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Mitochondrial recombination increases with age in *Podospora anserina*

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ABSTRACT

With uniparental inheritance of mitochondria, there seems little reason for homologous recombination in mitochondria, but the machinery for mitochondrial recombination is quite well-conserved in many eukaryote species. In fungi and yeasts heteroplasmons may be formed when strains fuse and transfer of organelles takes place, making it possible to study mitochondrial recombination when introduced mitochondria contain different markers. A survey of wild-type isolates from a local population of the filamentous fungus *Podospora anserina* for the presence of seven optional mitochondrial introns indicated that mitochondrial recombination does take place in nature. Moreover the recombination frequency appeared to be correlated with age: the more rapidly ageing fraction of the population had a significantly lower linkage disequilibrium indicating more recombination. Direct confrontation experiments with heterokaryon incompatible strains with different mitochondrial markers at different (relative) age confirmed that mitochondrial recombination increases with age. We propose that with increasing mitochondrial damage over time, mitochondrial recombination – even within a homoplasmic population of mitochondria – is a mechanism that may restore mitochondrial function.

1. Introduction

Many mechanisms may contribute to the overall process of ageing (Medvedev, 1990), but generally a key role of mitochondria in the ageing of eukaryote organisms is assumed. Harman's free radical theory of ageing (1956) stated that Reactive Oxygen Species (ROS) – predominantly generated in the electron transport chain in the mitochondria – are an important factor in the age-related decrease in mitochondrial function via damage of mitochondrial DNA (mtDNA), proteins and lipids. Whether through oxidative damage or not, a decrease in mitochondrial function and an increase in the number of defective mtDNA molecules have indeed been found to accompany ageing in many organisms (reviewed in Wallace, 1999; Osiewacz, 2002), including mammals (Trifunovic et al., 2004; Kujoth et al., 2007; Passos et al., 2007). Many cellular mechanisms may influence the ageing process by either preventing and limiting damage or by removal and repair of accumulating damage.

Protection against oxidative stress and related cell damage can for instance be found both in enzymatic antioxidants (*e.g.* superoxide dismutase, catalase, glutathione peroxidase) (for reviews see *e.g.* Mates et al., 1999; Landis and Tower, 2005) and in non-enzymatic antioxidants (*e.g.* Vitamin C, Vitamin E, carotenoids, lipoic acid and others) (for reviews see *e.g.* Valko et al., 2006), but these processes seem to diminish with age. Yeast, fungi and plants possess an alternative oxidase that can bypass complexes III and IV, typically resulting in an electron transport chain that exhibits reduced energetic efficiency as well as reduced ROS formation. Fungal strains in which complexes III and/or IV are absent or pharmacologically blocked, exhibit an increase in life span (Dufour et al., 2000; Lorin et al., 2001; Sellem et al., 2007; Maas et al., 2009).

Removal of damaged molecules and organelles can take place by autophagy, through sequestration and delivery of cargo to the lysozomes. Special genes involved in mitophagy – removal of mitochondria – have been found (see *e.g.* Kim et al., 2007). Also autophagous processes seem to decline with age (see *e.g.* Cuervo et al., 2005). Specific repair mechanisms exist like for instance the base excision repair (BER) pathway for the repair of small DNA modifications, a pathway that also shows an age-related decline with age (Chen et al., 2002; Soerensen et al., 2009). Homologous recombination in mitochondria has been observed in mammals (Thyagarajan et al., 1996). In this paper we focus on recombination in mitochondria as a mechanism to maintain a population of functional mitochondria with less age-accumulated damage.

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The filamentous fungus *Podospora anserina* is a model organism for ageing since the 1950s: mycelial growth rate and fertility decrease with age and the pigmentation of the distal mycelium changes when the ageing pigment lipofuscin accumulates. All wild-types of *P. anserina* senesce and the species shows genetic variation for ageing with a strain specific life span (Rizet, 1953; van der Gaag et al., 1998). In fact *P. anserina* was the first organism in which mitochondria were shown to be involved in ageing (Rizet, 1953; Marcou, 1961; for a complete history see Lorin et al., 2006). The mitochondrial role in ageing is also exemplified by the accumulation of major rearrangements of the mitochondrial genome (Griffiths, 1992; Bertrand, 2000; Osiewacz, 2002) which in turn is likely causally linked to respiration (Dufour et al., 2000).

The mitochondrial genomic instability in *P. anserina* has long been attributed to the activity of a single mitochondrial group-II intron (*Cox1*i1, senDNA α or intron α), but although removal of the intron α leads to life span extension and partial stabilization of the mtDNA, it does not abolish senescence altogether (Begel et al., 1999). Several other separate regions of the mitochondrial genome have also been found to be involved in mitochondrial rearrangements and these are the β, γ, ε, φ and ψ senDNA regions (Belcour et al., 1981; Wright et al., 1982; Cummings et al., 1985; Turker et al., 1987). Also a linear mitochondrial plasmid can be involved in mtDNA rearrangements (Osiewacz et al., 1989; Hermanns and Osiewacz, 1992; Maas et al., 2004). These pAL2-1 plasmid homologs occur in high frequency (45%) in natural isolates and shorten life span by approximately 30% (van der Gaag et al., 1998; van Diepeningen et al., 2008).

Mitochondria show a strict uniparental inheritance, also in filamentous ascomycetes, but these fungi provide the unique opportunity that heteroplasmons may be formed in the contact zone between mycelia with unlike mitochondria (Silliker et al., 1996, 1997; Xu, 2005). When heteroplasmons are formed between strains with different deleterious mitochondrial mutations, recombination yielding wild-type mitochondria is very frequently observed in *P. anserina* (Silliker et al., 1996, 1997). *P. anserina* has a relatively large mitochondrial genome of approximately 100 kb (Cummings et al., 1990) and analysis of ten different strains of the species has shown that there is much size difference in mitochondrial genomes (from 87 to 101 kb) due to optional introns. These are considered to be neutral as there is no correlation between life span and mitochondrial genome size (Salvo et al., 1998).

In this paper we want to test the role of (homologous) mitochondrial recombination as a mechanism to yield functional mitochondria during mitochondrial ageing. We first made a short inventory of genes with putative functions in homologous recombination in mitochondria in *Saccharomyces cerevisiae*. With these genes we searched for homologs in the genomes of both *P. anserina* and *Homo sapiens*, to know how relatively well these genes are conserved. Next, we made a survey of 112 natural *P. anserina* isolates from a local population for the presence of (neutral) optional mitochondrial introns. Linkage disequilibrium studies with these data show that the mitochondrial genome of *P. anserina* does recombine in nature. Splitting the natural population in two differentially ageing subpopulations based on the presence of mitochondrial linear plasmids and bio-assays of life span, indicates that especially faster-ageing strains have higher mitochondrial recombination rates. Finally, direct confrontation experiments to test for mitochondrial recombination were done with heterokaryon incompatible strains with different mitochondrial markers at different stages in life. The fact is mitochondrial recombination did indeed increase with age. Thus, we propose that with increasing mitochondrial damage over time, mitochondrial (homologous) recombination – even within an originally homoplasmic population of mitochondria – is a repair mechanism that may restore mitochondrial function.

2. Materials and methods

2.1. Sequence analyses

A selection of *S. cerevisiae* proteins from the *Saccharomyces* genome database at http://www.yeastgenome.org/was made (Table 1) that either have a known function in mitochondrial DNA recombination or potentially so based on functions elsewhere in the cell (nucleus) and cellular localization (in mitochondria). Homologs in the genomes of *P. anserina* and *H. sapiens* were identified by protein blasts (blastp) (Altschul et al., 1997) in the NCBI database at http://blast.ncbi.nlm.nih.gov/Blast.cgi and for *P. anserina* also at the *P. anserina* genome site at http://podospora.igmors.u-psud.fr/blast.cgi (Espagne et al., 2008). Based on the protein sequence data subcellular localization was predicted using MITOPROT (Claros and Vincens, 1996) and WoLFPSORT (Horton et al., 2007).

2.2. Strains and culture conditions

The Wageningen collection of 112 wild-type *P. anserina* strains (*Wa1*–*Wa119*) was tested for linkage disequilibrium between 8 optional mitochondrial markers. These wild-type strains have been isolated between 1991 and 2001 from herbivore droppings collected in the neighbourhood of Wageningen, The Netherlands. Strains were designated *P. anserina* based on their morphology (Mirza and Cain, 1969) and on sexual compatibility with known *P. anserina* strains (van der Gaag et al., 1998).

Heterokaryon incompatible strains were used for the recombination experiments: two lines derived from wild-type *Wa32* (*cyt*bi3$^+$, COX1i1$^+$), one naturally containing the *pAL2-1* homolog (+*pAL*), the other plasmid-free (−*pAL*) were crossed with confrontational crosses with *mid26* (*cyt*bi3$^-$, COX1i1$^-$), derived from culture collection strain *S*, that has the COX1i1 (intron α) sequence precisely deleted and whose cytochrome *c* oxidase activity is identical to that of wild-type cells (Begel et al., 1999). Testing of single-mating-type lines was done in crosses with known mat+ and mat− lines derived from strain *S*.

Culture conditions and media have been described by Esser (1974). Cornmeal agar was used as the standard growth medium. For germination the cornmeal agar was supplemented with 0.06 M ammonium acetate, for sporulation with 0.5% dried and ground horse dung. For DNA isolation strains were grown for two days on cornmeal agar covered with a layer of cellophane for easy collection of mycelium material. The *P. anserina* synthetic medium (PASM) used in the bio-assays for pAL2-1 homologs is based on the minimal media described by Esser (1974) (van Diepeningen et al., 2008). As carbon source 2% (N) or 0.02% (CR) glucose is used. All cultures were grown in the dark at 27 °C. For sporulation, strains were kept in the dark for three days and then transferred to a light stove at the same temperature with a diurnal rhythm of 12 h of light and 12 h darkness.

2.3. Isolation and analysis of DNA

Small mycelial cultures grown on solid medium with a cellophane overlay were ground in liquid nitrogen with a bead beater, suspended in 100 μl 5% Chelex X-100 (BioRad) and 10 μl Proteinase K (20 mg/ml), and incubated for 30 min at 56 °C. The samples were then heated for 10 min at 95 °C to inactivate the Proteinase K, centrifuged for 1 min at 12,000 × *g* and then ready for use in PCR analysis. Standard methods were used for gel electrophoresis and PCR (Sambrook et al., 1989).

2.4. Detection of pAL2-1 homologs

Two methods were used for the detection of pAL2-1 homologs in *P. anserina*. The first is based on PCR-detection using oligonucleotide primers located in the terminal inverted repeats of the plasmid (Maas et al., 2004). The second is a bio-assay for active pAL2-1 homologs: strains are grown on PASM with 2% glucose (N) and 0.02% glucose (CR) respectively. A strain without the pAL2-1 homolog shows a spectacular life span extension under CR conditions. With the pAL2-1 homolog this life span extension is absent (Maas et al., 2004; van Diepeningen et al., 2008).

2.5. Optional introns

For detection of optional introns (Fig. 1) primer sets have been used located in the surrounding exons of the genes, these primers enable the discrimination between intron-free (small fragment) and intron-bearing (larger fragments) genes. The oligonucleotide primers for the optional introns LSUr-i1, ND3i1, ATPase6-i1, *cyt*bi3, COIiA, COIi5 and gene duplication ND4L have been described before (Matsuura et al., 1986; Cummings et al., 1990; Belcour et al., 1997; Salvo et al., 1998). For the detection of the COX1i1 intron primers COX1i1fw TATTCGCCATGCTCCTCTTT and COX1i1rv AACCGTCAGCCTCAACTAATC have been used. Southern analyses on the mitochondria of wild-type strains confirmed the PCR-detection of the optional introns. The wild-type population of *P. anserina* did not show any polymorphisms for the COX1i1 trait, but the *mid26* and *Wa32* derived strains used for the below mentioned recombination experiment varied in this and the *cyt*bi3 intron.

2.6. Recombination experiment

For the recombination experiments single-mating-type lines were obtained from strains *mid26*, *Wa32* + *pAL*, and *Wa32* − *pAL*. These lines were tested in crosses with