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Podospora anserina Does Not Senesce When Serially Passaged in Liquid Culture

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A procedure was developed for the prolonged growth of the ascomycete fungus *Podospora anserina* in liquid culture to determine the effects of such growth on the senescence phenotype. Senescence in *P. anserina*, which is maternally inherited and associated with the excision and amplification of specific mitochondrial plasmids, occurs when this species is grown on solid medium. In two independent experiments no evidence of senescence was observed as mycelia were serially passaged in liquid culture. Further, when separable mycelial masses, termed puff balls, from the liquid cultures were plated on solid medium, a significant increase in their average longevity was observed. The apparent immortality of *P. anserina* in liquid culture was not dependent upon mitochondrial DNA rearrangements, nor was it affected by the presence of a previously described senescence plasmid, αsenDNA. Evidence was obtained indicating that growth in liquid culture exerts selective pressure to maintain the wild-type mitochondrial genome.

The ascomycete fungus *Podospora anserina* provides a model system for the study of senescence at the molecular level. When mycelial plugs are placed on corn meal agar in race tubes, growth occurs at a constant rate for a genetically determined distance (19, 20). The sudden cessation in vegetative growth which follows is termed senescence. Age is therefore measured as a function of the distance grown in centimeters. There are at least 10 different races of *P. anserina*, each with a characteristic longevity when grown in race tubes (14).

Early investigations of senescence in *P. anserina* demonstrated that senescence is maternally inherited (19, 20). Ensuing studies have demonstrated that senescence is associated with the excision and amplification of specific mitochondrial DNA sequences and mitochondrial DNA rearrangements (5, 22). The resultant plasmids, termed senDNAs, were found in cultures derived from senescent mycelium. At least six different senDNAs have been reported (1, 5–7, 11, 22, 28), but one particular 2.6-kilobase-pair plasmid, αsenDNA, is the most common senDNA found in senescent cultures. The other senDNAs occur relatively infrequently, and some have only been reported once. Unlike the other senDNA plasmids, the αsenDNA plasmid is also found in young, nonsenescent mycelia of race A, although in lesser amounts (26). This plasmid consists of a complete group II intron of the highly mosaic COI gene, and it possesses an open reading frame with extensive homology to retroviral reverse transcriptase (18). A second class of mitochondrial plasmids, termed sMt-DNAs, have recently been described in *P. anserina* (M. S. Turker, J. M. Domenico, and D. J.Cummings, J. Biol. Chem., in press). These plasmids are associated with longevity mutants, which are defined as strains capable of growth renewal after one or more senescent crises on solid medium. Similar to senescent cultures, such mutants usually yield mitochondrial DNAs with extensive rearrangements (2, 15; D. J. Cummings, M. S. Turker, and J. M. Domenico, in press). Figure 1 shows the most current restriction map for *P. anserina* mitochondrial DNA and the locations from which several of the excision amplification plasmids are derived.

For this study we investigated the kinetics of *P. anserina* senescence in liquid culture by developing a procedure for the serial passage of homogenized mycelia. The most striking observation was that *P. anserina* did not senesce when grown continuously in liquid culture. Further, when small masses of the liquid culture-grown mycelia, termed puff balls, were returned to growth on solid medium their average longevity was significantly increased as compared with the same strain grown only on solid medium. In some cases, these puff ball-derived cultures were apparently immortal, i.e., they never underwent a senescent crisis when grown on solid medium, in contrast to the previously described longevity mutants (Turker et al., in press) which have undergone at least one senescence event. Unlike the longevity mutants and the one immortal strain examined herein, the liquid cultures did not show evidence for mitochondrial DNA rearrangements, nor did they reveal the presence of the sMt-DNAs. They did, however, display the αsenDNA plasmid. In genetic crosses the liquid culture mycelia were found to be male fertile, but female sterile. All progeny of successful crosses with a wild-type strain had short lifespans, consistent with maternal inheritance of senescence. Evidence was obtained indicating that continuous growth in liquid culture serves to maintain the wild-type mitochondrial genome.

MATERIALS AND METHODS

Growth of *P. anserina* on solid medium and isolation of DNA. Organisms were grown and DNA was isolated as described in Cummings et al. (4) and Wright and Cummings (27). All growth experiments were performed at 27°C. Race A was used for all experiments, with a single exception in which race s was used (see Fig. 5).

Growth of *P. anserina* in liquid culture. A stock plate of *P. anserina* mycelia was allowed to grow for 3 days at 27°C. These mycelia were then homogenized with a Sorvall Omni-Mixer (Du Pont Co., Wilmington, Del.) in the presence of 100 ml of corn meal medium (4), and the homogenized mycelia were poured into a 2-liter flask containing 1 liter of corn meal medium. The flask was then rotated at 27°C. After

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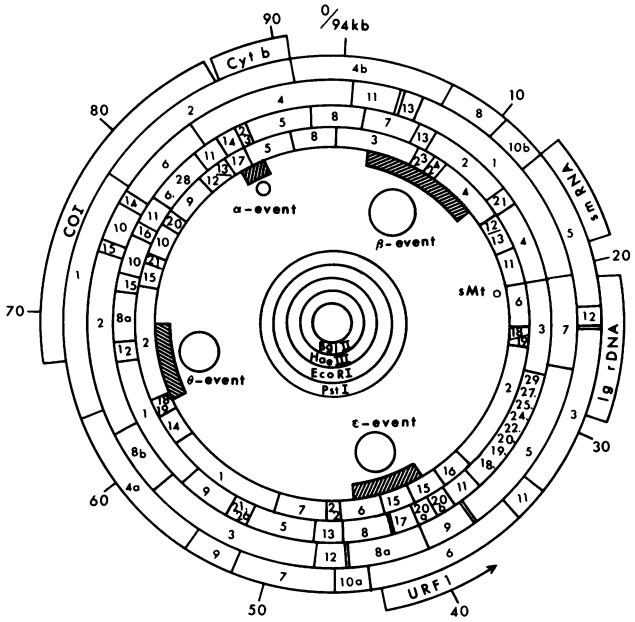

FIG. 1. Restriction and partial gene map of mitochondrial DNA of race s *P. anserina*. The origins of a number of excision-amplification plasmids are shown, including that for αsenDNA. The αsenDNA plasmid is derived from *Eco*RI restriction fragment E4 and from two *Bgl*II restriction fragments which are located in E4 (B5 and B17).

3 days, 100 ml of medium containing the mycelial puff balls was removed from the flask and homogenized. For the first experiment, which was maintained for 18 subsequent transfers, the remaining puff balls were filtered on cheesecloth and squeezed by hand to form a paste, and the paste weight was determined. For each transfer, 125 mg of homogenized mycelia, determined from the paste weight, was added. This resulted in a dilution factor of approximately 1:200. For the second experiment, which was maintained for 40 transfers, the first 20 transfers were performed at a 1:200 dilution. The second 20 transfers were performed at a 1:1,000 dilution.

Genetic analysis. For reciprocal crosses (i.e., male × female) puff ball-derived cultures from either transfer 14 or 40 of the second liquid culture experiment or mycelial plugs from the L14a2 longevity mutant were used as the male parent. All possess the "+" mating type. A wild-type A^- strain was used as the female parent. The male parent cultures were grown in the dark for 4 days on corn meal agar slants containing sorbose (1%). At the same time the female parent cultures were grown on corn meal agar plates for 4 days, also in the dark. Next, all cultures were grown in continuous fluorescent light for an additional 4 days to induce the formation of spermatia and ascogonia in the male and female parents, respectively. At that time the spermatia were washed from the sorbose slants with sterile distilled H_2O and poured over the agar plates to fertilize the ascogonia. Spore formation was allowed to occur for 3 to 5 days in the light, and the spores were collected on physiological agar plates (10 g of NaCl per liter). Ascus groupings containing five spores (~2%), rather than the more common four-spore groupings, were identified, and the two smallest uninucleate spores were selected for further study. Such spores are homokaryotic for mating type. All work was performed at 27°C.

The strains discussed above were also used for confrontational crosses. Mycelial plugs of the opposing mating types were plated on the same corn meal agar plates and allowed to grow toward each other for 4 days in the dark at 27°C. A confrontation boundary formed where the mycelia of the opposing mating type strains met. After this boundary had formed the cultures were exposed to continuous fluorescent light for 4 days to induce spore formation. Spores were then collected and identified as described above.

Restriction enzyme analysis and Southern blotting. Restriction enzyme analysis was performed as described previously (6). All enzymes were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, Md.). DNA-DNA hybridization was done by the method of Southern (21) as modified by Wright et al. (28).

RESULTS

Growth in liquid culture alters longevity. A relatively short-lived strain, A^+-84-11, was chosen for these studies. The average lifespan of this strain when grown on solid corn meal agar in race tubes was 12.2 ± 4.0 cm. Two independent liquid culture experiments were conducted to monitor the kinetics of senescence in liquid culture. For the first experiment the homogenized mycelia were passaged every 3 days at approximately a 1:200 dilution (see Materials and Methods) for a total of 18 transfers. The total amount of mycelia obtained at the end of each 3-day period increased gradually as a function of the transfer number (Table 1), indicating that the culture was growing vigorously when the experiment was terminated. To ensure that the apparent health of this culture was not due merely to its transfer to fresh medium every 3 days, the following control was performed. Four mycelial plugs were placed on corn meal agar plates, and growing edges were transferred to fresh agar plates every 3 days. All four cultures ceased growing within six transfers; the average longevity when summing the distance grown on each agar plate was 9.5 ± 1.0 cm.

Ten mycelial puff balls from every other liquid culture transfer were placed in race tubes to determine their longevity on solid medium. The average longevity decreased to 5.7 ± 1.6 cm when they were first transferred to liquid culture. It decreased further to 2.9 ± 1.2 cm by the second transfer. Longevity then began increasing steadily until transfer 14, at

TABLE 1. Effect of serial passage in liquid culture on *P. anserina* growth and longevity

Transfer no.	Paste (g/liter)[a]	Longevity[b] (cm)
Control[c]		12.2 ± 4.0
0[d]	14.4	5.7 ± 1.6
2	21.1	2.9 ± 1.2
4	19.4	4.7 ± 0.8
6	19.4	7.7 ± 3.6
8	23.3	16.2 ± 8.6
10	24.7	30.7 ± 19.7
12	26.7	43.8 ± 10.9
14	25.5	62.7 ± 15.9
16	26.7	45.1 ± 19.1
18	28.9	56.4 ± 16.2

[a] The corn meal liquid was removed by filtering through cheesecloth, and the mycelia were then squeezed by hand to form a paste.

[b] The distance grown in centimeters in race tubes before senescence. All numbers given are the average of 10 race tubes, except for the control, which is an average of 14 race tubes.

[c] Mycelial plugs from the A^+-84-11 strain which were plated directly into race tubes from a stock plate.

[d] An agar stock plate of the A^+-84-11 strain was homogenized and placed in a 1-liter flask of corn meal medium. This flask represents the 0 transfer.

which point it appeared to level off (Table 1, Fig. 2). The average longevity seen at transfer 14 (62.7 ± 15.9 cm) was nearly five times greater than that seen for the A^+-84-11 strain when it is grown exclusively on solid medium. All puff ball-derived cultures grew at a constant rate before exhibiting senescence, most at a rate of approximately 6.5 mm per day. This is identical to the growth rate for the A^+-84-11 strain, before senescence, when it is grown exclusively on solid medium. Several cultures grew somewhat slower (4.5 to 6 mm per day), although the growth for an individual culture still occurred at a constant rate. For these experiments senescence was defined as either a complete cessation in growth or a reduction in the growth rate.

The second liquid culture experiment was maintained for 40 transfers. Due to an increase in the growth vigor of the culture the last 20 transfers were passaged at a 1:1,000 dilution. Once again the liquid culture was growing vigorously when the experiment was terminated, and the longevity of most of the puff ball-derived cultures, measured periodically from transfers 14 to 40, was markedly increased. When the longevity results from this second experiment were considered, a bimodal, albeit markedly skewed, distribution for longevity was observed (Table 2). A similar result was seen when considering transfers 14, 16, and 16 from the first liquid culture experiment (data not shown). Occasional cultures never underwent senescence as defined above and have been tentatively termed immortal (Table 2).

Molecular analysis of altered longevity. To determine whether the apparent immortality in liquid culture was due to mitochondrial DNA rearrangements, DNAs were isolated from the young A^+-84-11 mycelia and from transfers 9, 11, 20, and 40 of liquid cultures. The DNAs were purified on CsCl-DAPI gradients (4) to produce three fractions enriched for (i) genomic mitochondrial DNA, (ii) heavy mitochondrial plasmid DNA, and (iii) nuclear DNA. We also isolated DNAs from a number of puff ball solid medium-derived cultures; two had grown 70 cm but had not yet undergone senescence, one was immortal after 140 cm of growth (L14c2Imm), and three cultures had undergone senescence and then spontaneously transformed into longevity mutants.

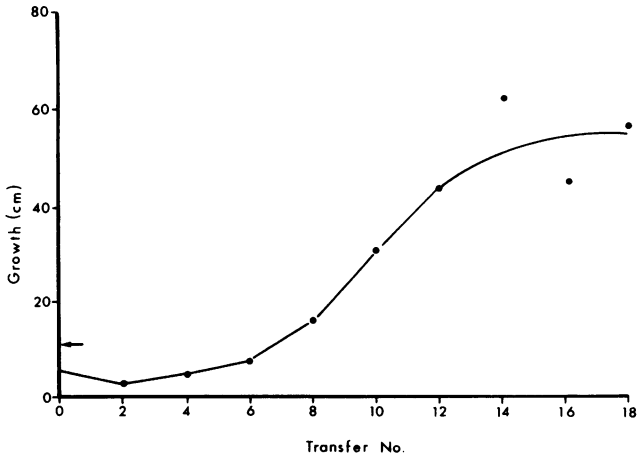

FIG. 2. Serial passage in liquid culture leads to an increase in *P. anserina* longevity on solid medium. Ten puff balls from every other transfer of the first liquid culture experiment were placed in race tubes. The average longevity achieved for each transfer tested is shown. The arrow points to the average longevity (12.2 cm) for the strain used for this experiment, A^+-84-11, when mycelial plugs were placed directly in race tubes without prior growth in liquid culture.

TABLE 2. Bimodal distribution in longevity for puff ball derived cultures after transfer 14 in liquid culture

Transfer no.	Longevity of cultures (cm)		
	Short lived[a]	Long lived[a]	Immortal[b]
14		83, 97, 137	>190 (1)
22	27, 39	91, 101	>175 (1)
26	29	76, 80, 98, 145	
30		107	>160 (3)
35		95, 96	>135 (2)
40	32, 40, 41	75, 82	>115 (3)

[a] Each number represents the growth of a single puff ball isolated from the indicated number in the second liquid culture experiment.

[b] Cultures which have not yet senesced. Numbers represent the distance grown at the time of submission. The numbers within parentheses represents the numbers of such cultures remaining for each transfer number.

Two of the longevity mutants (L9R2 and L18a7) had stopper phenotypes marked by multiple cycles of growth and senescence. The third longevity mutant (L14a2) had a continuous growth phenotype, although it had senesced once relatively early in its lifespan. Each longevity mutant was derived from an individual puff ball isolated from the indicated transfer (the number following "L"). L14a2 and L18a7 were isolated from the first liquid culture experiment, and L14c2/Imm was isolated from the second liquid culture experiment. The L9R2 culture was isolated from the ninth and final transfer of a preliminary experiment which we have not reported.

The mitochondrial DNAs were digested with the restriction enzyme *Bgl*II and separated by electrophoresis on 1.2% agarose gels. There was no apparent alteration in the restriction enzyme pattern for any of the liquid culture mitochondrial DNAs as compared with the young wild-type control (Fig. 3, lanes a through e). Further, no restriction enzyme pattern difference was seen for the DNAs isolated from the long-lived puff ball-derived cultures at 70 cm of growth, before senescence (data not shown). Marked differences in the restriction enzyme pattern (i.e., rearrangements) were seen, however, for the three longevity mutants, two of which

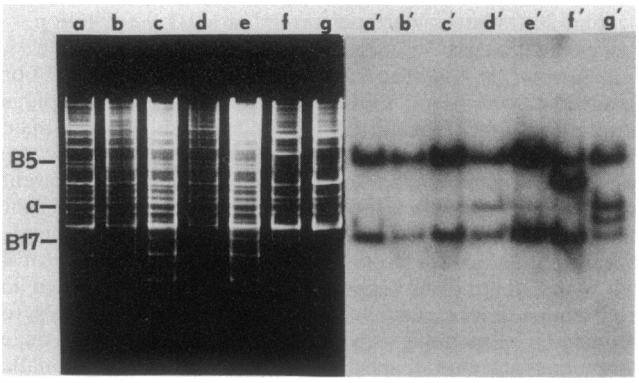

FIG. 3. Mitochondrial DNA from the liquid cultures appears identical to the young mitochondrial genome. Mitochondrial DNAs were isolated from (a) a young A^+ strain, culture transfers (b) 9, (c) 11, (d) 20, and (e) 40, and the (f) L9R2 and (g) L18a7 longevity mutants. These DNAs were digested with the restriction endonuclease *Bgl*II, separated by gel electrophoresis, and stained with ethidium bromide. They were then Southern blotted and hybridized to ^{32}P-labeled αsenDNA (lanes a' through g'). B5 and B17 correspond to the mitochondrial regions from which αsenDNA is derived (Fig. 1). The position of the αsenDNA plasmid is also shown. Note the extra hybridization bands in the longevity mutants (lanes f' and g').

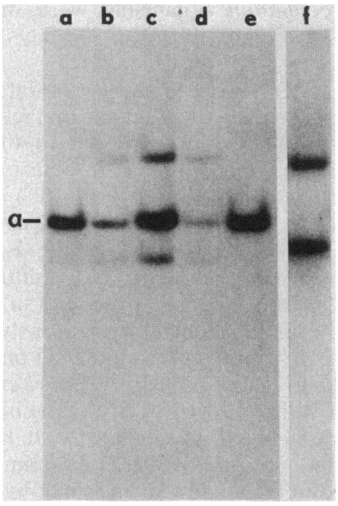

FIG. 4. The αsenDNA plasmid in the liquid cultures. Plasmid-enriched DNAs were isolated from liquid culture transfers (a) 9, (b) 11, (c) 20, and (d) 40 and from longevity mutant L14a2 (f). These DNAs were digested with the restriction enzyme *Bgl*II and, along with cloned αsenDNA (e), were separated by agarose gel electrophoresis, Southern blotted, and hybridized to ^{32}P-labeled αsenDNA. The two extreme bands (i.e., non-senDNA) represent B5 and B17 (Fig. 2).

are shown in Fig. 3 (lanes f and g). Rearrangements were also seen in the immortal mutant's mitochondrial DNA (data not shown). The DNAs shown in Fig. 3, lanes a through g were then blot hybridized to the αsenDNA plasmid to determine whether rearrangements involving the mitochondrial region giving rise to this plasmid, restriction enzyme fragments B5 and B17 (Fig. 1), could be detected (Fig. 3, lanes a′ through g′). No such alterations were detected for any of the liquid culture mitochondrial DNAs (Fig. 3, lanes a′ through e′), nor were they detected for the long-lived puff ball-derived cultures before senescence, the continuous growth longevity mutant L14a2, or the immortal culture L142/Imm (data not shown). Small amounts of αsenDNA were also seen in some of these DNAs due to contamination from the plasmid-enriched fractions. However, an extra hybridization band, in addition to B5, B17, and αsenDNA, was detected in the longevity mutants with stopper phenotypes, L9R2 and L18a7 (Fig. 3, lanes f′ through g′), indicating that there may be an association between rearrangement in the αsenDNA mitochondrial region and their longevity phenotypes.

The plasmid-enriched DNAs from the liquid cultures were also digested with *Bgl*II and separated on a 1.2% agarose gel. These DNAs were then blot hybridized with the αsenDNA plasmid. In all cases the liquid cultures contained the αsenDNA plasmid (Fig. 4). In comparison, no αsenDNA was detected in the continuous growth longevity mutant L14a2 (Fig. 4, lane f), but a significant amount of this plasmid was detected in the immortal culture L14c2/Imm, as well as another plasmid, βsenDNA (data not shown) (see Fig. 1 for mitochondrial location of the senDNA sequences). The plasmid-enriched liquid culture DNAs were also blot hybridized with the sMt-1 plasmid, which is found in many longevity mutants isolated on solid medium (Turker et al., submitted). No sMt-DNA was detected in any of these cultures (data not shown).

To determine whether continuous growth in liquid culture exerted any selective pressure on the mitochondrial genome, the following experiment was performed. An s$^-$ race longevity mutant, with a mixture of wild-type and rearranged mitochondrial DNAs, was identified. When digested with *Bgl*II endonuclease, mitochondrial DNA from this mutant revealed a prominent extra band (Fig. 5) which hybridized to the E4 region of the mitochondrial genome (Fig. 1), but not to αsenDNA (Cummings et al., in press). This s$^-$ longevity mutant was transferred at a 1:200 dilution in liquid culture, and mitochondrial DNA was isolated after nine transfers. After this extended growth in liquid culture the isolated mitochondrial DNA was essentially wild type (Fig. 5). Therefore continuous growth in liquid culture may selectively retain the wild-type mitochondrial genome, unlike growth on solid medium, which will tolerate extensive mitochondrial rearrangements in senescent and postsenescent cultures.

In mammalian cells there have been reports of quantitative changes in nuclear ribosomal DNA associated with the aging process (12). Therefore we used nuclear ribosomal specific probes to determine whether any such changes occurred in the nuclear DNAs from the liquid cultures. None were detected (data not shown).

Genetic analysis of altered longevity. To determine whether the increased longevity phenotype in liquid culture was heritable, we set up genetic crosses with puff ball-derived cultures from transfers 14 and 40. We attempted to cross the puff ball-derived cultures as both male and female parents in reciprocal crosses with wild-type A$^-$ strains, but they proved to be female sterile. Despite reduced fertility they were able to cross as males. Only uninucleate spores, homokaryotic for mating type, were tested for longevity (see Materials and Methods). When the puff ball-derived cultures were crossed as male parents, the progeny demonstrated longevity approximately equal to that of the A$^-$ female parent (Table 3). Crosses were also performed between the continuous growth longevity mutant L14a2 (Fig. 4) and the

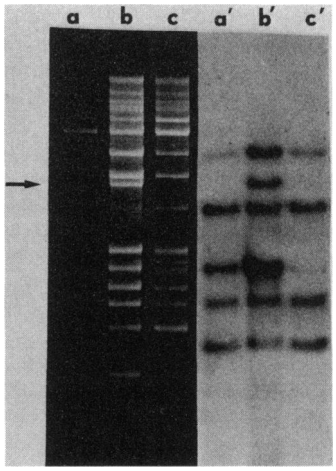

FIG. 5. Growth in liquid culture selects for the wild-type mitochondrial genome. Mitochondrial DNAs were isolated from (a) a young s$^-$ control, (b) the longevity mutant Psen1X grown on solid medium, and (c) the Psen1X strain after nine transfers in liquid culture at a 1:200 dilution per transfer. The DNAs were digested with the restriction enzyme *Bgl*II, separated by agarose gel electrophoresis, and stained with ethidium bromide. These DNAs were then Southern blotted and hybridized to ^{32}P-labeled mitochondrial fragment E4 (Fig. 1). The arrow points to an extra hybridization band present in Psen1X which disappeared after this strain was grown in liquid culture.

wild-type A^- strain. This mutant culture was also female sterile, but when the culture was crossed as a male parent three of the four progeny demonstrated a significant increase in lifespan before undergoing senescence. Further, the other spore-derived culture has not yet senesced after >125 cm of growth. Since the male parent does not contribute any cytoplasmic factors in *P. anserina* reciprocal crosses, this result indicates a nuclear component to the phenotype of the L14a2 longevity mutant. A comparison of this result with that obtained in the above crosses with the puff ball-derived cultures suggests that the increased longevity resulting from growth in liquid culture was probably not due to a dominant change in the expression of one or more nuclear genes. Such a change, however, may result in the female-sterile phenotype. We also note that although the analyzed spores were diploid (i.e., 2*N*) they should have been homokaryotic for all genetic loci. However, for this study we have not evaluated the potential effects of recombinational events between the two parental genomes before the formation of the uninucleate spores.

We also set up confrontational crosses (16), which allow for cytoplasmic mixing and mitochondrial fusions as well as nuclear DNA recombination. Once again fertility was reduced. In the confrontational crosses between the puff ball-derived cultures from both transfers 14 and 40 and the A^- strain, the progeny again demonstrated longevity approximately equal to that of the A^- parent. In confrontational crosses between the L14a2 mutant and the A^- wild type, three spores were isolated; all are still growing at 125 cm (Table 3).

DISCUSSION

Despite the well-documented ability of *P. anserina* to senesce when grown on solid medium (19, 20), we have shown here that this organism does not senesce when grown continuously in liquid culture. This observation is similar to that reported for the plasmodia of the slime mold *Physarum polycephalum*. This organism also senesces when grown on solid medium, but displays apparent immortality when grown in liquid culture (D. Poulter, Ph.D. thesis, University of Leicester, Leicester, United Kingdom, 1969). However, initial growth in liquid culture (70 to 100 days) appears to suspend senescence rather than alter it, in contrast to our result with *P. anserina*, which demonstrates an increase in longevity with serial passage in liquid culture. When *Physarum polycephalum* organisms were maintained for very long times in liquid culture (>700 days) a significant decrease in longevity was observed (10).

TABLE 3. Genetic analysis of longevity in puff ball-derived cultures

Cross[a]	Longevity[b]
A^- parent	24.9 (8)
L14 × A^-	24.9 (3)
L40 × A^-	28.5 (1)
L14a2 × A^-	74.7 (3), >125 (1)
L14 × A^- (confrontational)	23.7 (4)
L40 × A^- (confrontational)	27.2 (3)
L14a2 × A^- (confrontational)	>125 (3)

[a] Puff ball-derived cultures from transfers 14 and 40 of the second liquid culture experiment and the longevity mutant L14a2, all possessing the + mating type, were crossed with a wild-type A^- strain. They were crossed either as the male parent in a sexual cross or in a confrontation cross.

[b] Each number is an average derived from the number of spores tested. Numbers represent the distance grown at time of submission. These spore-derived cultures have not yet senesced. The number of spores tested is shown within parentheses.

Although our longest experiment was maintained for only 40 transfers (120 days), the total expansion factor of 200^{20} (or 10^{49}) + $1{,}000^{20}$ (or 10^{60}) from transfer 1 to 40 is extremely large. In comparison, human diploid fibroblasts senesce after a total expansion factor of only 2^{50} (or 10^{15}) (9). A further comparison between our results and growth kinetics of mammalian cells in vitro proves interesting. For example, it has been long known that a primary culture of mouse fibroblasts will initially undergo a decline in growth vigor followed by the establishment of a vigorously growing immortal culture (24). This result is similar to our observation of a decline in the average longevity of liquid culture derived *P. anserina* puff balls followed by a gradual increase resulting in very long lived cultures (Fig. 2). It had also been demonstrated that immortal cultures of mammalian cells are actually mixtures of short-lived and long-lived cells with bimodal growth distributions (17). We have shown that the liquid cultures of *P. anserina* contained mixed populations of puff balls, most with very long lifespans, but a significant minority of cultures senesce relatively rapidly. Therefore, the apparent immortality of *P. anserina* in liquid culture is not due to the establishment of a pure population of immortal mycleia. Instead it appears to be due to a subpopulation of very long lived mycelia which dominate the culture. Since short-lived cultures were found as late as transfer 40, we speculate that they are derived from the long-lived mycelia.

The longevity of *P. anserina* on solid medium has been previously shown to be increased by inhibitors of mitochondrial function (8, 25). These agents include ethidium bromide, tiamulin, and streptomycin. Although we also found an increase in longevity with ethidium bromide, we found no effect with streptomycin (unpublished). It has been shown that ethidium bromide can rejuvenate senescent cultures of *P. anserina*, presumably by allowing for the preferential replication of the wild-type mitochondrial genome (13). In this respect rejuvenation with ethidium bromide is similar to our observations that the liquid cultures displayed only the wild-type mitochondrial genome and that growth in liquid culture will preferentially select for a wild-type mitochondrial genome over a mutant one. Such preferential selection could occur either at the population level or at the molecular level. At the population level it is possible that after homogenization and dilution those myclelial fragments containing wild-type mitochondria would have a growth advantage over those fragments which contain altered mitochondrial genomes. Consistent with this speculation is our observation that on solid medium most cultures with altered mitocondrial genomes grow substantially slower than wild-type cultures (Turker et al., submitted). At the molecular level it is possible that altered mitochondrial genomes are less efficiently replicated or preferentially degraded, particularly in the nonsenescent state which is maintained in liquid culture. Although at this time we do not know the mechanism or preferential mitochondrial DNA selection, we can speculate that the apparent immortality in liquid culture is due to such selection for the wild-type mitochondrial genome. Whereas on solid medium senescence is associated with rearrangements of this genome (5), such events would quickly be lost in liquid culture. Longevity mutants isolated on solid medium would be due to those rare events which favor, rather than inhibit, growth. An example of such an event was presented in Fig. 3 and elsewhere (2; Cummings et al., in press), demonstrating a relationship between longevity and

rearrangement involving the αsenDNA mitochondrial region.

A significant difference remains, however, between the observations of "rejuvenation" with ethidium bromide and immortality in liquid culture. When cultures grown on ethidium bromide, which are immortal in the presence of this drug (25), are removed from its presence the cultures resume a wild-type longevity pattern (13). When we removed *P. anserina* organisms from liquid culture and allowed the puff balls to resume growth on solid medium, their average longevity was significantly increased. Since all young *P. anserina* cultures contain the wild-type mitochondrial genome, our observation that the wild-type mitochondrial genome persisted in the liquid cultures, which may explain immortality in liquid culture, cannot by itself explain the increased longevity of the puff ball-derived cultures on solid medium. We note that even after 70 cm of growth two puff ball-derived cultures still had the wild-type mitochondrial genome. These cultures eventually underwent senescence. An exceptional strain, L14c2/Imm, which we defined as immortal because it has yet to undergo senescence on solid medium, had mitochondrial DNA rearrangements. Since the mitochondrial DNA from this strain was not isolated until growth of 140 cm and considering our data indicating selective pressure for the wild-type genome in liquid culture, it is likely that its mitochondrial rearrangements occurred on solid medium and that they led to its immortal phenotype. An extensive study of mitochondrial DNA from a number of strains considered immortal will be necessary to determine whether an immortal phenotype on solid medium is possible in the absence of mitochondrial DNA rearrangement. We have also provided evidence against a dominant change in nuclear gene expression or the elimination of the αsenDNA plasmid during growth in liquid culture (see below) as causal to increased longevity of the puff ball-derived cultures on solid medium. Therefore, at this time we can offer no consistent explanation as to why the puff ball-derived cultures show the dramatic increase in longevity.

The αsenDNA plasmid has often been associated with senescence, although an absolute cause-and-effect relationship has not yet been established. In recent work we have found that the L14a2 continuous growth mutant, which lacks αsenDNA, has a temperature-sensitive phenotype for senescence (unpublished observations). When induced to senesce at 34°C the αsenDNA plasmid appears, further strengthening the association between this plasmid and senescence. Superficially, two results presented in this work would appear to contradict this association. We have shown that the liquid cultures do not senesce despite the presence of αsenDNA. However, immortality in liquid culture in the presence of αsenDNA would not be surprising if this plasmid were related to the mitochondrial rearrangements which occur concomitant with senescence. Such rearrangements would be selected against, negating the effect of the αsenDNA plasmid. The possibility of gene expression from the αsenDNA plasmid has been raised elsewhere (23). The presence of αsenDNA in the immortal strain L14c2/Imm is more difficult to explain, but assuming that there is a relationship between this plasmid and senescence a more complete analysis of this mutant might prove quite revealing. One possibility would be a point mutation (i.e., base pair substitution, frameshift mutation, or small deletion) in the αsenDNA plasmid produced by this strain. Another possibility is that a second site mutation rendered this mutant immune to the presence of mitochondrial plasmids, explaining the simultaneous presence of α- and βsenDNA.

Our genetic analysis of the increased longevity of *P. anserina* after growth in liquid culture was frustrating, since such growth led to female sterility. If we had been able to use the puff ball-derived cultures as female parents we may have been able to distinguish between a genetic or epigenetic component to this increase in longevity. Nonetheless our evidence demonstrated that the long-lived puff ball-derived cultures were recessive to a wild-type strain when crossed as a male parent and in confrontational crosses. Young plasmodia (i.e., long lived) of a second slime mold with a senescent program, *Didymium iridus*, were usually recessive to old plasmodia (i.e., short lived) in heterokaryons (3), as was an immortal mutant of *P. polycephalum* (10). In marked contrast to this latter finding, the L14a2 mutant was dominant when crossed as a male, indicating a nuclear component to its phenotype, and in confrontational crosses.

Finally, the fact that *P. anserina* does not senesce in liquid culture should not be taken as evidence that the well-documented phenomenon of senescence in this organism is an artifact of its usual growth on solid medium. Instead we believe that thorough analysis of this observation as well as nonsenescent strains we have identified may allow us to identify the underlying basis for senescence in *P. anserina*. In this regard we note that the continuous growth mutant L14a2, which does not normally yield the αsenDNA plasmid, and the immortal strain L14c2/Imm, which does not senesce despite the presence of both α- and βsenDNA, may be particularly important for a further analysis of *P. anserina* senescence.

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