Curr Genet (1994) 26: 184–186

Current Genetics

Short communications

Reversion of a long-living, undifferentiated mutant of *Podospora anserina* by copper

Karin Marbach, Juan Fernández-Larrea, Ulf Stahl

Fachgebiet Mikrobiologie and Genetik, Technische Universität Berlin, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany

Received: 28 January 1994

Abstract. The *Podospora anserina* nuclear mutant *grisea* displays an undifferentiated growth phenotype (diminished production of aerial hyphae), is female sterile (lack of perithecia), has a prolonged life-span compared to the wild-type strain, and lacks detectable phenoloxidase (laccase and tyrosinase) activity. Reversion of all of these characteristics to those of the wild-type phenotype was accomplished by supplementing the growth medium with extra amounts of copper salts. These results indicate that the primary defect of the *grisea* strain is in its copper uptake and/or distribution in the cells.

Key words: *Podospora anserina* – Copper metabolism – Pleiotropic mutation – Phenoloxidases

Every wild-type strain of the ascomycete *Podospora anserina* displays a race-specific life span on solid media; i.e., after growing for a distinct time and distance the front hyphae show an aberrant morpholoy and pigmentation and eventually die (Rizet 1953). This so-called ageing phenomenon is controlled by both nuclear and mitochondrial genes as demonstrated by the existence or short- and long-living strains with either nuclear of mitochondrial mutations (Tudzynski and Esser 1979; Schulte et al. 1988; Belcour et al. 1991; Marbach and Stahl 1994).

One of the nuclear mutations which results in longevity is the recessive mutation *grisea* (*gr*) located on linkage group IV. It displays a life span of 39±1 days in contrast to the 24±1 days of the isogenic wild-type strain. In addition, *gr* has impaired female sexual development (does not form mature perithecia), is temperature sensitive (hardly grows at 37 °C), and lacks detectable activities of the phenoloxidases laccase I, II and III and tyrosinase. In fact, *gr* was originally isolated and characterized as a laccase-negative clone. However, it still produces cross-reacting material (CRM) against laccases I and III, showing that these enzymes are synthesized but inactive (Prillinger and Esser 1977). Combining *grisea* with another long-living mutant, namely *vivax* (*viv*), gives rise to immortal double mutants (Esser and Keller 1976).

As laccase and tyrosinase are copper-containing enzymes, and as the pleiotropic effect of *gr* suggests a broad-acting metabolic defect, the possibility that these defects were related to a deficiency in copper availability, which would affect numerous copper-dependent enzymes, was investigated. For this purpose, the response of the *P. anserina* wild-type *s1* strain (ATCC 26003; Esser 1956) and an isogenic *gr* strain to copper salts was compared (Table 1). Different end concentrations of $CuSO_4$, $CuCl_2$ and $Cu(NO_3)_2$ were added to the growth medium; $FeSO_4$, $FeCl_2$, K_2SO_4, $CaCl_2$, $MgSO_4$, $MgCl_2$, $ZnSO_4$ and NaCl were also added with the same end concentrations to a series of control media.

Table 1. Influence of different copper concentrations on *grisea* and wild-type strains of *P. anserina*. Cultures were maintained at 27 °C. For physiological experiments and longevity tests, strains were grown using synthetic medium II with 0.5% glucose (Prillinger and Esser 1977), solid in Petri-dishes or racing-tubes. Crosses were performed on synthetic medium I with 1.5% fructose (Prillinger 1976). Extracellular laccase-activity was monitored qualitatively by adding 5 µl of 1% (v/v) guiacol in phosphate buffer (100 mM; pH 6.0) to solid medium. nt, not tested; ++, strong positive reaction; +, moderate positive reaction; (+), slight reaction, –, no reaction

Item	$CuSO_4$							
	No addition		1 µM		10 µM		100 µM	
	wt	gr	wt	gr	wt	gr	wt	gr
Laccase activity	–	–	++	–	++	(+)	++	++
Protoperithecia	–	–	++	–	++	+	++	++
Mature perithecia (ascospore formation)	–	–	++	–	++	(+)	++	++
Aerial hyphae	+	–	++	(+)	++	+	++	++
Live span (days)	29±1	>70	24±1	39±1	nt	nt	24±1	24±1
Growth at 37 °C	+	–	+	(+)	nt	nt	+	+

Correspondence to: U. Stahl

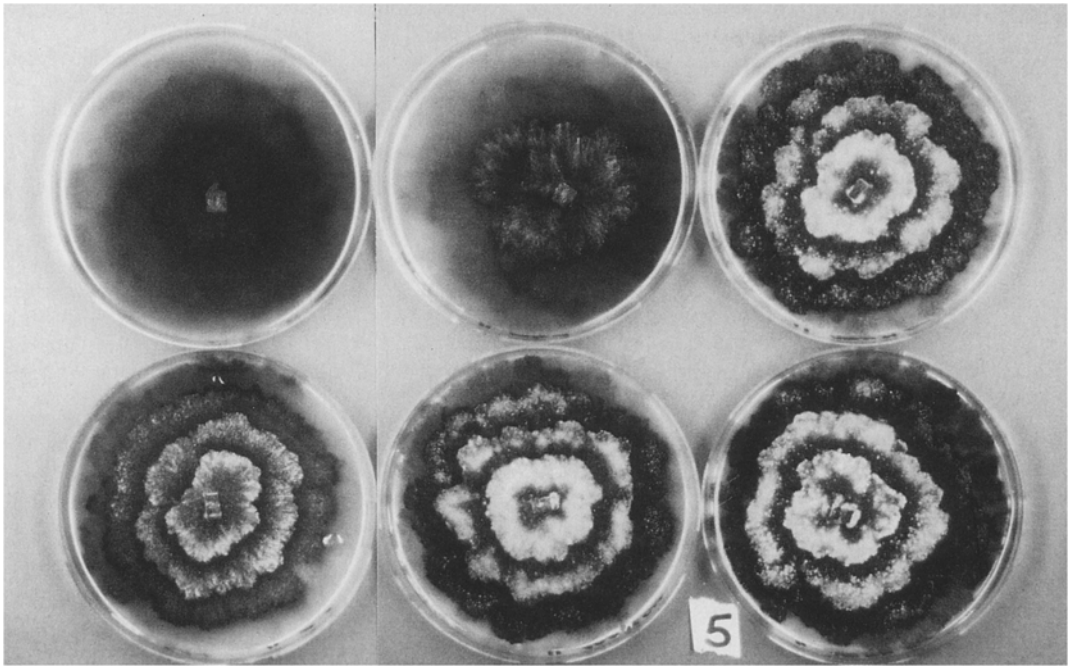

Fig. 1. Growth phenotype of the mutant *grisea* (upper row) in comparison to the wild-type strain (lower row) of *P. anserina* grown on synthetic medium II (Prillinger and Esser 1977) at different copper concentrations. From left to right: omission of copper (much less than 1 μM), 1 μM copper, 100 μM copper

Only the copper-containing salts counteracted the pleiotropic defects of *gr*. The degree of reversion of *gr* to the wild-type phenotype increased gradually with elevated copper concentrations, from being only partial with 10 μM Cu^{++} to complete reversion with 100 μM of additional Cu^{++} (Table 1 and Fig. 1).

Conversely, depletion of Cu^{++} salts from the medium accentuated the undifferentiated morphology and increased the longevity of *gr* and induced a *gr*-like phenotype in the wild-type strain. A similar effect was accomplished by the addition of 100 μM of $ZnSO_4$ to the medium. The initial pH of the medium also influences the expression of the *gr* phenotype. Both the *gr* and the wild-type strains showed similar phenotypes when cultivated at pH values of 5.0 or less, exhibiting a more-accentuated rhythmical growth, darker pigmentation, and a higher extracellular laccase activity than the wild-type strain grown at a standard pH of 6.5. With pH values ≥pH 7.0, *gr* grew very slowly and produced no aerial hyphae at all, whereas the wild-type strain still formed aerial hyphae and protoperithecia and excreted moderate amounts of laccase. Only when grown at pH 8.0 did the wild-type strain show diminished aerial hyphae and protoperithecia formation and a very low extracellular laccase activity.

The results presented clearly show that the *gr* strain is dependant on elevated extracellular copper concentrations for normal growth and development. The effect of zinc on *gr* and the wild-type strain might be due to a competition between copper and zinc uptake, as described for *Saccharomyces cerevisiae* (White and Gadd 1987; Lin et al. 1993). The influence of pH on the expression of the *gr* phenotype could also be explained, at least in part, by the higher solubility of copper salts at low pH values (and its decreased solubility at higher pH).

As CRM against laccases I and III is produced by *gr* when cultivated at a standard (1 μM) copper concentration (Prillinger 1976) on could conclude that this CRM corresponds to the inactive copper-depleted forms of these enzymes, produced as a result of a diminished intracellular availability of copper. Synthesis and stability of apolaccases I and III would therefore be independent of copper. By contrast, no CRM against laccase II is produced by *gr*, suggesting that the synthesis and/or stability of this enzyme is copper dependent.

A behaviour similar in many respects to that of the *P. anserina gr* strain has been described for the *Aspergillus nidulans* mutants *ygA* (recessive) and *yB* (semi-dominant) (Clutterbuck 1972; Kurtz and Champe 1981; Hermann et al. 1983). Both these mutants are deficient in cleistothecial laccase II and conidial laccase I and their sexual development is blocked at an early stage; but they revert to the wild-type phenotype when additional copper is added to the growth medium or when grown at low pH. Active laccases could be recovered in mycelial extracts after dialysis against copper salts, showing that the apo-forms of the enzymes are synthesized by these copper-deficient mutants.

The diminished intracellular copper availability in *gr* when grown on media containing normal amounts of copper (1 μM), could result from a diminished net copper uptake; this might be due to a defective copper transport system specific for low copper concentrations. Indeed in *N. crassa*, the presence of a copper-binding component within the cell-wall which controls the transfer of copper

ions across the cell membrane has been proposed (German and Lerch 1987). A mutation in such a copper-binding cell-wall component, resulting in a lower affinity for copper, would explain not only the recessiveness of the *gr* mutation but also the fact that higher copper concentrations completely restore the *gr* phenotype.

The defects of *gr* mutation could also result from an alteration in the storage or distribution of copper in the cells. It has been shown in *N. crassa* that the synthesis of active laccase and tyrosinase depends on intracellular storage of copper ions during the active growth phase. Metallothionein (MT) and glutathione (GSH) are likely candidates for this function (German and Lerch 1987; Huber and Lerch 1987). The ability of Cu-MT to donate copper ions to the apo-forms of other copper proteins, such as laccase (Morpurgo et al. 1983) and tyrosinase (Beltramini and Lerch 1982), has been demonstrated in vitro; the transfer of copper between MT and GSH, and from MT to copper-zinc superoxide dismutase, has also been shown to occur in vivo (Freedman and Peisach 1989).

When compared to the wild-type strain, *gr* mutants show a 30% reduction in growth, which might be due to a reduced activiy of cytochrome-c oxidase (COX) as a consequence of the mutant's impaired copper homeostasis. This has been shown to occur in copper-limited cultures of yeast (Keyhani and Keyhani 1975) and sycamore (*Acer pseudoplatanus*) cells (Bligny et al. 1986). Whatever the underlying mechanism, it is known that, similarly to the *gr* strain, mitochondrial mutants of *P. anserina* defective in COX activity display undifferentiated growth, female sterility and longevity (Kück et al. 1985; Schulte et al. 1988).

As an essential co-factor of numerous oxidases, copper plays a central role in oxidative metabolism. Copper deficiency, which seems to be the cause of reduced phenoloxidase activity and an undifferentiated growth phenotype in *gr* mutants, might also result in a distorted oxidative, metabolism. The fact that oxidative stress seems to be connected with differentiation (Sohal et al. 1987; Orrenius et al. 1992; Hansberg et al. 1993) could also explain the undifferentiated growth of *gr*. Thus it may be proposed that *gr* is a good model system for studying copper metabolism and the uptake, distribution and influence of copper and copper-dependent enzymes on physiological processes including respiration, free radical generation, growth and differentiation.

References

Belcour L, Begel O, Picard M (1991) A site-specific deletion in mitochondrial DNA of *Podospora anserina* is under the control of nuclear genes. Proc Natl Acad Sci USA, 88: 3579–3583

Beltramini, Lerch K (1982) FEBS Lett 142: 219–222

Bligny R, Gaillard J, Douce R (1986) Excretion of laccase by sycamore (*Acer pseudoplatanus L*) cells. Biochem J 237: 583–588

Clutterbuck AJ (1972) Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J Gen Microbiol 70: 423–435

Esser K (1956) Die Inkompatibilitätsbeziehungen zwischen geographischen Rassen von *Podospora anserina* (Ces.) Rehm. I. Genetische Analyse der Semi-Inkompatibilität. Z induckt Abstramm- u Vererbl 87: 595–624

Esser K, Keller W (1976) Genes inhibiting senescence in the ascomycete *Podospora anserina*. Mol Gen Genet 144: 107–110

Freedman JH, Peisach J (1989) Intracellular copper transport in cultured rat hepatoma cells. Biochem Biophys Res Comm 164: 134–140

German UA, Lerch K (1987) Copper accumulation in the cell-wall-deficient slime variant of *Neurospora crassa*. Chem J 245: 479–484

Hansberg W, DeGroot H, Sies H (1993) Reactive oxygen species associated with cell differentiation in *Neurospora crassa*. Free Rad Biol Med 14: 287–293

Hermann TE, Kurtz MB, Champe SP (1983) Laccase localized in Hulle cells and cleistothecial primordia of *Aspergillus nidulans*. J Bacteriol 154: 955–964

Huber M, Lerch K (1987) The influence of copper on the induction of tyrosinase and laccase in *Neurospora crassa* FEBS Lett 219: 335–338

Keyhani E, Keyhani J (1975) Cytochrome c oxidase biosynthesis and assembly of *Candida utilis* yeast cells: function of copper in the assembly of active cytochrome c oxidase. Arch Biochem Biophys 167: 596–602

Kück U, Osiewacz HD; Schmidt U, Kappelhoff B, Schulte E, Stahl U, Esser K (1985) The onset of senescence is affected by DNA rearrangements of a discontinuous mitochondrial gene in *Podospora anserina*. Curr Genet 9: 373–382

Kurtz MB, Champ SP (1981) Dominant spore color mutants of *Aspergillus nidulans* defective in germination and sexual development. J Bacteriol 148: 629–638

Lin C-M, Crawford BF, Kosman DJ (1993) Distribution of ^{64}Cu in *Saccharomyces cerevisiae:* kinetic analyses of partitioning. J Gen Microbiol 139: 1617–1626

Marbach K, Stahl U (1994) Senescence of fungal mycelia. In: Esser K, Lemke PA (eds) The Mycota, II. Growth differentiation and sexuality. Springer Verlag, pp 196–210

Morpurgo L, Hartmann H-J, Desideri A, Weser U, Rotilio G (1983) Yeast copper-thionein can reconstitute the Japanese-lacquer-tree (*Rhus vernicifera*) laccase from the type 2-copper-depleted enzyme via a direct copper (I)-transfer mechanism. Biochem J 211: 515–517

Orrenius S, Burkitt MJ, Kass GEN, Dypbukt JM, Nicotera P (1992) Calcium ions and oxidative cell injury. Ann Neurol 32: 33–42

Prillinger H (1976) Genetische Kontrolle der Phenoloxidase "Laccase" des Ascomyceten *Podospora anserina*. Biblio Mycol 51. J Cramer Verlag, Vaduz

Prillinger H, Esser K (1977) The phenoloxidases of the ascomycete *Podospora anserina*. XIII. Action and interaction of genes controlling the formation of laccase. Mol Gen Genet 156: 333–345

Rizet G (1953) Sur l'impossibilité d'obtenir la multiplication végétative ininterrompue et illimitée de l'ascomycète *Podospora anserina*. CR Acad Sci 237: 838–840

Schulte E, Kück U, Esser K (1988) Extrachromosomal mutants from *Podospora anserina:* permanent vegetative growth in spite of multiple recombination events in the mitochondrial genome. Mol Gen Genet 211: 342–349

Sohal RS, Allen RG, Nations C (1987) Oxygen free radicals play a role in cellular differentiation: a hypothesis. J Free Radicals Biol Med 2: 175–181

Tudzynski P, Esser K (1979) Chromosomal and extrachromosomal control of senescence in the ascomycete *Podospora anserina*. Mol Gen Gent 173: 71–84

White C, Gadd GM (1987) The uptake and cellular distribution of zinc in *Saccharomyces cerevisiae*. J Gen Microbiol 133: 727–737

Communicated by K. Wolf