responded to light in very different ways. Continuous white light increased the β-carotene content of *Phycomyces* 5.6-fold and the total carotene content 3.7-fold while the ubiquinone content remained unchanged (Table 2). Light decreased the carotene content of *Blakeslea* by two thirds relative to the value in the dark and increased the ubiquinone content by about 30%. The results with *Blakeslea* in the dark were different from

Table 2 Effect of light on ubiquinone and carotene content

Culture		Content, μg/g dry mass				
		β-carotene	γ-carotene	Lycopene	Phytoene	Ubiquinone
Blakeslea	dark	113±20	42±6	21±2	78±18	185±15
	light	28±14	20±4	15±4	16±4	243±10
Phycomyces	dark	87±4			49±5	251±15
	light	490±12			19±1	256±16

Wild-type *Blakeslea* and *Phycomyces* were grown for 4 days at 22°C in the dark or under white light (5 W m^{-2}). The means and their standard errors of 2 to 15 determinations in two to four independent experiments are shown

other results in this report because cultures were grown at 22°C in these experiments.

Oxidative stress

The role of ubiquinone in mitochondrial electron transport induced us to investigate if the ubiquinone content was modified in the presence of oligomycin A. This inhibitor of mitochondrial functions was very toxic to both fungi, which did not grow on minimal agar with 1 mg/l oligomycin, independently of whether the inocula consisted of spores or mycelial fragments.

Large fragments of mycelia of both fungi (about 3–4 mm in diameter, 1 mm thick) expanded slowly on minimal agar with 0.1 mg/l oligomycin. After 4 days, the carotene content was lower than in the controls, while the ubiquinone content was about the same in *Blakeslea* but larger in *Phycomyces* (Table 3).

A small fraction of the spores of *Phycomyces* (about one in a million), but no *Blakeslea* spores grew on minimal agar with 0.1 mg/l oligomycin. The resulting *Phycomyces* colonies suffered a severe loss of spore viability and were unstable. Few of the spores visible under the microscope formed colonies on minimal agar (3.6×10^{-4}, average of two determinations) and only about one tenth of the viable spores formed colonies on minimal agar with 0.1 mg/l oligomycin. When cultured in the absence of the inhibitor, the presumed mutants reverted to the wild-type phenotype and lost their oligomycin resistance.

Color tests for ubiquinone

A color test for ubiquinone that stains single fungal colonies would facilitate greatly the search for mutants with altered ubiquinone content. To be useful, a color test would have to detect about 1 μg ubiquinone, the amount estimated to be present in a colony. Chemicals that are known to yield colored products with ubiquinone or other quinones (Crane and Barr 1971; Fritz 1966; Pasto and Johnson 1981) were tested with samples that contained 0 to 40 μg ubiquinone-10. The fluorescence from the reaction products of rhodamine 6G and ubiquinone (Crane and Barr 1971) was seen clearly in the spots containing 40 ng ubiquinone or more. We tested the following reagents in various ways but without success: phloroglucinol, *orto*-phthalaldehyde, *N*,*N*'diphenylbenzidine, 2,4-dinitrophenylhydrazine, and ethyl cyanoacetate.

Discussion

Although the pathways leading to carotene and the terpenoid moiety of ubiquinone share the vast majority of their biosynthetic reactions, we have found that they are

Table 3 Ubiquinone and carotene content of colonies obtained after incubating a mycelial fragment of wild-type *Blakeslea* and *Phycomyces* on minimal agar with 0.1-mg/l oligomycin for 4 days

Culture	Oligomycin	Content, μg/g dry mass		
		β-carotene	Total carotene	Ubiquinone
Blakeslea	no	244±13	416±17	321±17
	yes	140±17	240±22	303±23
Phycomyces	no	76±5	147±22	229±27
	yes	17±3	45±6	429±10

The total carotene content is the sum of the contents of β-carotene, γ-carotene, lycopene, and phytoene in the case of *Blakeslea* and of β-carotene and phytoene in the case of *Phycomyces*. The means and their standard errors of 2 to 15 determinations in two to four independent experiments are shown

regulated independently. We found that the large variations in carotene content caused by mutations and sexual stimulation were not accompanied by changes in the content of ubiquinone. The opposite might be expected for a branched pathway, with variations in the flow of substrates into one branch modifying the flow into other branches, at least when mutations disrupt the regulatory mechanisms. The absence of coregulation is consistent with the strict separation of the two pathways, from acetyl-CoA to their end products, in subcellular compartments that do not exchange their intermediary metabolites (Kuzina et al. 2006).

Our cultures of various strains of *Blakeslea* and *Phycomyces* contained ubiquinone at about 0.3 mg/g dry mass (about 3 mg/l) under experimental conditions that are far from optimal for industrial production. This value is similar to those found in many microorganisms, but far lower than claims of up to about 10 mg/g dry mass (0.8 g/l) after genetic manipulation of bacteria and optimization of the culture conditions (Choi et al. 2005).

Our results with various nutrients should be considered in the design of production media for ubiquinone and carotene. Some conditions that increased the ubiquinone content decreased mycelial growth to a larger extent. L-Leucine represents a useful addition because it increased the ubiquinone content with little effect on growth. High initial glucose concentrations seemed to inhibit the accumulation of ubiquinone; thus, a gradual addition of glucose to the media would probably lead to higher ubiquinone contents.

Continuous white light illumination had varied effects on the mycelial content of terpenoids. The ubiquinone content of *Phycomyces* was not affected, but that of *Blakeslea* was increased by about one third. More substantial effects on the carotene content, an increase in *Phycomyces* (Bejarano et al. 1991) and a decrease in *Blakeslea* (Sutter 1970), were confirmed.

The oxidative stress due to oligomycin modified the ubiquinone content in *Phycomyces* and the carotene content in both fungi. The *Phycomyces* mutants obtained because of their resistance to oligomycin were unstable, had no practical interest, and were not further characterized. They are reminiscent of the *petite* mitochondrial mutants of *Saccharomyces*, but *Phycomyces* is strictly aerobic (De Boer 1928), while *Saccharomyces* can live anaerobically and tolerates the loss of functional mitochondria. The growth of *Phycomyces* drops to a half of its maximal value in the presence of 2% O_2 and is abolished with less than 0.5% O_2 present (Galland and Russo 1979).

Mutants with altered ubiquinone content would be useful in the study of metabolism and to industry. In the absence of an obvious phenotype, we tried to develop a color test with reagents that yield colored products with ubiquinone. A similar approach allowed the isolation of mutants nearly devoid of gallic acid (Weinkove et al. 1998). The color reactions that we tried, except one, were not sufficiently sensitive to detect the ubiquinone present in a single wild-type colony. Rhodamine fluorescence is very sensitive but has several drawbacks. It is lethal to the fungi and would require duplication of the colonies before the test. The main problem with rhodamine is its lack of specificity. Sterols would be detected (Kemp and Mercer 1968) and these are very abundant in *Phycomyces* and *Blakeslea* (Barrero et al. 1998, 2002; Kuzina et al. 2006), some 20 times more abundant than ubiquinone. Only very large increases in ubiquinone content could be detected, and these are likely to be infrequent. In the search for very large increases in ubiquinone contents, rhodamine staining of single colonies does offer an alternative to individual chemical analyses by chromatography or other means.

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