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EXPERIMENTAL ARTICLES

A Mitochondrial Respiratory Mutant of *Podospora anserina* Obtained by Short-Term Submerged Cultivation of Senescent Mycelium

O. A. Kudryavtseva[a, 1], O. V. Kamzolkina[a], I. S. Mazheika[a], and C. Sellem[b]

[a] *Moscow State University, Faculty of Biology, Moscow, 119992 Russia*
[b] *Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France*

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Abstract—A spontaneous long-lived isolate of *Podospora anserina* obtained by relatively short-term submerged cultivation of the wild-type senescent culture and conventionally termed "immortal" was shown to be a *cox1* mutant. As a respiratory mutant, the isolate in question is characterized by dysfunction of the cytochrome respiratory chain, activation of alternative respiration leading to a low level of reactive oxygen species production, and the lack of accumulation of α-senDNA, the specific factor of *P. anserina* senescence. Absence of visible vegetative incompatibility was shown in the fungal mutants carrying respiratory defects. It was discovered that the *P. anserina* female sex organs could be fertilized not only by microconidia but also by the fragments of vegetative mycelium. Partial nonobservance of monoparental female inheritance of mitochondria associated with fertilization by vegetative mycelium was also revealed.

Keywords: *Podospora anserina*, respiratory mutant, factors of male fertility, mitochondrial inheritance

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The ascomycetous fungus *P. anserina* is one of the few fungal species whose wild strains are susceptible to pronounced replicative senescence and death [1]. *P. anserina* aging is defined as an impaired capacity of the cells for proliferation and/or differentiation [2]. Under the standard laboratory conditions, the average life span of the wild strains of *P. anserina* (from ascospore germination to dying off of the mycelium) is 25 days [1] for the colonies grown on agarized medium in special tubes (30–40 cm long and ~2 cm in diameter) or with continuous serial transfers on petri dishes.

As in other organisms, the life span of *P. anserina* is controlled by many different factors, both genetic and environmental. However, mitochondria play the key role in the regulation of its lifespan [2]. In *P. anserina*, their contribution is realized in two interrelated ways: by the accumulation of numerous age-dependent deletions and rearrangements in the mitochondrial genome, and by the oxidative stress caused by reactive oxygen species (ROS), which are continuously formed in the process of functioning of mitochondria [1, 3]. The fact that mitochondria are directly involved in the senescence processes is quite obvious; their most important function is to produce energy, i.e., these organelles directly control the vitally important functions [4].

The senescence of *P. anserina* always correlates with the changes in the mitochondrial DNA (mtDNA), which are absent at the early stages of the fungus developmental cycle. In the process of mycelium senescence, *P. anserina* cells accumulate mutant mtDNA molecules carrying numerous deletions and rearrangements; the elimination of the wild-type mtDNA molecules occurs in parallel. The loss of the wild-type mtDNA is supposed to be the immediate cause of death of *P. anserina* cells. Such modifications of *P. anserina* mtDNA are usually linked to the action of the "senescence factor". However, this problem is far from being understood in detail, and the nature of the senescence factor has not yet been established definitively [5]. The plasmid-like sequences called α-senDNA, which are accumulated in the mitochondria in all senescent wild-type strains of *P. anserina*, are conventionally considered to be one of the main factors of their senescence. The α-senDNA sequence is a mobile intron, the first intron (*Cox1*-i1, or α-intron), of the mitochondrial gene *cox1* encoding the first cytochrome *c*-oxidase (COX) subunit. In the process of mycelial aging, the α-senDNA sequence is released and amplified in the form of covalently closed DNA ring molecules (2.5 kb). The α-senDNA transposition into different mtDNA sites with the subsequent recombination leads to deletions and the loss of functionality of the mitochondrial genome [1, 4, 5]. As a result, the genes encoding the respiratory chain proteins are compromised, i.e., the respiratory chain is blocked. Therefore, in the growing hyphal tips of senescent cultures, the restoration of defective mito-

[1] Corresponding author; e-mail: for-ol-ga@yandex.ru

chondria is slowed down, as is the biogenesis of new organelles. As a consequence, the hyphal tips are not able to grow due to the lack of energy resources, which results in the manifestation of the "senescence syndrome" in this part of the mycelium [1, 6].

On the other hand, the mitochondria are the main source producing ROS inside the cell. As a result of accumulation of lesions in the mitochondria in the process of *P. anserina* senescence, ROS production increases in these organelles. In young *P. anserina* cultures, mitochondria produce a relatively small amount of ROS. Accumulation of the errors and the generation of ROS are the processes mutually enhancing each other. The increase in the rate of ROS production by mitochondria contributes to the increase in the oxidative modification of proteins, lipids, and nucleic acids leading to different metabolic disorders, due to which the cellular control over ROS production decreases and a vicious circle closes—the ROS generation increases more and more, which, in the final analysis, results in the cessation of mitochondrial functioning [7].

The existence of a close relationship between the respiration type and the lifespan of *P. anserina* is demonstrated by different mutants with defects of one or another complex of the main (cytochrome *c*-dependent) respiratory pathway. They are all characterized by a considerable lifespan increase [3, 4, 8]. In the literature, the term "immortal" is applied to such a type of *P. anserina* strains [1]. Immortality in relation to fungal organisms implies the potential capacity for the unlimited proliferation of their vegetative cells [9]. The cessation of functioning of the main respiratory pathway is not lethal for *P. anserina* due to the presence of alternative oxidase (AOX) in this fungus: when the main respiratory chain is compromised, respiration is carried out at the expense of the alternative pathway [5].

The alternative respiratory pathway is characterized by a decrease in the number of the ATP molecules synthesized, and by a decrease in the ROS level, which is essential [7]. In active alternative respiration, the electrons are transferred from the respiratory chain to oxygen immediately after ubiquinone bypassing the respiratory complexes III and IV. This results in the blockage of the transmembrane proton transfer mediated by complex III, thus decreasing ATP synthesis. As a consequence, the intensity of damage to the intracellular molecules decreases. Slowing down of ROS production by mitochondria can explain the increased lifespan in *P. anserina* respiratory mutants [5].

P. anserina mutants with a defective respiratory chain demonstrate not only a complete or partial repeal of pronounced replicative senescence, but also a change in certain morphological and physiological characteristics of a culture (the rate and morphology of growth, fertility, etc.) [3, 4, 8]. Therefore, such mutants are of interest for studying both the mechanisms of senescence and other cellular processes. Earlier, we investigated the phenomenon of increasing the lifespan of *P. anserina* mycelium after long-term cultivation in the aerated submerged culture [10]. Along with the thus obtained long-lived isolates, which had a different nature of repeal of pronounced replicative aging, the isolates, which were probably respiratory mutants, as judged by the phenotypic manifestations, were obtained.

The goal of the present work was to reveal the possible mtDNA modifications of one of the *P. anserina* isolates obtained, candidates for respiratory mutants, as well as to study the characteristic features of the functioning of its electron transport chain and to carry out reciprocal crossings with *P. anserina* wild strains.

MATERIALS AND METHODS

Strains and conditions of cultivation. The *P. anserina* strains—s, S, *cox5, mex16*, and Δ*rse2*—were kindly provided by Annie Sainsard-Chanet and Carole H. Sellem (Département Biologie Cellulaire et Intégrative, Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France).

The cultivation of *P. anserina* was carried out in the dark at 27 ± 1°C under the surface and submerged conditions on the standard synthetic M2 medium [11]. The composition of the cultivation medium (g/L) was as follows: 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; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2$), 0.001; $CuSO_4 \cdot 5H_2O$, 2.5×10^{-4}; $MnSO_4$, 5×10^{-5}; H_3BO_3, 5×10^{-5}; $Na_2MoO_4 \cdot 2H_2O$, 2×10^{-5}; thiamin, 5×10^{-5}; biotin, 2.5×10^{-6}. Surface cultivation was carried out in petri dishes on agarized M2 medium (20 g/L agar); submerged cultivation, in 750-mL Erlenmeyer flasks with 100 mL of he medium on a rotary shaker (200 rpm). Long-term storage of fungal cultures was carried out on M2 agar slants in a refrigerator at 4°C.

The *P. anserina* cultures obtained by transfer of the mycelium samples from the liquid onto agarized medium, whose continuous growth was maintained by surface cultivation, will be referred to as isolates.

The lifespan of *P. anserina* was determined using the method of serial transfers on petri dishes, which were performed at a period of three days (the mycelium for inoculations was always taken from the colony margin) or by growing the mycelium without transfers in special tubes with agarized medium. The lifespan was accordingly expressed as a period of time (days of growth) elapsed from the moment of strain isolation from the ascospore until the mycelium stopped growing completely or as the length (cm) by which the mycelium had grown over this period of time. The lifespan of the isolates was determined from the moment of their plating on the agarized medium. The *P. anserina* isolates with a constant growth rate, i.e., those that did not experience the so-called senes-

cence crises (the periods accompanied by a temporary growth rate decrease and the decline in aerial hypha formation) in the process of cultivation on agarized medium and did not cease growing over the cultivation time, which was several times longer than the average lifespan of *P. anserina* wild strains, will be referred to as immortal.

Strain s is one of the most widely used wild strains of *P. anserina* [12]. The haploid generative descendant of strain s designated as s1 with the "+" mating type and an average lifespan of 18.5 ± 2.1 days or 12.1 ± 0.6 cm was a stock culture for obtaining the immortal isolate studied. Other haploid strains, descendants of the wild strain s, were used for hybridizations and as controls.

The *P. anserina* respiratory mutants *cox5, mex16*, and Δ*rse2* were used for comparison with the isolate obtained. *cox5* is an immortal mutant strain obtained from the wild strain s by inactivating the nuclear gene *cox5* responsible for the COX subunit 5 synthesis [4]. *mex16* is a spontaneous immortal mitochondrial mutant obtained from strain s by selecting the sectors, which sometimes continue growing after the onset of aging of *P. anserina* wild strain colonies. *mex16* carries an insertion accompanied by a short mtDNA site deletion, which incorporates the intron α and the neighboring exon *cox1e1*, i.e., similar to *cox5*, it suffers a COX dysfunction [4, 8]. The mutant Δ*rse2* does not differ morphologically and in growth rate from the wild type when it is grown on the standard nutrient medium; however, due to deletion of the gene *rse2*, which encodes the gene *aox* transcription factor, Δ*rse2* is not able to synthesize AOX and to grow in the presence of the antibiotic antimycin A, an inhibitor of complex III of the main respiratory pathway [13]. It is therefore used as a marker of the activity of antimycin A added to the cultivation medium.

The wild strain S of *P. anserina* is characterized by vegetative incompatibility in relation to strain s [12, 14]: when the agarized medium is inoculated in combination with the colonies of incompatible strains, the lysis band seen with a naked eye and called barrage is formed in the zone of their contact.

Southern blot hybridization of *P. anserina* mtDNA. Analysis of the *P. anserina* mtDNA was carried out using the standard methods [15, 16]. The total DNA was isolated with the phenol–chloroform mixture by sequentially adding phenol, phenol : chloroform (1 : 1), and chloroform to each homogenized mycelium sample. After each extraction cycle, the samples were centrifuged (11500 *g* for 5–8 min) with the subsequent sampling of the supernatant. The DNA was precipitated by adding to each sample 50 μL of 3 M sodium acetate and 1200 μL of 100% ethyl alcohol (for better precipitation, the mixture was incubated at –20°C for 20–30 min). The precipitate was separated by centrifugation (60 min, 14000 *g*), washed with 1.5–2 mL of 70% ethyl alcohol, recentrifuged (5 min, 14000 *g*), and dried in a vacuum desiccator.

Restriction of the isolated *P. anserina* DNA was carried out with the *Hae*III endonuclease overnight at 37°C. Electrophoretic separation of the DNA fragments was carried out in 1% agarose gel. The DNA transfer to the membrane (blotting) was performed according to the standard method [17]. The Amersham Hybond™-N PVDF-charged nylon membrane was used.

In order to prepare the P^{32}-labeled DNA probe targeted at the *P. anserina* mtDNA Eco4 site, the recombinant plasmid pbR322 with the DNA fragment complementary to *P. anserina* mtDNA Eco4 site (10.895 kb) [16] cloned earlier by the researchers of Département Biologie Cellulaire et Intégrative, Centre de Génétique Moleculaire, CNRS, France, was used in this work. The Eco4 sequence was cut out from the recombinant plasmid pbR322 by means of the highly specific restriction endonuclease *Eco*RI; the mixture of the two plasmid fragments obtained was separated electrophoretically. DNA extraction from the gel strip containing the DNA fragment with a molecular mass of 10.895 kb was performed with the QIAquick® Gel Extraction Kit (250). The radioactive label was included into the DNA using the nick-translation method with the Prime-a Gene® Kit (Promega), including three unlabeled deoxyribonucleoside triphosphates (dATP, dGTP, dTTP) and one labeled: dCTP(P^{32}); the reaction was carried out at room temperature for 1 h. DNA–DNA hybridization was carried out for 18–20 h. The hybridization products were visualized on the Kodak BioMax film (18 × 24 cm) by means of autoradiography in an X-ray cassette with an intensifying screen.

The test for *P. anserina* resistance to antimycin A. The tested isolate of *P. anserina* was grown for 96 h on the agarized M2 medium supplemented with antimycin A (10 μg/mL), an inhibitor of complex III of the cytochrome *c*-dependent respiratory pathway. Comparison of the diameters of the colonies formed under the standard conditions and in the presence of antimycin with the diameters of the colonies of *P. anserina* wild strains and of the mutant Δ*rse2* strain that is unable to produce AOX made it possible to determine the respiration pathway of the tested isolate. The colony diameter of a *P. anserina* culture grown over 96 h on antimycin A-free medium was taken to be 100%.

Quantitative RT-PCR of the *P. anserina aox* gene. The level of the AOX gene expression in *P. anserina* was determined using the method of quantitative polymerase chain reaction with reverse transcriptase (quantitative RT-PCR) [13]. In order to isolate the total RNA from *P. anserina* mycelium, the RNeasy® Plant Mini Kit (Qiagen) was used. The synthesis of single-chain complementary DNA (cDNA) to the isolated RNA was carried out by the standard method of using the reverse transcription reaction.

For each *P. anserina* cDNA sample, three genes were amplified: the test gene *aox* and two reference

genes, *gpd* and *pdf2*, that were constitutively expressed in all the earlier studied fungal strains under different growth conditions [13]. The level of the *aox* gene expression was calculated in relation to their levels of expression. The gene *gpd* encodes glyceraldehyde-3-phophate dehydrogenase of *P. anserina* [18]; *pdf2* supposedly encodes the regulatory phosphatase subunit PP2A [19]. Amplification was carried out with PRC in the real-time mode on a LightCycler® (Roche) amplifier according to the following protocol: 10 min of preincubation at 95°C followed by 30 amplification cycles according to the regimen: the melting temperature 95°C (30 s); the annealing temperature 60°C (30 s); the amplification temperature 72°C (15 s). The PCR mixture contained cDNA dilutions (10-fold), synthetic oligonucleotide primers, forward and reverse (0.25 μg each), and the LightCycler® FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche) components. The efficiency of amplification compared to the theoretical efficiency for each pair of primers was determined by making a series of dilutions of the initial cDNA samples: 1/40, 1/160, and 1/640. The PCR results were processed in real time, and the level of the AOX gene expression in *P. anserina* was calculated using the REST-MCS software package. For the strain/isolate and the gene, the result was expressed as the level of expression of this gene in relation to the level of its expression in the wild strain of *P. anserina*.

Isolation of protoplasts, and measurement of their respiration rate and ROS production. The protoplasts of *P. anserina* mycelium were isolated according to the well-known technique used for this fungus [20]; for this purpose, the biomass was grown under static conditions in the flasks containing 100 mL of the liquid MR medium (50 g/L of corn flour) for 48 h for the wild control strain and for 72 h for the immortal isolate studied. The mycelium was separated from the culture liquid, washed with sterile water and TPS1 buffer (0.6 M of sucrose, 5 mM of Na_2HPO_4, 45 mM of KH_2PO_4; pH 5.5). The volume of the TPS1 buffer equal to the mass of the mycelium harvested and containing 0.6 g of the enzyme β-glucanase (Glucanex® 200G) per 10 mL of the buffer was added to each sample. The incubation was carried out for 3 h at 37°C on a shaker. The mycelium was then separated and washed with TPS1. The protoplast suspension thus obtained was centrifuged (15 min, 3200 *g*). The protoplasts were resuspended in 50 mL of TPS1; their concentration was determined by direct count in a Goryaev chamber.

The protoplast respiration was recorded polarographically by the rate of consumption of dissolved oxygen using the Oxytherm respirometer (Hansatech Instruments) fitted with a Clark cell. The protoplast suspension (1 mL) at a concentration of 7×10^7/mL was introduced into the respirometer cell. In order to determine the contribution of the main and alternative respiratory pathways to the overall respiration of *P. anserina*, we observed how the protoplast respiratory rate changed after the addition of 1 mM of potassium cyanide, a COX inhibitor, and 1 mg/mL of salicylhydroxamic acid (SHAM), an AOX inhibitor. The measurement results were processed using the Oxyg32V2.17 software package.

The production of ROS (including hydrogen peroxide, the peroxyl-radical, and the peroxynitrite anion) by *P. anserina* protoplasts was detected cytofluorometrically by the kinetics of dichlorofluorescein (DCF) formation, for which purpose the compound H_2DCFDA, which is a fluorogen, was added to the protoplast suspension (1.4×10^6/mL) to the end concentration of 80 μM [4, 21]. The fluorescence intensity was recorded with a Partec PAS III flow cytofluorimeter (Germany) in the sample of 50000 protoplasts using a 488 D/NG3E filter and the Partec FloMax software package. The fluorescence of each protoplast sample was measured several times for 4.5 h.

Hybridizations. Reciprocal hybridizations between *P. anserina* strains were carried out using the standard method of washouts from the surface of the colonies [10, 22] grown on M2 slanted agar. In such a hybridizing mode, the colonies from which the washout is made play the role of the male parent, while those onto which the washout containing the fertilizing units is transferred act as the female parent. Washing out was carried out with 20 mL of sterile MR medium. To separate the microconidia, the washout was passed through a filter with the pore diameter corresponding to the diameter of *P. anserina* microconidia.

Petri dishes with the fertilized colonies were incubated in the light until the fruiting bodies appeared and the ascospores began to shoot off. Under a dissection microscope, the ascospores were isolated with a sharply pointed sewing needle from the inner surface of the lid of a petri dish on which a layer of starvation agar (30 g/L) was applied. The ascospores were isolated only from the five-spore asci containing three binuclear and two mononuclear spores. The ascospores were grown on M1 medium [11].

RESULTS AND DISCUSSION

Obtaining of the Immortal P. anserina Isolate

The mycelium of the senescent, surface-grown *P. anserina* s1 strain, which ceased growing completely, was transferred to the liquid medium (zero transfer), in which its capacity for development of new hyphae and continuation of growth was restored after 9 days of cultivation (the resuscitation process) [10]. Further growth of the submerged culture of strain s1 was maintained by transfers every three days. V-s1-IV(3), one of the *P. anserina* isolates obtained as a result of inoculating the agarized medium with mycelium from the restored submerged culture on the 18th day of submerged cultivation (the fourth transfer), appeared to be long-lived: its continuous growth

Table 1. Southern blot analysis of *P. anserina* mitochondrial DNA

Strain/isolate	Physiological age at the time of DNA isolation	Sizes of the *Hae*III-mtDNA fragments hybridizing with the DNA probe Eco4, kb						New mtDNA fragments, kb
		4.769	3.393	2.539 (α-senDNA)	1.889	1.188	0.841	
s14(+)	Young	+	+	Trace	+	+	+	–
s13(–)	Young	+	+	+	+	+	+	–
s20(+)	Senescent	–	+	+ (Intense)	–	–	–	–
s21(–)	Senescent	–	+	+ (Intense)	–	–	–	–
V2-s1-IV(2)	Young	+	+	–	–	–	–	0.9

Note: "+" and "–" indicate the presence and absence of the fragment, respectively.

on petri dishes was maintained for 252 days using the method of serial transfers, which were performed with a period of three days (the corresponding total mycelium length was 82.0 cm). The V-s1-IV(3) growth rate was constant (3.3 mm/day).

After 66 days of surface cultivation, the isolate V-s1-IV(3) was transferred again into liquid medium where it was maintained for nine days (three transfers), after which the agarized medium was inoculated with the samples of the secondary submerged culture thus obtained (5 replicates). Without exception, all secondary isolates from the V-s1-IV(3) culture designated as V2-s1-IV(1–5) were also long-lived: they did not cease growing until the serial transfers on plates with agarized medium for 180 days were stopped (the average total length of the mycelium of the secondary isolates in question was 60.0 ± 1.8 cm). When they grew under the surface conditions, both the primary long-lived isolate V-s1-IV(3) and its vegetative descendants V2-s1-IV(1–5) were characterized by poor development of the aerial mycelium, stable growth at a constant, relatively low rate, as well as the complete absence of senescence crises throughout their cultivation.

After eight months of storage at 4°C, the isolate V-sl-IV(3) lost the capacity for growth; however, one of these secondary isolates, V2-s1-IV(2), again showed stable growth (at a rate of 4.1 mm/day) and the absence of any signs of senescence for over 100 days under the conditions of continuous cultivation on the agarized M2 medium in the tubes for determination of the lifespan of *P. anserina*. By convention, we will call the isolate V2-s1-IV(2) immortal.

Molecular Characterization of the Immortal Isolate V2-sl-IV(2) of P. anserina

Analysis of the V2-s1-IV(2) mtDNA. Since amplification and accumulation of the intron α sequence accompanies senescence in the wild-type *P. anserina* strains, the integrity of the 10.895 kb mtDNA Eco4 site containing it was of the greatest interest to us [16]. Southern blot hybridization of the radioactive Eco4 DNA probe with the total DNA isolated from the immortal isolate V2-s1-IV(2) and treated with *Hae*III restriction endonuclease revealed differences between the sequences of the wild-type mitochondrial chromosome and this isolate. In the autoradiogram of V2-s1-IV(2), the bands corresponding to the 1.889-, 1.188-, and 0.841-kb mtDNA fragments were completely absent. No accumulation of the α-senDNA sequence was revealed. Along with this, a new 0.9-kb restriction fragment absent in the wild strains was detected in the isolate studied (Fig. 1, Table 1).

In the autoradiogram of *P. anserina* wild strains, the Eco4 probe usually detects five *Hae*III-mtDNA bands. Two fragments with a close molecular mass, 1.157 and 1.188 kb, are usually not separated by electrophoresis and therefore look like a single band in the autoradiogram. Accumulation of the α-senDNA sequence in the wild strains results in the emergence of an additional sixth 2.539 kb band. The α-senDNA is known to be present at the earliest stages of growth of the young mycelium [16], as was well seen in the control young strain s13(–) (Fig. 1). Owing to α-senDNA amplification, the corresponding band becomes much more intense in the process of mycelium aging. Simultaneously, certain other bands decrease their intensity, i.e., the corresponding mtDNA sites are lost during senescence. Two restriction fragments which overlap with the intron α, namely, the 0.841- and 1.889-kb fragments, undergo such changes most vividly and systematically, although the nearby fragments (Fig. 1) do also noticeably loose intensity [16].

Our findings, as well as the knowledge of the sequence of gene location in the *P. anserina* mitochondrial chromosome [16], gave evidence of the V2-s1-IV(2) mitochondrial genome having a deletion incorporating the gene *cox1*. The presence of a deletion in the *P. anserina cox1* gene suggested that the main respiratory chain complex IV was inactive and the mutant respired due to AOX functioning [4, 16],

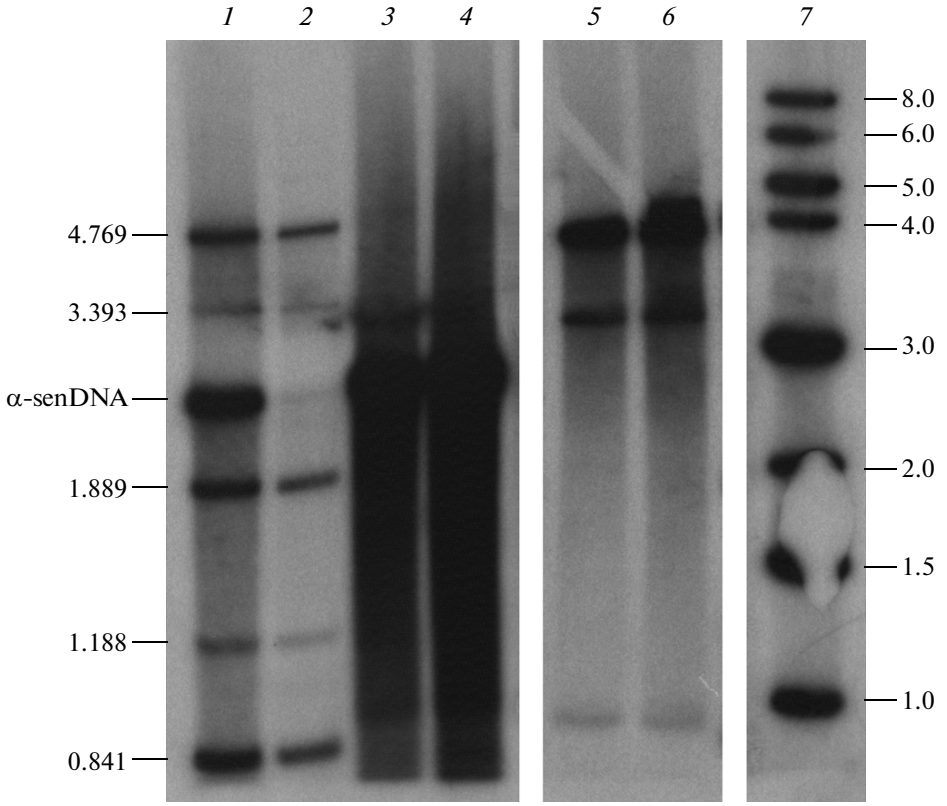

Fig. 1. Southern blot analysis of *P. anserina* mitochondrial DNA. On the right, the sizes of fragments of the molecular marker (kb) are indicated; on the left, those of the *Hae*III-mtDNA fragments (kb) hybridizing with the radioactive Eco4 DNA probe. Restriction of mtDNA was carried out with the *Hae*III enzyme. The wild strain s13(–), young culture (*1*); the wild strain s14(+), young culture (*2*); the wild strain s20(+), senescent culture (*3*); the wild strain s21(–), senescent culture (*4*); the isolate V2-s1-IV(2) (*5*, *6*); the molecular marker (*7*).

which was experimentally confirmed in this work in relation to V2-s1-IV(2) by other methods of investigation.

Analysis of the functioning of the alternative respiratory pathway in the isolate V2-s1-IV(2). As seen from Fig. 2 and Table 2, the immortal isolate V2-s1-IV(2) was virtually insensitive to antimycin A, V2-s1-IV(2) showing even greater resistance than the respiratory mutant *cox5*, which testifies to the active functioning of the alternative respiratory pathway in the immortal isolate obtained by us. In the wild strains of *P. anserina*, the inactivation of the main respiratory pathway leads to AOX induction. Alternative respiration enables the wild strains to grow on the medium with antimycin; however, their growth is delayed, which is necessary for a sufficient amount of *AOX* to be synthesized. *P. anserina* mutants with a constitutive *AOX* expression grow on such a medium without delay. On the contrary, the mutants with disrupted *AOX* gene expression (such as Δ*rse2*) are not capable of growing on medium with antimycin A even after the time required for the wild strains to induce AOX had elapsed [13].

As it would be expected, quantitative RT-PCR revealed a high level of the *aox* gene expression in the isolate V2-s1-IV(2). When the data obtained were normalized by the constitutive gene *gpd* as the reference one, the level of AOX expression in V2-s1-IV(2) exceeded 37-fold the AOX expression in *P. anserina* wild strain; when normalized by gene *pdf2*, an increase was 75-fold. These values were approximately at the same level as in two known *P. anserina* respiratory mutants analyzed in parallel in the same experiment, namely, in the nuclear mutant *cox5* and the mitochondrial mutant *mex16* (Fig. 3).

In contrast to the *P. anserina* wild-type strain, the respiration of V2-s1-IV(2) was absolutely insensitive to the action of KCN but was virtually completely inhibited by SHAM (Table 3); hence, it respired only at the expense of AOX; the main respiratory pathway in it was absolutely nonfunctional. Oxygen consumption by V2-s1-IV(2) was almost twice as high as in the wild type. Other *P. anserina* respiratory mutants—*cyc1-1, PaCox17::ble*, and *ex1*—investigated in this relation were also characterized by an increased oxygen consumption rate [3, 23, 24]. The cause of such an increase is, however, not clear [24]. The organism whose main respiratory chain does not function probably tries to compensate for the required ATP yield in such a way.

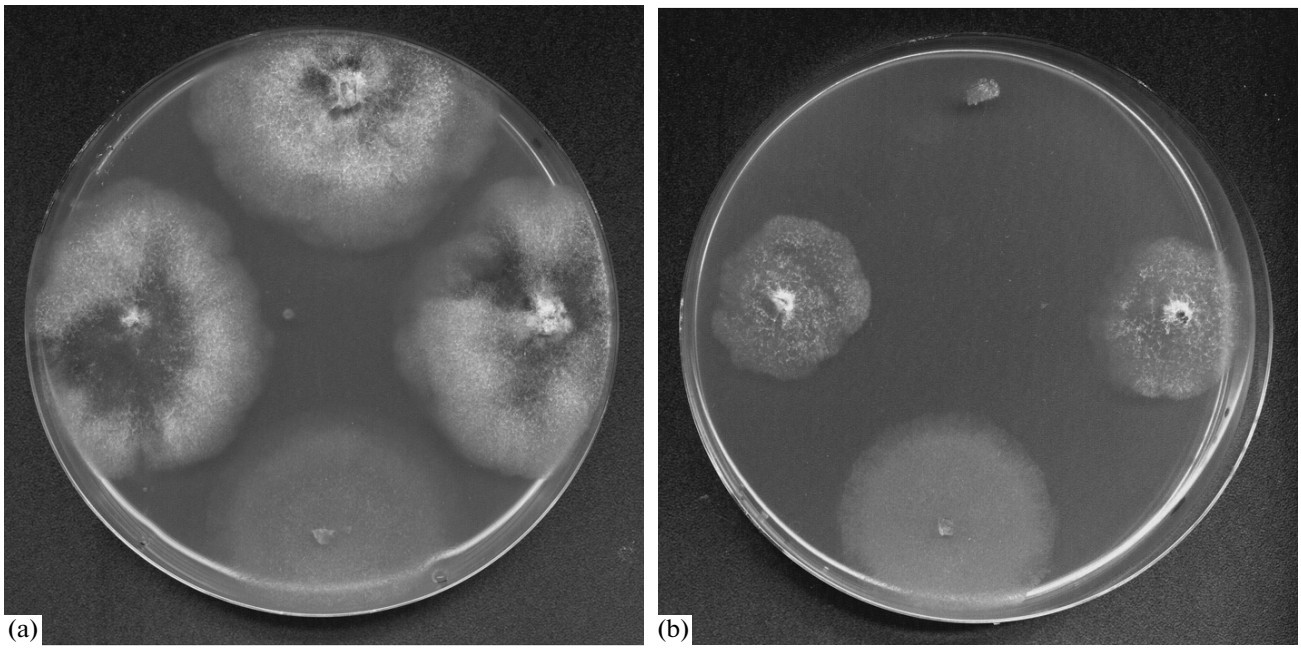

Fig. 2. Growth of the isolate V2-s1-IV(2) on agarized M2 medium in the absence (a) and presence (b) of main pespiratory chain inhibitor antimycin A compared to *P. anserina* wild type strains s14(+), s13(−) and rse2, the mutant with the defective AOX respiratory pathway. On both photoes: above, Δ*rse2*; on the left, s14(+); on the right, s13(−); down, isolate below, V2-s1-IV(2).

ROS production in the immortal isolate V2-s1-IV(2) was approximately 35% lower than in the wild strain of *P. anserina* (Fig. 4). It is this circumstance that is supposed to allow the respiratory mutants to stabilize, to a significant extent, the mitochondrial genome and not to be susceptible to replicative senescence [3, 25].

Hybridizations with the Involvement of the P. anserina Isolate V2-s1-IV(2)

Partial loss of fertility in V2-s1-IV(2). Reciprocal fertilizations of the mutant isolate V2-s1-IV(2) and the wild strain s13(−) carried out by washing from the colony surface showed this isolate to have female sterility: acting as a maternal parent, it did not form the

Table 2. Resistance of the *P. anserina* isolate V2-sl-IV(2) and its sexual descendants to antimycin A

	Strain/isolate	Colony radius at 4 days of growth, mm		Resistance to antimycin, %
		On M2 medium	On M2 medium + antimycin	
Controls	s13(−)	20.9 ± 0.9	9.8 ± 0.4	47.0 ± 2.0
	s14(+)	21.4 ± 0.6	9.3 ± 0.3	43.5 ± 1.5
	Δ*rse2*	20.3 ± 0.6	0	0
	cox5	7.9 ± 1.0	6.3 ± 0.5	80.0 ± 6.7
Tested strains*	V2-s1-IV(2)	16.6 ± 0.4	14.7 ± 0.6	88.3 ± 3.5
	IVN13B2	12.0 ± 1.3	8.5 ± 0.9	70.8 ± 7.6
	IVN13B3	11.8 ± 1.5	8.3 ± 0.8	70.1 ± 6.7
	IVN13S1(+); wild type	21.0 ± 1.3	8.8 ± 1.5	41.7 ± 7.3
	IVN13S2(−); wild type	21.0 ± 1.6	8.3 ± 0.8	39.3 ± 3.8
	IVN30B1	15.0 ± 0.9	11.0 ± 1.3	73.3 ± 6.8
	IVN30B2	13.0 ± 1.3	10.0 ± 1.3	76.9 ± 7.9
	IVN30S1(+)	12.8 ± 1.5	11.0 ± 1.3	85.9 ± 9.6

* In the names of the test strains, V2-sl-IV(2) descendants, the designations N13 and N30 are the ordinal numbers the asci were awarded; B, large binuclear ascospores; S, small mononuclear ascospores.

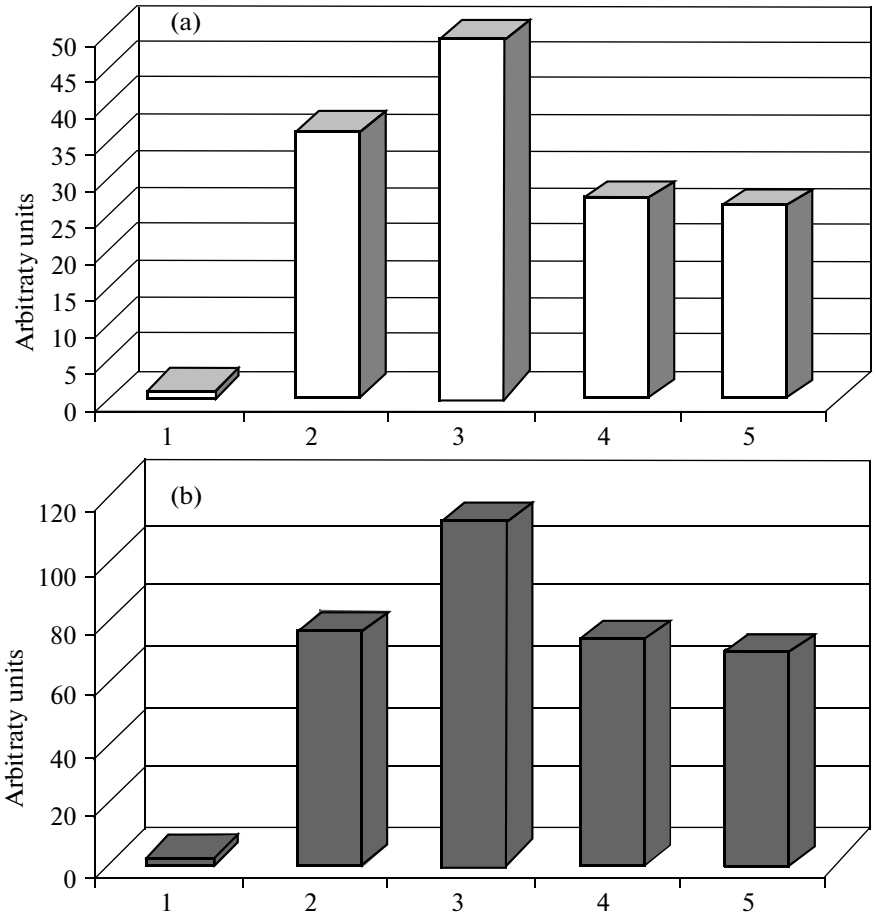

Fig. 3. Level of the gene *aox* expression in different *P. anserina* respiratory mutants with the use of *gpd* (a) and *pdf2* (b) genes as the reference genes. The level of *aox* expression in the control wild strain s14(+) was accepted as 1. The wild strain s14(+) (1); the immortal isolate V2-s1-IV(2) (2); the homokaryotic strain IVN30S1, a generative descendant of V2-s1-IV(2) (3); the mitochondrial respiratory mutant *mex16* (4); the nuclear respiratory mutant *cox5*(+) (5).

fruiting bodies. The data cited agree with the results obtained in relation to other *P. anserina* respiratory mutants among which are *cox5, cyc1-1*, and the *mex* group mutants: they are all sterile when used as a female partner in hybridization [3, 7, 26].

The male fertility of the isolate V2-s1-IV(2) was not lost completely: when it acted as a paternal parent in fertilization, i.e., when the washout from the surface of its colonies was carried out without filtration, the colonies of the wild maternal strain of *P. anserina* formed scanty fruiting bodies. If, prior to its application to the maternal strain colonies, the washed out liquid was passed through a special filter with the pore diameter corresponding to the diameter of *P. anserina* microconidia, the isolate V2-s1-IV(2) was incapable of fertilization. Moreover, microscopy of the V2-s1-IV(2) mycelium did not reveal the formation of microconidia, which are known to act as spermatia in *P. anserina*, i.e., they serve for fertilizing protoperithecia [1]. It follows from the data obtained that fertilization in *P. anserina* is possible not only by microconidia but also by the fragments of nonspecialized vegetative hyphae, which may also be removed during washout but are effectively held back by filtration. However, in the vast modern literature pertinent to the fungus *P. anserina*, it is only microconidia (spermatia) that are conventionally mentioned as the fertilization factors, while the information concerning whether the mycelium could be involved in this process is absent; this is of no small importance to take into account when reciprocal hybridizations are performed.

Table 3. Respiration of *P. anserina* protoplasts

Strain/isolate	Respiratory rate*	% of inhibition	
		KCN	SHAM
s14(+)	3.8	86.0	33.4
V2-s1-IV(2)	6.6	0	93.4

* The oxygen consumption rate in nmol/mL min × 10^7 protoplasts.

Only one work, dating back to 1936, in which its author, B. Dodge, studied fertilization in *P. anserina*

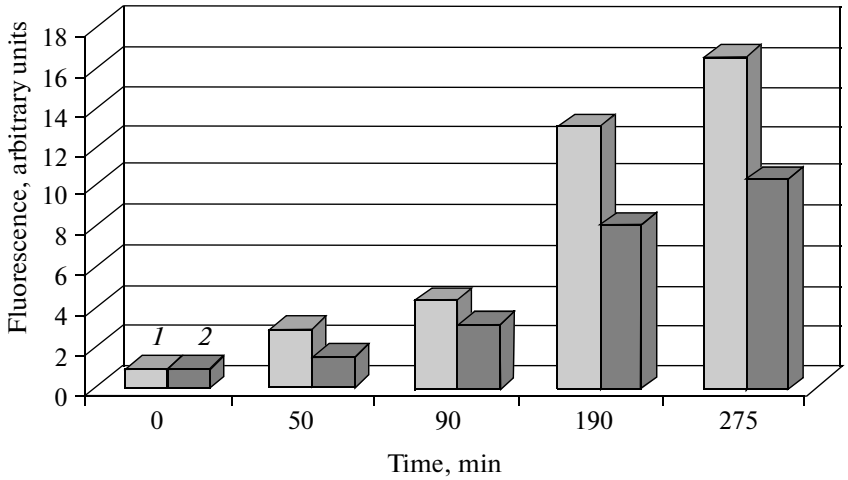

Fig. 4. ROS production by the protoplasts of *P. anserina* wild type s14(+) (*1*) and respiratory mutant V2-s1-IV(2) (*2*).

(the then *Pleurage anserina*) in the absence of microconidia, is available [27]. However, this publication is not quoted by the present-day authors using *P. anserina* as a model subject of molecular genetic investigations. Dodge selected the strains that did not produce microconidia and showed that, if such strains of the opposite mating types were inoculated onto the agarized medium, they formed fruiting bodies despite the absence of microconidia [27]. However, in the subsequent years, researchers did not refer to this work, and the fact that *P. anserina* could be fertilized without the involvement of microconidia was not gotten proper attention.

Confirmation of fertilization by the vegetative mycelium in *P. anserina* wild strains. In order to establish whether fertilization by nonspecialized vegetative hyphae occurs in the representatives of *P. anserina* on the whole, the approaches enabling *P. anserina* wild strains to be fertilized were developed, which did not involve the microconidia. Complete absence of microconidia was revealed in strain s14(+) at 24 h of growth (in the dark); the washout made from such colonies and passed through the filter did not fertilize the maternal strain with the opposite sign. However, fertilization by washout from the 24-h colonies of this strain carried out without filtration demonstrated a high capacity for fertilization (Table 4). In addition, another method of fertilization, pointwise application of small samples of the mycelium thoroughly premicroscoped for the absence of microconidia, was used. After the paternal mycelium samples had been transferred in the drops of sterile water onto the corresponding maternal strain, clusters of fruiting bodies with ascospores were formed at almost all points of introduction (Table 5).

Thus, *P. anserina* vegetative hyphae take part in the fertilization of protoperithecia with the same effectiveness as microconidia. The decreased male fertility of V2-s1-IV(2) (when washouts without filtration were used) probably resulted from the poor development of aerial mycelium in this isolate and, hence, from the decreased concentration of mycelial fragments performing the function of fertilization.

The inheritance of V2-s1-IV(2) mutation. Hybridizing V2-s1-IV(2) with the wild strain s13(–) was carried out by combined cultivation on petri dishes. A total of 152 homokaryotic and 258 dikaryotic descendants originating from 116 asci of mononuclear ascospores were obtained (the share of germination was 66 and 74% for mononuclear and binuclear ascospores, respectively). Among them, the phenotype of the parent isolate V2-s1-IV(2)—slow growth and poor development of the aerial mycelium—was noted only for six strains originating from two different sacs: IVN13B2, IVN13B3 and IVN30B1, IVN30B2, IVN30B3, and IVN30S1. The ascospores producing them germinated according to the *cox* type, i.e., with a 72–96-h delay compared to the wild variant; the nascent mycelium was exclusively a substrate one and formed an irregular margin. Strain IVN30B3 appeared to be short-lived; its growth stunted as early as several days after the isolation. The remaining five strains were immortal, the immortal descendants of V2-s1-IV(2) being resistant to antimycin A, similar to their parent (Table 1). For one of them, the level of AOX expression was determined; it exceeded the AOX expression of the parent isolate (Fig. 3). In the aggregate, the data obtained allow a suggestion that strains IVN13B2, IVN13B3, IVN30B1, IVN30B2, and IVN30S1 are respiratory mutants.

Since the loss of the functionality of the main respiratory chain in V2-s1-IV(2) is associated with the presence of a deletion in the mitochondrial gene *cox1*, this mutation should, theoretically, be inherited only from the maternal parent. Earlier, it was shown that monoparental maternal inheritance of the mitochondria takes place in *P. anserina* as in many other organisms [28]. When the wild-type strain was hybridized

Table 4. Fertilization of wild *P. anserina* strains with fragments of vegetative mycelium using the washout method without filtration

Paternal strain*	Maternal strain	Washout with filtration	Washout without filtration
s13(–)	Colony s14(+)	Single fryiting bodies	Abundant development of fruiting bodies
	Sterile medium M2	No mycelium development	*P. anserina* mycelium
s14(+)	Colony s13(–)	No fruiting bodies	Abundant development of fruiting bodies
	Sterile medium M2	No mycelium development	*P. anserina* mycelium

* Paternal strains were cultivated in the dark for 24 h.

Table 5. Fertilization of wild *P. anserina* strains with the vegetative hyphae using the method of point application of mycelium samples on the maternal colony surface

Paternal strain, age, days	Maternal strain, age, days	Number of points of introduction of mycelium	Number of points at which fruiting bodies were formed
s8(–), 18	s9(+), 18	8	7
s11(+), 22	s10(–), 22	13	12
s12(–), 22	s11(+), 22	16	16

with the isolate V2-s1-IV(2), possessing absolute female sterility and not forming microconidia, the inheritance of the mitochondrial mutation occurred in 1.7% of the cases. The inheritance of V2-s1-IV(2) mitochondrial mutation by sexual descendants is mediated only by the vegetative mycelium, which, as we have established, is able to fertilize protoperithecia per se. In other words, it is not ruled out that, in *P. anserina*, the monoparental inheritance of mitochondria is not strict; transmission of mitochondria from the somatic hyphae of the paternal partner into the female sex organs of the maternal one is possible. No failure of the monoparental mitochondrial inheritance in *P. anserina* has been described in the scientific literature so far.

Disruption of Vegetative Incompatibility in P. anserina Respiratory Mutants

P. anserina strains have the property of vegetative incompatibility. This fungal species has two groups of vegetative incompatibility: s and S [12, 14]. It was established in the course of the present work that, despite the fact that the respiratory mutants *cox1* and V2-s1-IV(2) were both obtained from strain s, they did not form a barrage with S, with which they, theoretically, should be vegetatively incompatible.

Moreover, when *cox5*(+) was hybridized with the wild-type strains of both classes of vegetative incompatibility, s13(–) and S10(–), the mycelium of the wild hybridization partner germinated between the *cox5*(+) mycelium, so that the fruiting bodies were formed across the whole surface of the *cox5*(+) colonies, not only in the zone of contact, as in the case of hybridization between *P. anserina* wild strains. Importantly, *cox5*(+) was not capable of penetrating into the zone that had already been occupied by the colony of the wild strain s13(–) or S10(–): fruiting bodies were not formed on their surface.

The low growth rate, female sterility, a change in the mycelial morphology, and the capacity for germination in the mutants with the compromised cytochrome oxidase pathway are all the consequences of insufficient ATP synthesis in the cases when only the alternative respiratory pathway functions [5]. We may suggest that, lacking energy resources, the respiratory mutants are neither able to prevent another fungal organism from penetrating the territory occupied by them, nor to realize the program of cell death upon an encounter with a vegetatively incompatible strain.

It should be noted that the isolate V2-s1-IV(2) studied in this work was obtained as a vegetative descendant, or the secondary isolate, of the long-lived, slowly growing primary isolate V2-s1-IV(3) subjected to short-term repeated cultivation under rotary submerged conditions. It should be suggested that the latter is also a mitochondrial mutant. This isolate, unlike the other long-lived *P. anserina* isolates obtained in the course of our investigations [10], was obtained by reseeding onto the agarized medium the mycelium samples from the submerged culture that was in the earliest (first) adaptation phase. The submerged culture was inoculated with *P. anserina* senescent mycelium (strain s1). The initial stage (phase I) of submerged cultivation of the senescent *P. anserina* mycelium was characterized by slow growth and poor biomass accumulation in all the experiments [10], which was apparently caused by the presence of the

senescence factors in the wild-type cells. We must suggest that the slowly growing mutant subpopulations, which are not susceptible to aging and have defects in the main respiratory chain, if they were present at this stage of submerged cultivation, could gain access to the substrate and become competitive and, consequently, accessible to isolation only in such a situation when the bulk of the mycelium consisting of the wild-type cells manifested the signs of senescence and was not able to utilize the substrate efficiently and to surpass the mutant subpopulations in growth. It was shown that more prolonged submerged cultivation implemented by serial transfers enabled the senescent cultures of *P. anserina* to overcome the senescence crisis and to return again to the physiologically young state accompanied by rapid biomass accumulation (phases II and III). At these stages of submerged cultivation of *P. anserina*, no respiratory mutants were obtained [10]. In this period of cultivation, the mutant cells with defects in the main respiratory chain, even if they did occur in the culture, seemed to lose in the competition with the cells with completely functional respiration and therefore did not form the subpopulations the agarized medium could be inoculated with.

Thus, spontaneous respiratory mutations occur in the process of submerged cultivation of *P. anserina* and may be selected by creating the specific conditions related to the inhibition of vital activity of the normal cells in the culture (those without respiratory functional defects). Being a respiratory mutant, the immortal isolate studied is characterized by an activation of alternative respiration, affording a low ROS yield, and the absence of accumulation of the senescence factor α-senDNA. Moreover, it was shown that the program of vegetative incompatibility was not realized in the mutants carrying defects of the main respiratory chain. It is the first time that the fact of not strict monoparental inheritance of mitochondria in *P. anserina* has been described. It might be linked to the involvement in fertilization of the vegetative mycelium, which was established to be able to spermatize not only the mutant studied but also the wild type. The data obtained are important for further studies of the principles of cytoplasmic inheritance and should be considered when reciprocal hybridizations are performed.

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