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EXPERIMENTAL ARTICLES

Genetic Instability of the Short-Living Ascomycetous Fungus *Podospora anserina* Induced by Prolonged Submerged Cultivation

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Abstract—Investigation of genetic variability of the short-living filamentous fungus *Podospora anserina* during its adaptation to conditions of prolonged submerged cultivation has been carried out for the first time. Cultivation of *P. anserina* under aeration (on a shaker) provides pronounced selective pressure, which makes it possible to obtain isolates with specific features, which are well adapted to cultivation in liquid media and have a life span several times exceeding that of the original strain. Static cultivation did not prevent the ageing of *P. anserina*. Repeated transfers in the shaker culture resulted in formation of mycelium deprived of the dark pigment melanin and actively producing carotenoids under illumination. The qualitative composition of *P. anserina* carotenoids was the same as in the closely-related species *Neurospora crassa*. The features obtained during the shaker cultivation (including changes in the colony morphology and decreased capacity for melanin synthesis) are inherited by their hybrids with the wild type strains, i.e., they resulted from the intragenomic rearrangements occurring during submerged cultivation of the fungus.

Keywords: Podospora anserina, submerged cultivation, genetic instability, phenotypic variants selection, carotenoids.

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Genetic instability is one of the mechanisms broadening the ranges of inherited variability and thus resulting in increased adaptive capacities of the population [1–3]. The fungal genome is highly dynamic and capable of rapid accumulation of various modifications [4]. For example, amplifications and deletions were shown to be a normal response of *Saccharomyces cerevisiae* under conditions of "experimental evolution", i.e., under strong selective pressure during prolonged continuous cultivation [3]. Numerous references to genetic instability of the fungal strains may be found in the literature, including its manifestations under natural [1] and laboratory conditions, e.g., during numerous transfers on growth media [5, 6], chemostat cultivation [7], and cryoconservation [2].

The current interest in microbial instability is associated with development of biotechnology, since the variants differ in activity, growth rates, physiological characteristics, and affect on production of the biologically active compounds [8, 9]. In some cases, genetic instability of mycelial fungi creates difficulties for industrial cultivation, especially in chemostats, since continuous cultivation often results in emergence of the variants which are more competitive and stable, but less productive than the original phenotype [7]. On the other hand, the selection of bacterial and yeast variants with improved characteristics is often achieved by prolonged selection in batch or continuous submerged culture [10]. Few works deal with random or purposeful submerged cultivation of mycelial fungi resulting in the isolates exceeding the original variants in some biotechnologically important characteristics. For example, Swift et al. obtained an *Aspergillus niger* isolate with higher glucoamylase yield than the original strain [11]. Heat-tolerant strains of the entomopathogenic fungus *Metarhizium anisophilae* were obtained by continuous submerged cultivation [12]. The mechanisms responsible for the variability of fungal cultures, are, however, studied insufficiently. Development of the model system for efficient enhancement of genomic instability and obtaining the phenotypes with different characteristics is therefore an urgent task.

The ascomycetous fungus *Podospora anserina* (Rabenh.) Niessl is a well-known model object. Since the 1950s, this species has been widely used for investigation of such fundamental biological phenomena as cell differentiation, meiosis, mitochondrial functioning, prion properties, ageing, vegetative incompatibility, cell death, etc. [13, 14]. *P. anserina* is among the few fungal species with the wild type strains subjected to pronounced replicative ageing and death [15]. The ageing of *P. anserina* is defined as a decreased cell capacity for proliferation and/or differentiation. Similar to fibroblasts and yeast cells, ageing occurs in the mycelial cells undergoing the highest number of divisions [16]. Under standard laboratory conditions (continuous growth in special tubes with agar medium

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or transfers on petri dishes), the average life span of *P. anserina* wild strains (from ascospore germination to mycelial death) is 25 days. On rich agar medium, the young and mature *P. anserina* mycelium grows at a linear rate of ~7 mm/day. During the following ageing stage, the growth rate gradually decreases and phenotypic changes occur: pigmentation intensifies and formation of aerial hyphae becomes suppressed. Finally, the vegetative growth ceases completely and the peripheral hyphae die off. This effect is a stable feature of the *P. anserina* life cycle [15].

The ageing of *P. anserina* is controlled by a number of various endogenous and environmental factors. It is therefore an integrated process, affected by many factors [16]. Mitochondria play the central role in life span regulation of *P. anserina*. They act in two mutually-related ways: by age-dependent accumulation of deletions and rearrangements in the mitochondrial genome, which in this species are associated with amplification of the α-senDNA mobile element; and through the action of oxidative stress caused by reactive oxygen species produced in the course of the functioning of mitochondria [15, 17, 18].

P. anserina was mainly studied in surface culture (on agar media). In a single work published in 1987, Turker and Cummings found that in a shake flack (agitated) submerged culture maintained by periodic serial transfers, *P. anserina* mycelium was capable of unlimited vegetative growth. Moreover, the isolates obtained by plating mycelial fragments after prolonged submerged cultivation to agarized medium became long-lived and, in some cases, "immortal" [19]. The "immortality" of fungal organisms implies potential capacity of their vegetative cells for unlimited proliferation [17]. However, the behavior of *P. anserina* in submerged cultures was not studied further. In particular, its effects (apart from the increased lifespan) and their probable mechanisms are unknown.

As a model object for investigation of genetic instability, *P. anserina* has certain advantages, including ease of cultivation, the thoroughly studied life cycle, rapid sexual reproduction (the time from ascospore germination to a new generation of ascospores is about a week), the possibility to obtain homokaryotic strains from a single ascospore, and a completely sequenced genome [13, 14]. Moreover, while the sexual process is present in *P. anserina*, asexual reproduction is completely absent. The microconidia of *P. anserina* are almost incapable of germination and act as spermatia, i.e., are used for fertilization of protoperithecia [15].

The goal of the present work was to investigate the variability of *P. anserina* under prolonged submerged cultivation and to carry out the morphological, physiological, and genetic investigation of the surface-growing isolates obtained by plating the mycelium from submerged cultures onto agar medium.

MATERIALS AND METHODS

Objects and cultivation conditions. *P. anserina* strains s and GFP were kindly provided by Annie Sainsard-Chanet and Carole H. Sellem, Département Biologie Cellulaire et Intégrative, Centre de Génétique Moéculaire, CNRS, Gif-sur-Yvette Cedex, France. Strain s is among the most widely used wild type *P. anserina* strains [20]. Strain GFP is a transformant of strain s bearing the green fluorescent protein (GFP) as the mitochondrial label. The *gfp* gene in this strain is bounded to the constitutive promoter of the *gpd* gene [18], making it possible to observe directly the mitochondrial morphology and their distribution in the cytoplasm. Haploid generative descendants of these strains obtained by germinating of single monokaryotic ascospores, s1(+) and GFP1(+), respectively, were used for experiments in prolonged submerged cultivation.

Life span of *P. anserina* was determined by serial transfers on petri dishes with 3-day intervals (for inoculation, the mycelium from the edge of a colony was always taken). Life span was expressed in days from isolation of a strain to complete cessation of mycelial growth. In some experiments (specifically mentioned in the text), life span was determined by cultivating *P. anserina* in special tubes with agar medium, 30–40 cm long (i.e., exceeding the maximal length of mycelium for *P. anserina* strains with a standard life span). In this case, life span was expressed in cm.

P. anserina was grown in the dark in surface and submerged cultures on the standard synthetic medium M2 [21]. The medium contained the following (g/l): dextrin, 10; KH_2PO_4, 0.25; K_2HPO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.25; urea, 0.5; ascorbic acid, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 2.5×10^{-4}; $MnSO_4 \cdot H_2O$, 5×10^{-5}; H_3BO_3, 5×10^{-5}; $Na_2MoO_4 \cdot 2H_2O$, 210^{-5}; thiamine, 5×10^{-5}; and biotin, 2.5×10^{-6}. Surface cultivation was carried out on agarized (20 g/l) M2 medium in petri dishes; submerged cultivation was carried out in 750-ml Erlenmeyer flasks with 100 ml of the medium at 27 ± 1°C. For inoculation, 2–3 blocks of agar medium (5 × 5 mm) with *P. anserina* surface mycelium were added to the liquid medium. Both the young and the ageing mycelium were used for inoculation (6–11 and 20–23 days after the ascospore germination, respectively).

To vary the aeration conditions, two types of submerged cultivation were used: static (without mixing) and shaken (on a 200-rpm rotor shaker). Every 3 days submerged cultures were transferred to fresh medium. During submerged growth, mycelium samples were plated on the agar medium and their life spans were determined by transfers on petri dishes. These surface cultures will henceforth be termed "isolates." Depending on the character of growth, the following inocula were used: blocks of agar medium (5 × 5 mm) with mycelium from the edge of a colony for surface cultivation; fragments of mycelial mats (5 × 5 × 5 mm)

for static submerged cultivation; and 2–3 pellets of average size (5–10 mm in diameter) for shaken submerged cultivation.

The initial surface cultures of *P. anserina*, submerged cultures, and the surface cultures of the isolates obtained from submerged cultures were grown in 10–15, 2–3, and 5 repeats, respectively.

Cytological observations were carried out under an Axioskop 40 FL light microscope (Zeiss) equipped with an AxioCam MRc digital camera (Zeiss), using the ×100 lens.

Analysis of carotenoid pigments. To stimulate the synthesis of carotenoids, *P. anserina* cultures were incubated in the light (60 μE/(m^2 s) for 3–6 days. The carotenoids were extracted with acetone or with the Folch mixture (chloroform : methanol 2 : 1) [22]. Spectral measurements in the 350–750 nm range were carried out on a Hitachi 150-20 spectrophotometer (Japan) in a 1-cm cuvette against the relevant solvents (acetone or chloroform).

For comparative investigation of *P. anserina* carotenoids, *Neurospora crassa*, a closely related fungal species with a thoroughly studied pigment composition, was used. The wild type strain *N. crassa* (RL3-8a)[FGSC 2218] was obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas City, United States. The mycelium was grown in 200 ml of the Vogel synthetic medium [23] in 750-ml flasks in the dark on a rotary shaker (200 rpm) at 28ºC for 22 h. The mycelium was then separated from the culture liquid, placed on filter paper disks in petri dishes, and exposed to light for 5 h in order to induce carotenoid synthesis. The pigments were extracted with the Folch mixture.

Qualitative analysis of the pigments was carried out by high-performance liquid chromatography (HPLC) on a Knauer chromatograph (Germany) equipped with a K-5002 embedded degasser, two K-501/1 MiniStar pumps, a Prontosil RP C-18 reversed-phase column (150 × 4.5 mm, Upchurch Scientific, United States), and a K-2500/1 UV detector. The following system was used for separation: acetonitrile : water (85 : 2) as component A and ethyl acetate as component B at 1 ml/min flow rate. Gradient elution was carried out in three stages: 0–30% (18 min), 30–100% (6 min) of component A, and 100% (6 min) of component B. Detection was carried out at 455 nm.

Crossing experiments. Reciprocal crossing was carried out between the selected *P. anserina* isolates and the wild-type tester haploid strains (sexual descendants of strain s) having the "–" mating type. The sexual descendants were obtained for the following isolates: V-s1-IV(3), V-GFP1-43(1), V-GFP1-51(1), V-GFP1-XXXVIII(4), V-sl-46(4,5), and V-sl-22(1,2)light (the latter variant was grown under light after plating from the relevant rotation culture).

The crossing was carried out according to the standard procedure [19]. The colonies of each isolate and each wild partner were grown on M2 medium in two variants: on petri dishes (acting as a maternal parent) and on agar slants (acting as a paternal parent). The mycelium was initially grown in an incubator for 3–6 days depending on the growth rate, and then incubated under illumination for 4–5 more days in order to facilitate formation of the sexual structures (microconidia and protoperithecia). Prior to the crossing, mycelial samples from all the dishes and test tubes were examined microscopically in order to determine the presence of the sexual structures. For fertilization, the test tubes were filled with sterile water (10–15 ml), stirred vigorously to remove the microconidia (10–15 min by hand or 1 min on a shaker), and poured over the petri dishes with the colonies of the relevant maternal partners. Excessive water was removed after 10–40 min. Each variant of crossbreeding was carried out in 2–8 repeats.

The plates with fertilized colonies were incubated in the light until the formation of the fruiting bodies and onset of ascospores shot. Under a dissecting microscope, the ascospores were isolated with sharpened needles from the lids of the petri dishes covered with water agar (30 g/l). The ascospores were isolated only from the five-spore asci containing three binuclear and two mononuclear spores. The ascospores were germinated on M1 medium [21]. The phenotype of the descendents was described and the life spans of some of them were determined.

RESULTS

Comparative Dynamics of the Growth Parameters of P. anserina Mycelium under Prolonged Rotational and Static Cultivation

Transfer of the wild type surface-cultivated *P. anserina* mycelium at the last stages of ageing (after complete cessation of growth) into liquid medium was found to result in resumption of growth. After 6–9 days of incubation in a shaken or static submerged conditions, the mycelium started to form new hyphae. These restored cultures transferred every 3 days behaved similar to the regular submerged cultures obtained from the young mycelium. The restoration of growth of the ageing *P. anserina* mycelium in liquid medium will be subsequently termed reanimation. The ratio of successful reanimations in the shaken and stationary cultures was 75 and 100%, respectively.

Morphological and physiological characteristics of *P. anserina* in shaken culture. Among four parallel shaken cultures obtained from the young and ageing mycelium of strains s1 and GFP1 *P. anserina*, the highest number of transfers for submerged s1 and GFP1 cultures was 50 and 62, respectively. The total life duration from ascospore isolation to the termination of the experiment was 161 and 200 days, respectively, while the same strains grown exclusively on solid

Table 1. Duration of the adaptive phases of shaken cultivation of *P. anserina* (for the cultures obtained from young mycelium, the numbers of transfers are given in Arabic numerals, for reanimated cultures, in Roman numerals)

Strain	Transfer no. in liquid medium and the day of cultivation corresponding to growth cessation in the transfer		
	Initial phase (phase I)	Transitory phase (phase II)	Phase of stable growth (phase III)
GFP1	0–8 (14–38 days of growth)	9–11 (41–47 days of growth)	starting with 12 (starting with 50 days of growth)
Reanimated GFP1	I–XXX (32–119 days of growth)	XXXI–XXXIII (122–128 days of growth)	starting with XXXIV (starting with 131 days of growth)
s1	0–8 (11–35 days of growth)	9–18 (38–65 days of growth)	starting with 19 (starting with 68 days of growth)
Reanimated s1	I –IX (32–56 days of growth)	X–XIV (59–71 days of growth)	starting with XV (starting with 74 days of growth)

medium lived for 18.5 ± 2.1 and 20.5 ± 1.7 days, respectively.

By a cumulation of different characteristics, three phases of *P. anserina* adaptation to submerged conditions of shaken cultivation may be discerned. While the duration of these phases varied for the shaken cultures obtained from the young and ageing colonies of strains GFP1 and s1 (Table 1), their succession and general characteristics remained stable.

Phase I: the initial phase. Its main characteristics were the following. Mycelial growth occurred as spherical pellets 5–10 mm in diameter. The outer hyphal layer of the pellets consisted of the cells with the thickened cell walls and had dark pigmentation (sometimes black) apparently due to melanin synthesis [13, 14]. The outer layer was often crustlike. The inner part of the pellets consisted of loose nonpigmented hyphae. The outer layer cells contained numerous spherical mitochondria and exhibited intense fluorescence of protein GFP expressed in the mitochondria of strain GFP1. Such features are also characteristic of the chondriome of the ageing surface mycelium of *P. anserina* (Figs. 1b and 1d).

Phase II: the transitory phase. The mycelium gradually lost pigmentation, becoming light brown, yellowish, or even white in the case of this phase was longer (for the culture s1). The pellets became smaller (4–7 mm in diameter), with a loose outer layer, which was still formed by thick-walled cells. The mitochondria were mostly rod-shaped but still exhibiting intense fluorescence.

Phase III: the phase of stable growth. Intense biomass accumulation occurred, 3–5 times up to the values exceeding the biomass of the initial cultures (Fig. 2). The mycelium became homogeneous in color and consistency, almost white with a slight yellowish hue, very loose, sometimes pasty (in mycelium of strain s1). The pellets were numerous, formed by the mycelium of single type, and varying in size from large (5–10 mm) to very small (2–5 mm in diameter). Diffuse, not-pellet-forming mycelium was also always present in phase III. The chondriome acquired the characteristics of the young surface mycelium: all the mitochondria were rod-shaped, exhibiting weak GFP fluorescence (Figs. 1a and 1e).

During phases I and II of shaken *P. anserina* cultivation, numerous typical microconidia were formed (2.24 ± 0.05 μm), while during phase III microconidia either did not occur at all or were formed rarely as individual, somewhat larger cells. After the serially transferred cultures acquired all the properties of phase III, mycelium morphology did not change during subsequent transfers, and the biomass accumulation per transfer became relatively stable (Fig. 2). Rapid mycelial growth at a constant rate is the major physiological criterion of the youthful state of *P. anserina* cells.

On transition to phase III, the average life span of the isolates obtained by plating of mycelial samples from submerged cultures on agar medium began to increase (Fig. 3), becoming several times (from 1.5 to >8) longer than the life span of the control strains. Among the simultaneously transferred repeats, single very long-lived isolates were revealed, with their life duration several times higher than the average for this transfer (if such isolates were present, they are shown on Fig. 3 to the right from the average life span). Some isolates did not stop growth to the end of the experiment (marked with an arrow on Fig. 3). Their growth was maintained for 147 to 252 days from plating of the shaken culture to the termination of the experiment.

On solid M2 medium, the original wild type strains s1 and GFP1 had the growth rates of 6.3–6.9 mm/day and formed broadly growing lobed felted colonies with a well-developed aerial mycelium, gradually acquiring intense dark-gray pigmentation with a slight greenish hue, which intensified under light (Fig. 4). The isolates from phase I and II of shaken cultures usually formed weakly-pigmented, slowly-growing (2.5–5.5 mm/day) colonies, which partially or completely lost their aerial mycelium. The isolates from shaken cultures of phase III formed well-developed nonpigmented aerial mycelium of accumbent, felt-accumbent, or tufted-accumbent types, with the average growth rate of 5.5–6.7 mm/day.

Morphological and physiological features of *P. anserina* in stationary culture. Growth characteris-

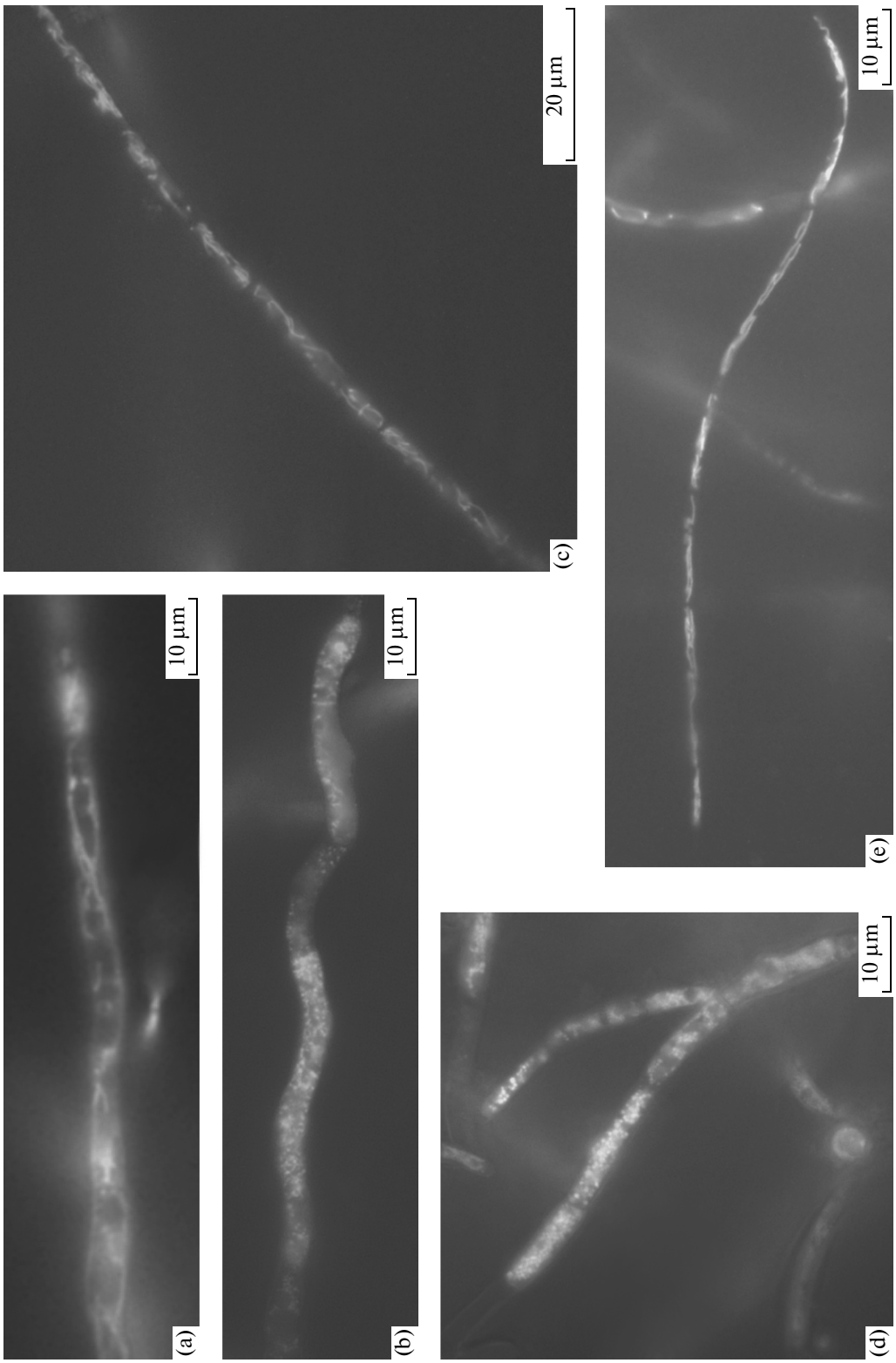

Fig. 1. Micromorphology of *P. anserina* GFP1 strain with fluorescent mitochondria under different cultivation conditions: surface culture, young mycelium, 11 days of growth (a); surface culture, old mycelium, 23 days of growth (b); static submerged culture, the first transfer (c); shaken submerged culture, phase I of adaptation (the 7th transfer) (d); and shaken submerged culture, phase III of adaptation (the 17th transfer) (e).

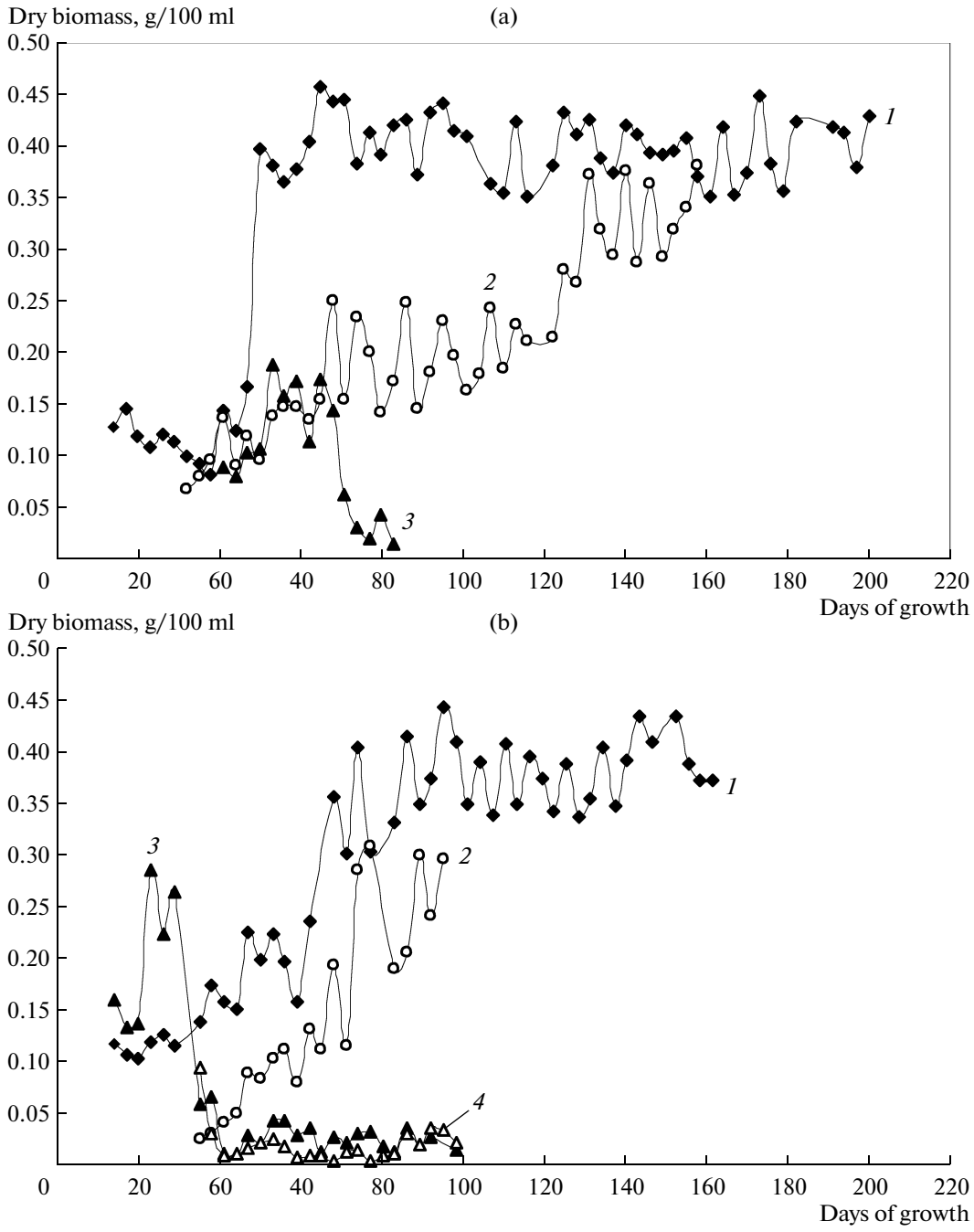

Fig. 2. Dynamics of biomass accumulation by *P. anserina* mycelium in submerged cultures per one transfer (3 days of growth). Strain GFP1: shaken culture (*1*), reanimated strain, shaken culture (*2*), and stationary culture (*3*) (a). Strain s1: shaken culture (*1*), reanimated strain, shaken culture (*2*), stationary culture (*3*), and reanimated strain, stationary culture (*4*) (b).

tics of *P. anserina* in the shaken and static cultures were different. In the latter case, the mycelium formed submerged mycelial mats growing radially from the pieces of inoculum (which precipitated to the bottom of the flask) and merging together. The mitochondria were of both spherical and rod-like shape, and the fluorescence was low or absent (Fig. 1c). In the cultures transferred under stationary conditions and in the resulting surface-grown isolates, the dark pigment was not lost; its production increased under illumination. The static cultures were maintained for a shorter period (29 and 15 transfers for strains s1 and GFP1, respectively), which was also much higher than the life span of the surface original cultures of the fungus. Under these conditions, however, the mycelium suffered pronounced replicative ageing: after 8–11 transfers of the static cultures obtained from young strains, the mycelium became slowly growing, the biomass

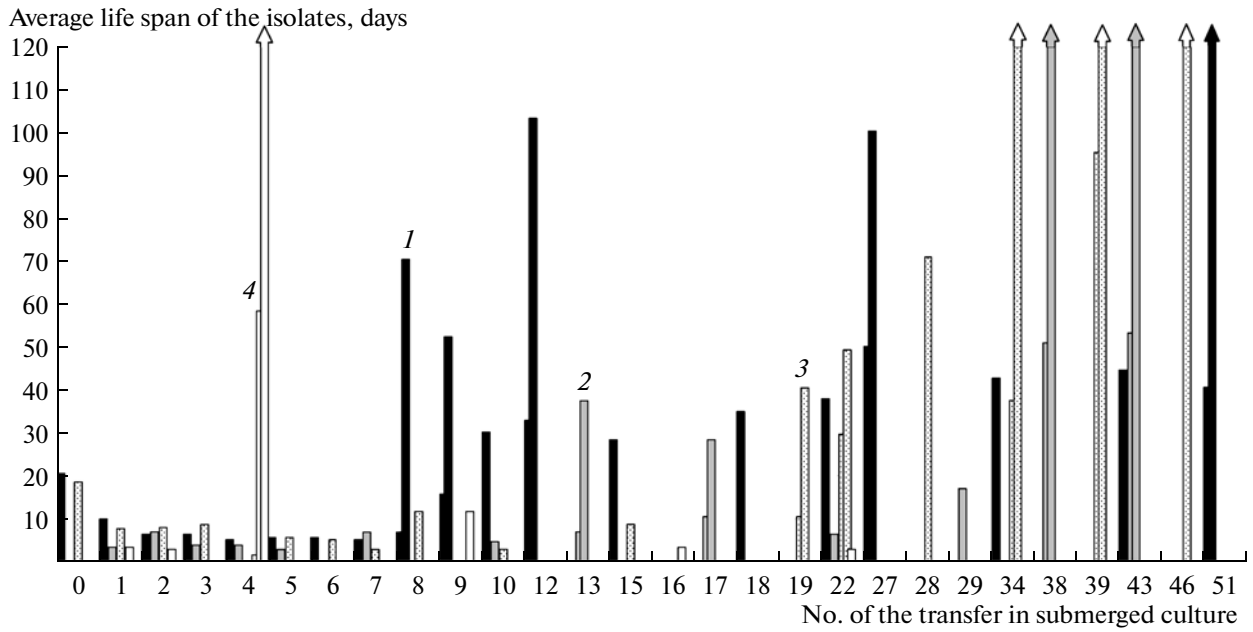

Fig. 3. Life span of *P. anserina* isolates obtained from shaken cultures: V-GFP1 isolates (*1*), V-GFP1 isolates from the reanimated culture (*2*), V-s1 isolates (*3*), V-s1 isolates from the reanimated culture (*4*), initial surface-growing GFP1 and s1 strains, respectively (0).

accumulation per transfer decreased sharply, and the life span of the isolates decreased below the control values (Figs. 2 and 5). The reanimated mycelium grown under static conditions produced ageing, slowly-growing cultures from the very beginning of subwerged growth. Unlike the surface cultivation of *P. anserina*, in spite of pronounced ageing in submerged cultures without stirring, complete growth cessation and mycelial death did not occur.

Thus, unlimited vegetative growth of *P. anserina* in submerged aerated cultures was confirmed and its relation to the cultivation conditions was established.

Identification and qualitative composition of carotenoid pigments of P. anserina

As was mentioned above, the mycelium of *P. anserina* grown by serial transfers in the shaken conditions stopped melanin synthesis at transition to phase III. The surface-cultured isolates obtained form these cultures also lost capacity for melanin synthesis. Under illumination, all melanin-free isolates acquired pink or pink–orange coloration, which did not depend on the number of transfers carried out on solid medium and retained invariable intensity until the end of the life span of the isolate or until the termination of the experiment. The phase III cultures incubated on a shaker under illumination also acquired pink coloration.

Absorption spectra of all the studied extracts from *P. anserina* mycelium had a typical carotenoid spectrum with the maxima and a shoulder in the blue-green range (400–550 nm). In the spectra of acetone extracts from the submerged and surface mycelium, the main maximum was observed at 476 and 474 nm, respectively; in chloroform extracts, the main maximum shifted to 489 nm for surface-growing micelium. The spectra of the extracts from mycelia of *P. anserina* and the closely related species *N. crassa* were similar in shape, amplitude ratio, and position of the maxima.

Chromatographic analysis revealed that the qualitative composition of the major carotenoids synthesized by *P. anserina* coincided with the carotenoid composition of *N. crassa* (Fig. 6). Comparison with the literature data [24–26] indicates high probability of identification of the peaks as neurosporoxanthin, torulin, γ-carotene, and β-carotene. In both fungal species under the experimental cultivation conditions, torulin was the predominant carotenoid, with neurosporoxanthin being the second most abundant (except for the surface culture of *P. anserina* where it was present in insignificant amounts) (Fig. 7). The qualitative composition of *P. anserina* carotenoids did not depend on the isolate and cultivation conditions.

The fact that *P. anserina* is a carotenogenic fungus was originally established by Strobel et al. in 2009 [27]. In that work, however, the carotenoid yield of *P. anserina* was very low, lower than in *N. crassa*. This is understandable, since under normal conditions melanin is the main light-protecting pigment of *P. anserina* [13, 14]. In the melanin-free, surface-growing *P. anserina* isolates, bright carotenoid coloration was observed, similar in intensity to that of the *N. crassa* wild type strain. The same is true for the shaken cultures of *P. anserina* which were grown in the dark for 3 days in order to induce carotenoid synthesis (during

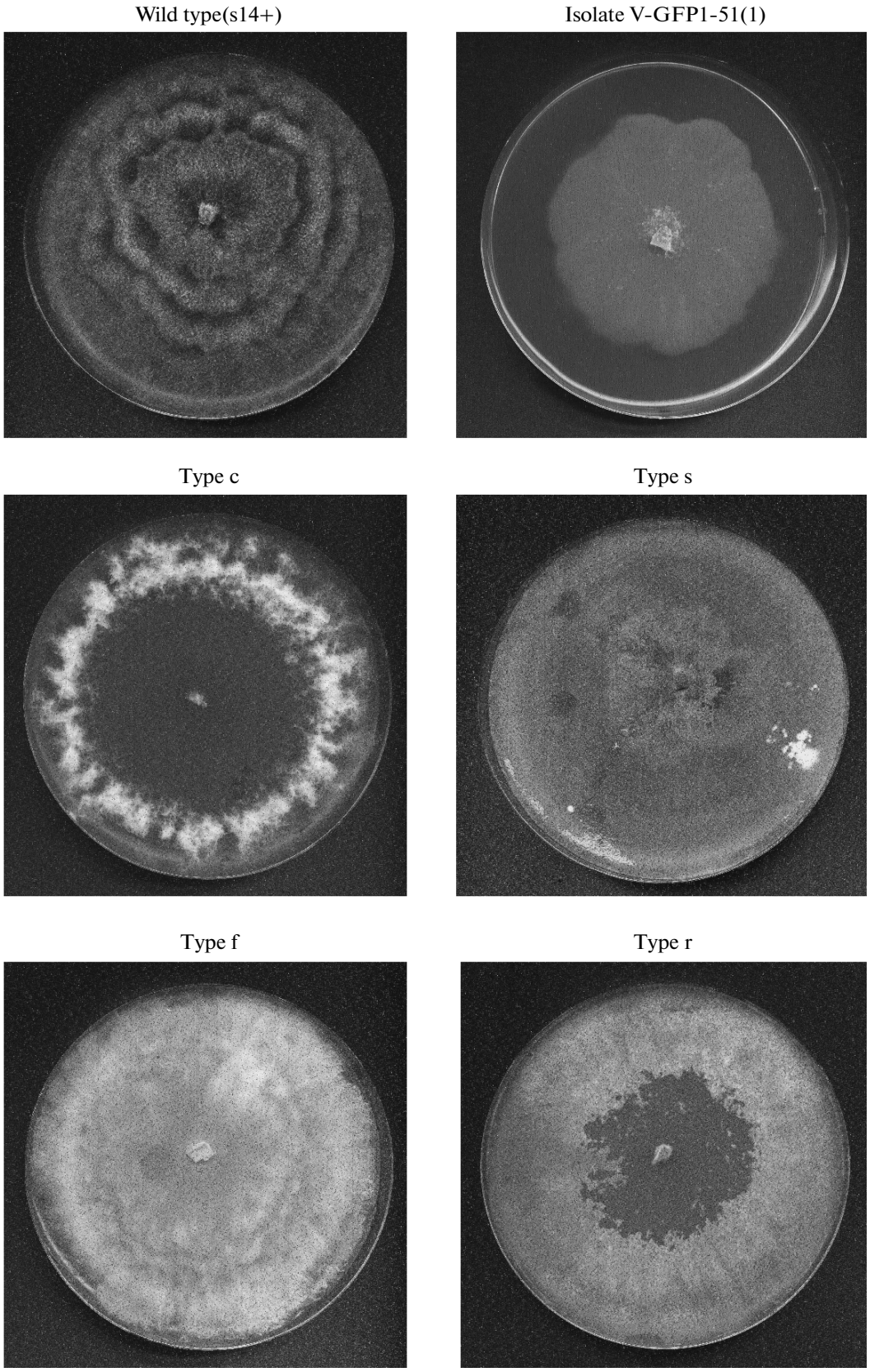

Fig. 4. The main morphological types of *P. anserina* colonies.

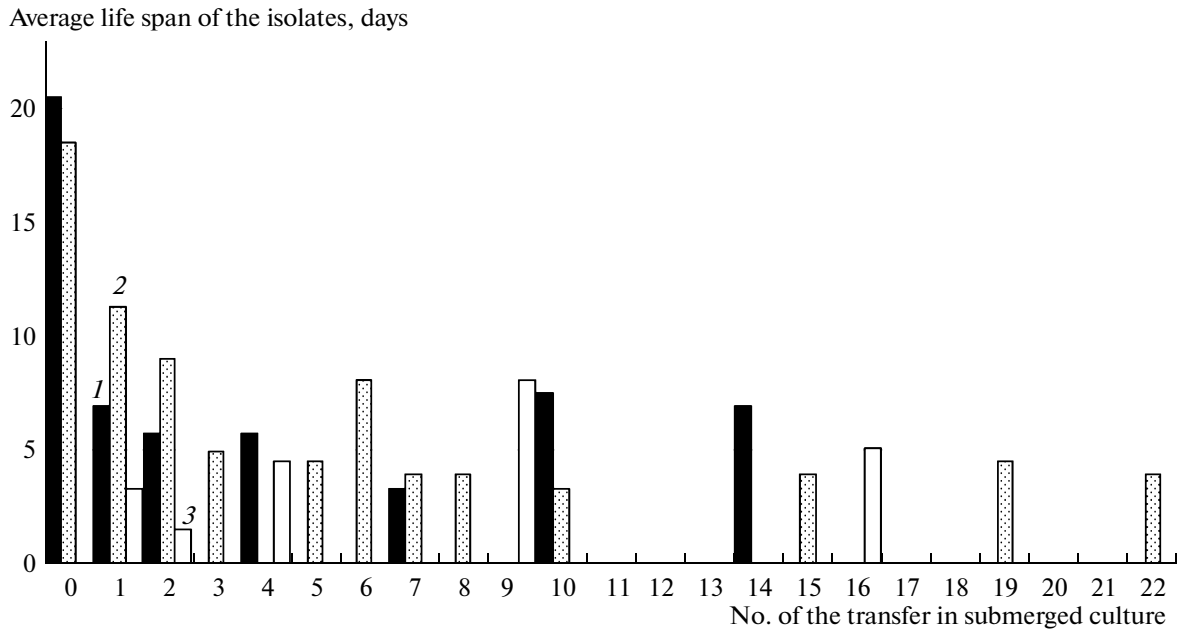

Fig. 5. Life span of *P. anserina* isolates obtained from static cultures: V-GFP1 isolates (*1*), V-s1 isolates (*2*), V-s1 isolates from the reanimated culture (*3*), initial GFP1 and s1 strains, respectively (0).

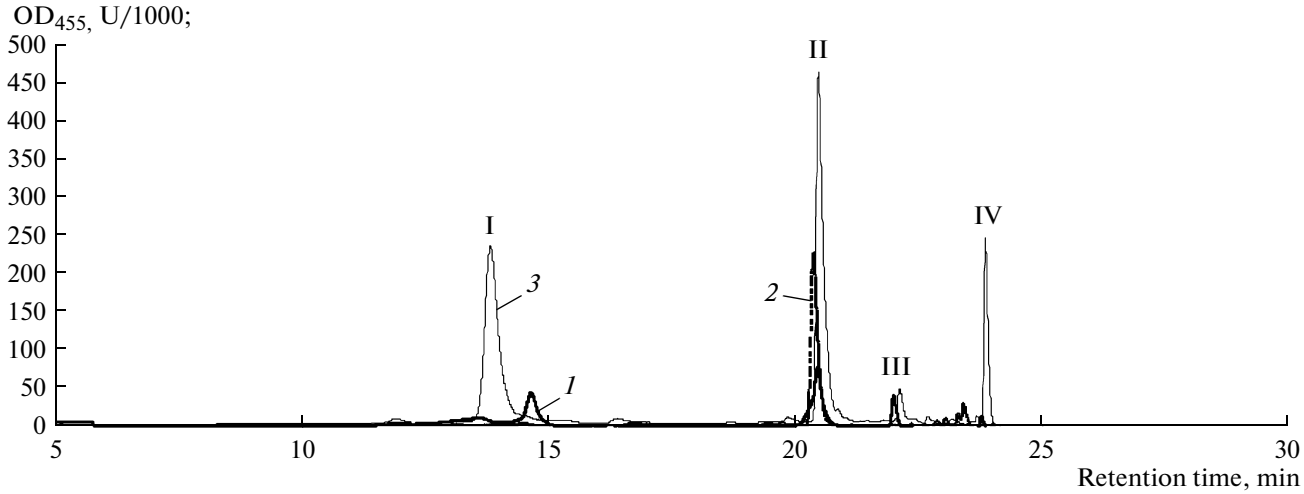

Fig. 6. Chromatograms of mycelial extracts from *P. anserina* and *N. crassa*. Detection at 455 nm: *N. crassa*, submerged mycelium (chloroform) (*1*); *P. anserina*, isolate V-GFP1-22, 17 days of growth, surface mycelium (chloroform) (*2*); and *P. anserina*, culture s1, 152 days of growth, submerged mycelium (acetone) (*3*). Neurosporoxanthin (I), torulin (II), γ-carotene (III), and β-carotene (IV).

this period, the biomass reached the plateau) and then incubated for 3 more days under light. Such a procedure for induction of carotenoid synthesis resulted in their concentrations in submerged mycelium of 149 and 187 μg/g dry mycelium for s1 and GFP1, respectively. When the shaken cultures of *P. anserina* were grown under light for 6 days right after inoculation, carotenoid content increased drastically to 2844 and 790 μg/g dry mycelium for s1 and GFP1, respectively. Under these conditions, the mycelium became brick-red. Intensive brick-red coloration of submerged aerated mycelium growing under light developed immediately after starting of new hyphae growth.

Stimulation of carotenoid synthesis under illumination with blue-violet light is known for many fungi and some bacteria [28]. Formation of carotenoids by the nonpigmented *P. anserina* mycelium under light is probably a form of photoprotection and compensates the absence of melanin. Light-induced accumulation of carotenoid pigments in the cells of *N. crassa* is known to be an oxygen-dependent process. Accumulation of carotenoids by this fungus intensifies in the

atmosphere of pure oxygen and drops sharply in oxygen-free atmosphere compared to the control (the standard oxygen content in air). Growth of *N. crassa* mycelium in liquid medium without stirring, where contact with oxygen is limited, also suppresses accumulation of carotenoids [29]. In a similar manner, submerged shaken cultures of *P. anserina*, which were potentially capable of carotenoid synthesis, remained nonpigmented even under illumination when incubated under static conditions, which provided for decreased oxygen supply.

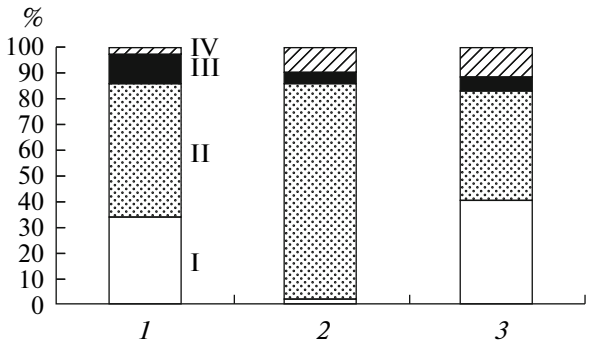

Fig. 7. Carotenoid composition of mycelia of *P. anserina* and *N. crassa* (% of the total carotenoid content): *N. crassa*, submerged mycelium (*1*); *P. anserina*, isolate V-GFP1-22, 17 days of growth, surface mycelium (*2*); and *P. anserina,* cuilture s1, 152 days of growth, submerged mycelium (*3*). Neurosporoxanthin (I), torulin (II), γ-carotene (III), and β-carotene (IV).

Crossbreeding of the Long-Living P. anserina Isolates with the Wild Type Strains

Reciprocal crossing of long-living *P. anserina* isolates with wild type strains that was carried out by the method of microconidia washing revealed the loss of female fertility in the isolates. When they acted as maternal parents, they usually did not form fruiting bodies, rarely producing single fruiting bodies. Most of the *P. anserina* isolates could therefore be used only as male parents (Table 2). These data are in agrement with the results of Turker and Cummings [19] who also observed the loss of female fertility in *P. anserina* after submerged cultivation.

The total number of sexual descendants was 452, including 189 dikaryotic and 263 homokaryotic strains. All descendants of the isolate V-s1-IV(3), which originated from the phase I shaken culture, had wild type colonies, i.e., they reproduced only the phenotype of the wild type parent. Other *P. anserina* isolates used for crossing were obtained from phase III submerged cultures, and their descendants (both homokaryotic and dikaryotic) exhibited a variety of morphological characteristics (Fig. 4). Among the latter, some variants completely reproduced the parental phenotypes (both of the wild type strain and of the isolate). Apart from the parental phenotypes, however, a broad spectrum of features was observed, which were not present in crossed parents. Importantly, most of the asci contained spores producing the strains of more than two different morphological types.

Among the first generation of sexual descendants obtained as a result of crossing of the long-living *P. anserina* isolates with the wild type strains, four morphological groups were determined, which differed in the type of the aerial mycelium formed on M2 agar medium (Fig. 4). Type c was characterized by shaggy aerial mycelium formed in the center of the colony, at its edge, or over its whole surface. Type s had brownish aerial mycelium of specific morphology: the colonies looked floury of powdery due to overproduction of microconidia. In our experiments, only the isolates V-s1-22 and V-s1-22light were found to overproduce microconidia. Such an overproduction was not observed in the original control strains, in the crossing tester strains, or in other surface-growing *P. anserina* isolates, including those used for crossbreeding. Type f was a heterogeneous group differing from type c in formation of dense, accumbent or felt-accumbent aerial mycelium, more or less uniform over the surface of a colony. Unlike type f, type r formed spreading, partially reduced aerial mycelium. The descendant strains with the colonies of types c, s, and r exhibited no melanin coloration. Under illumination, the colonies of types c and r synthesized the pigments responsible for pink coloration. The colonies of the s type were brown. Type f had an intermediate pigmentation with weak melanin synthesis combined with carotenoid production. The morphological types of the descendants of *P. anserina* isolates, their share in the total amount, and average life span are listed in Table 2.

DISCUSSION

The genotypic variability of *P. anserina* based on the adaptive reactions of the mycelium during prolonged submerged cultivation was studied for the first time. Crossing of the long-living *P. anserina* isolates obtained by plating of mycelial samples from the shaken cultures of phase III of adaptation and the wild type strains revealed a variety of morphological types among the first generation descendants, including those not found in any of the parents. This is an indication of selection of various mutations under such conditions. Consequently, the morphological characteristics of the isolates of phase III resulted from intragenomic rearrangements.

The mycelial culture of *P. anserina* should probably be considered as a population of relatively independent cells where the microevolution processes occur, which efficiency depends on growth conditions. In the shaken variant of cultivation, *P. anserina* mycelium experienced both selective pressure and efficient selection. Efficient selection probably resulted from the spatial organization of the mycelium as pellets. Under static conditions, no factors exist causing the

Table 2. Main characteristics of the first generation descendants from breeding of long-living *P. anserina* isolates with wild-type strains

Isolate	Crossing variants	Main characteristics of the descendants			
		Total number	Morphological types[1], %	Number of homokaryotic strains	Average life span[2], cm
Wild type testers		4	Wild – 100	4	12.8 ± 1.0 (4)
V-GFP1-43(1)[3]	♀ V-GFP1-43(1)	6	Wild – 16.7 Type V-GFP1-43 – 16.7 Type f – 33.3 Type r – 33.3	6	13.0 ± 1.5 and 58.1 (6)
	♂ V-GFP1-43(1)	5	Wild – 40 Type V-GFP1-43 – 20 Type s – 40	5	18.5 ± 7.0 and 51.5 (5)
V-GFP1-51(1)	♂ V-GFP1-51(1)	99	Dikaryons: Wild – 48 Type f – 52 Homokaryons: Wild – 35.1 Type V-GFP1-51 – 33.3 Type f – 21.1 Type r – 10.5	57	10.4 ± 1.4 (9)
V-GFP1-XXXVIII(4)	♂ V-GFP1-XXXVIII(4)	28	Wild – 71 Type V-GFP1-XXXVIII – 29	28	–
V-s1-22(1, 2) light[4]	♂ V-s1-22(1.2) light	197	Dikaryons: Wild – 3.8 Type V-s1-22 light – 28.3 Type s – 30.2 Type f – 6.6 Type r – 3.8 Type c – 27.3 Homokaryons: Wild – 31.9 Type V-s1-22 light – 12.1 Type s – 28.6 Type f – 14.3 Type r – 3.3 Type c – 9.8	91	12.0 ± 1.2 (36)
V-s1-46(4, 5)	♂ V-s1-46(4.5)	100	Dikaryons: Wild – 0 Type V-s1-46 – 34.1 Type s – 19.5 Type f – 24.4 Type r – 17.1 Type c – 4.9 Homokaryons: Wild –16.9 Type V-s1-46 – 10.2 Type s – 33.9 Type f – 27.1 Type r – 6.8 Type c – 5.1	59	14.9 ± 2.5 (3)
V-s1-IV(3)	♀ V-s1-IV(3)	2	Wild – 100	2	15.5 and 16.6 (2)
	♂ V-s1-IV(3)	15	Wild – 100	15	15.0 ± 1.5 (8)

[1]The morphological types of the descendants are described in the text.

[2]Life span was determined only for the homokaryotic strains belonging to different morphological groups. Continuous growth of descendants of the isolates V-GFP1-43(1) and V-s1-IV(3) was maintained by serial transfers in petri dishes every 3 days, in other cases, the descendants were grown in the tubes with solid medium. The number of descendants for which the life span was determined is given in parentheses. For the cases when the scattering of the life span values was high, the results of a typical experiment are presented, rather than the average value.

[3]In the name of an isolate, its number corresponds to the number of the transfer for the original submerged culture. The transfers of the cultures obtained from the young original strain are numbered in Arabic numerals and the numbers for the transfers of reanimated cultures are in Roman numerals. The repeat number is given in parentheses.

[4]The petri dishes with the isolates were incubated under illumination (12 h day / 12 h night).

mechanical damage of the mycelium, pellet formation, and often pellet fragmentation, i.e., formation of numerous relatively independent subpopulations where the original wild type genotypes may be replaced by newly formed variants. Since during the first several transfers the static cultures accumulated more biomass than the shaken ones and the differences in their cells exhibited minimal morphological differences from the surface-growing control (Fig. 1), the absence of mixing in the case of static submerged cultivation is probably a favorable factor for initial adaptation of *P. anserina* to growth in liquid medium. Further transfers under static conditions resulted, however, in growth suppression. This is probably a certain variant of ageing of *P. anserina*, which is significantly extended in time. Perhaps in the case of submerged cultivation without mixing, the *P. anserina* mycelium forms an integrated continuous network where specific ageing factors (α-senDNA being the most important), which are amplified in the cells and participate in the ageing-related degradation of the mitochondrial DNA [15], may freely migrate and accumulate, causing growth retardation, i.e., the ageing of the culture as a whole.

The features common for most of the long-living isolates obtained in phase III of shaken submerged cultures included the absence of melanin synthesis, production of carotenoids under illumination, lost of capacity for microconidia formation, complete or partial loss of female fertility, and the changed structure of the aerial mycelium. Such phenotypic characteristics as the type of the aerial mycelium, its pigmentation, and intensity of microconidia formation were inherited as a complex. Some or all of the mutations acquired during the shaken cultivation probably have a pleiotropic character of phenotypic expression. It should be noted that in most of the experiments on prolonged chemostat cultivation of *A. niger*, stable nonpigmented variants became dominant, which did not form conidia or had a decreased capacity for conidia formation and had growth rates higher than that of the wild type [7]. Similar mechanisms may underlie the variability of *P. anserina* and *A. niger*.

The loss of capacity for melanin synthesis, which was genetically established in a specific genotype, however, hardly enhanced the adaptive characteristics of the culture. Melanins carry out a number of important functions in fungal cells and facilitate their survival in the environment [30]. The same applies to the loss of female fertility observed in the *P. anserina* isolates obtained from parallel cultures. However, the genetic material of an organism is an integrated system, so that selection according to a specific feature, even a more or less discrete one, is accompanied by the correlational rearrangements of other features [31]. Optimization of real systems is possible only as a compromise between the contradictory requirements for optimization of specific parameters [32]. It may therefore be concluded that the relatively rapid intragenomic rearrangements during shaken cultivation of *P. anserina* are directed to increased functional adaptability of some subsystems at the expense of the functioning of the other ones. Reduction of some functions is an inevitable result of this process.

The ageing of many species of living organisms is closely associated with genetic instability. Age-dependent changes in DNA include changes in the gene expression, the decrease in DNA repair efficiency, increased mutation rate, inactivation of a number of genes (sometimes due to insertion of the mobile elements), chromosomal rearrangements, chromosome fractures, etc. [33]. For example, the ageing of the individual cells of *S. cerevisiae* is associated with increased genetic instability, which is retained until cell death [34]. Efficient induction of genetic instability in *P. anserina* may be also associated with ageing. In the model system of serial transfers of *P. anserina* under conditions of submerged shaken culture, a synergistic effect of mycelial ageing associated with genome destabilization and selection of the cells, which for some reasons escaped ageing, may occur, resulting in the possibility of obtaining long-lived isolates by plating on solid media.

Further investigation of the variability of *P. anserina* will promote our understanding of specific aspects of the instability of fungal genomes, broaden the methodological base of biotechnological techniques for intensification of biomass accumulation and synthesis of the target products, and make it possible to obtain new strains with the features important for microbiological industry.

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