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0014-4754/90/050513-05$1.50 + 0.20/0

A spontaneous benomyl-resistant mutant of *Podospora anserina* exhibiting a diurnal growth rhythm

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Received 16 September 1988; accepted 19 December 1989

Summary. A spontaneous mutant of *P. anserina* isolated by screening for benomyl resistance exhibited a diurnal growth rhythm dependent on light-dark cycles. The rhythmic character, the benomyl resistance and a growth rate reduced to 50% of that of the wild type were inherited together over more than 10 generations. The locus was mapped on linkage group II, 0.35 map units distal to the locus *z* (= 0.81 map units from the centromere).
Key words. Podospora anserina; benomyl resistance; mutant; mapping; diurnal growth rhythm; light-dark cycles.

Benomyl resistance has been found in *Aspergillus nidulans*[1], *Verticillium malthousei*[2], *Neurospora crassa*[3] and *Venturia inaequalis*[4–6]. The resistance is attributed to alterations of the fungal tubulin[1,7,8]. Spontaneous mutagenesis occurs regularly in *Podospora anserina*. As a result, mutants resistant against D-galactose, a non-metabolized hexose[9] and similarly against benomyl[10] have been obtained. In *P. anserina*, the benomyl-resistant strains are interesting in that they exhibit a rhythmic mycelial growth[11]. Rhythmic growth in fungi is related to the permeability of hyphal membranes[12,13], and hence ionic fluxes[14,15] and energy metabolism[16].
One of these strains differs from other clock mutants of *P. anserina*[17], and from the remainder of the isolated benomyl-resistant variants, in that its rhythm is synchronized to light-dark-cycles. This paper reports on further investigations with this particular strain.

Materials and methods

Fungus. The benomyl-resistant mutant *mbc*^{r} of *P. anserina* was isolated from an agar culture containing an MBC gradient. In the gradient agar technique[18], a layer of minimal-thiamine agar supplemented with 2 µg/ml benomyl was allowed to solidify while the petri dish was held in an inclined position. An equal amount of benomyl-free agar was then added which hardened while the plate was level. The two complementary slanted layers established a graded MBC concentration across the plate. The resistant strain grew ahead of the inhibited front of the sensitive mycelium.
The wild type s_1 and the reference strains of *P. anserina* (Ces. ex Rbh.) Niessl were obtained from the collection of *Podospora* strains in Bochum, FRG.

Media and cultures. The following media of Esser[17] were used: corn meal agar for crosses; corn meal agar containing 4.4 g/l ammonium acetate for germination; minimal-thiamine medium with 0.1 mol/l fructose as carbon source in tests.
Colonies for SEM observation were grown on aluminium-coated coverslips, partially covered by 1.5% malt extract solution adjusted to pH 6.0 with potassium hydroxide.
Benomyl, of which MBC (= methyl-benzimidazol-2-yl-carbamate) is the hydrolyzed derivative in aqueous solution, was a gift or Dr P. Wallnöfer (Munich, FRG). It was first dissolved in a solution containing 80% (v/v) ethanol and 20% (v/v) propylene glycol acidified with 10 ml/l 10 M HCl. The benomyl stock was added after the medium was cooled down to 40 °C following autoclaving, to give final concentrations of 1 µg/ml

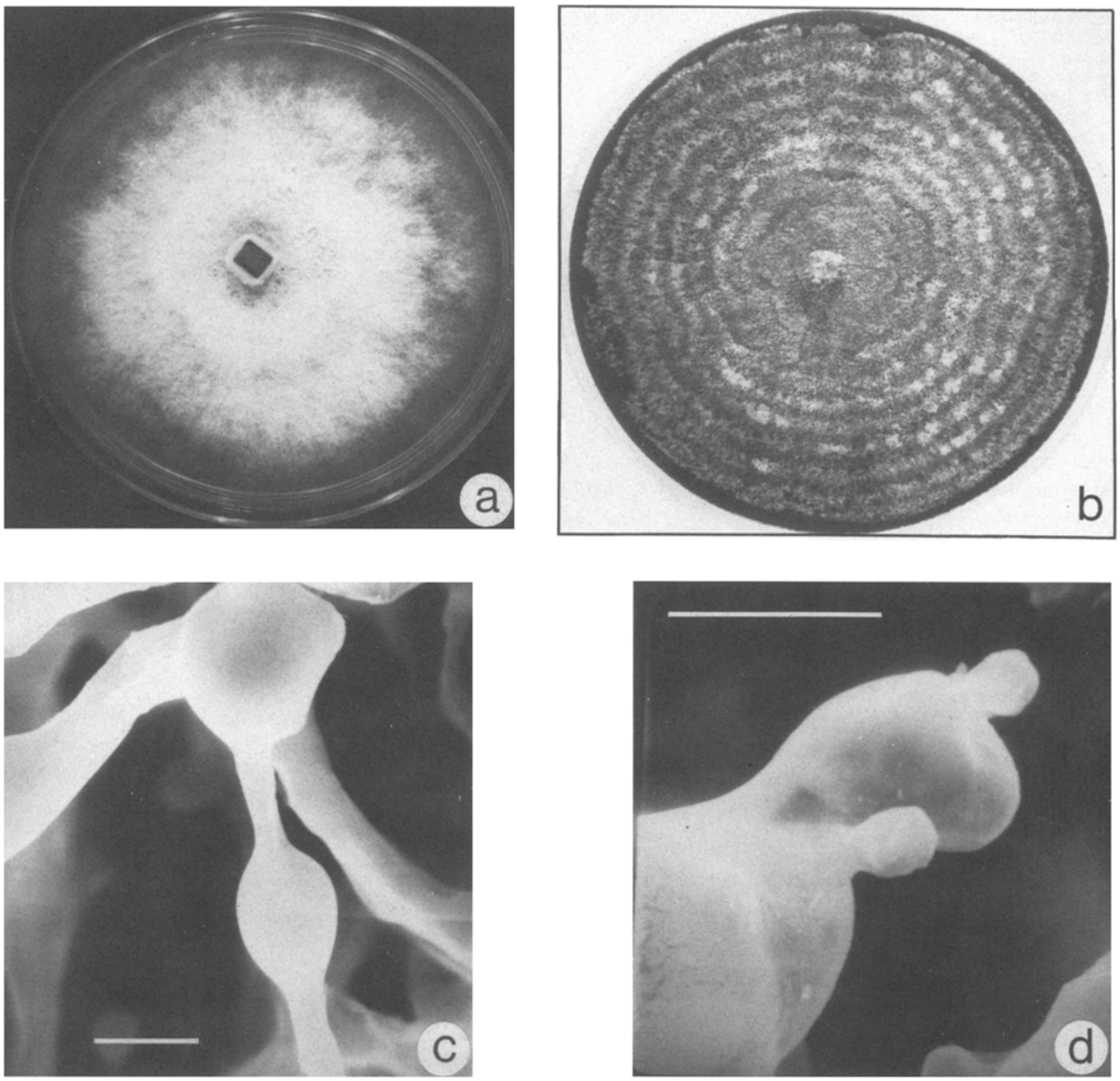

Figure 1. Agar cultures of the wild type (*a*) and the mutant *mbc*^{r} (*b*), grown at 27 °C under light-dark cycles of 10 h light and 14 h dark. (*c*) and (*d*) Scanning electron micrographs showing irregular hyphal shapes of the mutant *mbc*^{r}; the bar represents 5 µm.

($3.44 \cdot 10^{-6}$ mol/l benomyl), 10 µg/ml, 100 µg/ml and 1 mg/ml. The solvent without added benomyl had no antimicrobial effect up to a concentration of 1% (v/v) when added to the medium [11].

Scanning electron microscopy. The specimens were dehydrated through an acetone series, critical-point dried, and carbon coated. Observations were made on a Cambridge MK IIA SEM using an acceleration voltage of 20 kV.

Genetic analysis. Genetic analysis of the isolated strain was achieved by isolating ascospores in ordered tetrads [19] from crosses of the *mbc*^{r} strain. The reference mutants were mainly clock or incompatibility mutants. To detect the locus on a linkage group, additivity tests were performed according to Kuenen [20, 21] and Hohmeyer [22].

Results

Morphological description of mbc^r. A colony of *mbc*^{r} is shown in figure 1a in comparison to the wild type s_1 (fig. 1b). It can be seen that the pigmentation is similar to that of the wild strain, but the hyphae are more dense so that the mycelium looks slightly darker. This mutant is similar to the clock mutants *undulata, circulosa* or *tarda* of *P. anserina* [17]. As with these mutants, the linear growth rate of *mbc*^{r} is about 50% of the wild type.

Table 1. Periods of the endogenous growth rhythm in light-dark cycles (LD 10:14) and continuous dark (DD) of the benomyl resistant mutant *mbc*^{r} of *Podospora anserina*. Growth at 27 °C

Light regime	Medium	Bands/d	Period (h)	Linear growth rate (mm/d)
Light–dark 10:14	Minimal thiamine	0.98	24.4	2.36
	Malt extract	1.00	24.0	2.65
Constant dark	Minimal thiamine	0.34	72.0	1.94
	Malt extract	0.72	33.4	2.30

Figure 1 c and d show typical irregularities of these hyphae forming the outer region of the bands with conidia-like protrusions.

The bands formed by *mbc*^{r} are clearly visible. Their morphogenetic development is similar to that of other clock mutants of *P. anserina*[17] or the original mutant clock of *Neurospora crassa*[23]. Table 1 shows that the period is dependent on light-dark cycles. In permanent darkness the period depends on nutrient supply or temperature, as is found for other clock mutants. In LD-cycles the period is completely synchronized to these cycles. The rhythm is abolished in constant light.

Resistance to benomyl. The resistance to benomyl is given in figure 2: the *mbc*^{r} strain tolerated about 10 times more benomyl than the wild type. The rhythmically growing mutants *circulosa* and *undulata* of *P. anserina* were as sensitive to benomyl as the wild type (data not presented here) and exhibited a discoloring of the mycelium (Lysek, unpubl.). The mutant *mbc*^{r} did not, however, use the fungicide as a carbon source; this is so for other tolerant strains[11]. The resistance against benomyl is inherited together with the clock character and the reduced growth rate over more than 10 generations in crosses with the wild strain[11].

Mapping of the mbc^r *locus by tetrad analysis.* In tetrad analysis 1136 out of 1706 evaluable asci showed second division segregation (SDS) of the *mbc*^{r} locus, i.e. an SDS frequency of 0.67. In crosses with marker strains it became evident that *mbc*^{r} is situated on the one-armed linkage group II. But in contrast to the high interference on this arm, which prevents the occurence of non-parental-ditypes (NPD) with high efficiency, we frequently found such equivalents in crosses with mutant *circulosa* (*ci*) (table 2). A significantly low number of these NPDs after crossing with *zonata* (*z*) indicated that *z* and *mbc*^{r} belong to the same interference region, whereas *ci* and *mbc*^{r} do not.

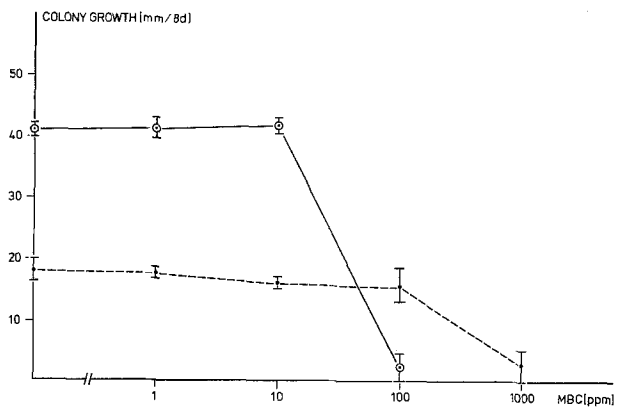

Figure 2. Linear growth of the wild strain s_1 and the mutant *mbc*^{r} on solid malt extract medium containing benomyl at the given concentrations (culture-conditions as in fig. 1).

Table 2. Tetrad analysis. Data of crosses of *mbc*^{r} with two marker strains of linkage group II, *ci* and *z*. * Data from conversion asci, i.e. 3:1 segregations were regarded half first and half second division segregation (SDS). For further details see Hohmeyer[21]

Ascus types	*ci* × *mbc*^{r} observed asci / calculated asci	*z* × *mbc*^{r} observed asci / calculated asci
A (SDS of both markers)	7.5* / 9.8	148.0 / 204.1
B (SDS of marker with lower SDS frequency)	6.0 / 4.8	80.5* / 41.8
C (SDS of marker with higher SDS frequency)	97.0 / 88.0	121.0 / 100.5
D_1 (diparental ditype)	24.0 / 21.7	15.5* / 10.3
D_2 (recombination ditype)	11.5* / 21.7	2.0 / 10.3
SDS frequency of tester strain	*ci*	*z*
observed	0.09	0.73
in literature[17]	0.1	0.83

Additivity tests[19] revealed that *mbc*^{r} is localized on the linkage group II distal *z*. Hence the arrangement is centromere – 0.46 units – *z* – 0.35 units – *mbc*^{r}. This indicates two independent interference zones on this arm. The first is situated between the centromere and the hitherto marked loci *377* and *z* (SDS frequency 0.84), where one cross-over occurs with high probability preventing further ones in this region. The second interference zone distal *z* is not affected by a cross-over in the first one. Similar distal regions exist for linkage groups I, III, and VII of *P. anserina*[24]. Furthermore, the SDS frequency of *z* is significantly reduced when crossed with *mbc*^{r} (table 2). With respect to this feature, *mbc*^{r} is also a meiotic mutant resembling those described by Simonet and Zickler[25]. This is also shown by a number of asci containing abnormal spores when *mbc*^{r} is crossed inter se[11].

The mutant *mbc*^{r} is deposited in the American Type Culture Collection, strains no. ATCC 46547 and ATCC 46558.

Discussion

While the isolation of clock-mutants after mutagenic treatment is well known in laboratory fungi[26], these mutations also arise spontaneously, as shown with benomyl or D-galactose resistance[10]. This corresponds to findings for populations of *Monilia fructigena*[27] and *Trichoderma*[41] that different types of growth and conidiation

rhythms occur regularly. These differences within populations are inherited and hence presumably caused by spontaneous mutations. These naturally occurring fungal clock mutants may help in the understanding of the evolution of biological rhythms[28], particularly since rhythmic events can be induced by a single mutation, regardless of whether they are endogenous, diurnal or circadian.

Benomyl is known to affect mitosis in *Aspergillus nidulans*[29], and the resistance to the fungicide is related to alterations in the tubulin[1,7]. A similar cause may also be assumed for *mbc*^{r}. This would easily explain the striking features of *mbc*^{r}, i.e. abnormal spore formation in self-crosses of *mbc*^{r}, significant reduction of SDS frequency in crosses with *z*, and hyphal irregularities.

On the other hand, rhythmic growth in *P. anserina* is thought to depend on alterations in the hyphal plasma membrane and consequently the ion distribution in and around the hyphae[12,14,15]. Experiments with *Neurospora crassa* and other fungi indicate that membrane events are likewise involved in the mechanism leading to rhythmic conidiation[16,30–32]. It is therefore possible that the mutation affects the membrane permeability for benomyl uptake. However, the internal ion concentrations of the mutant *mbc*^{r} are different from those in other clock mutants of *P. anserina*[15]. It is therefore likely that a different mechanism, involving altered tubulin, causes the rhythmic growth in this strain, since it has been shown that benomyl resistance is conferred by mutation in a structural gene for β-tubulin in *Aspergillus nidulans*[33], *Schizosaccharomyces pombe*[34], *Saccharomyces cerevisiae*[35,36], *Neurospora crassa*[37] and the slime mould *Physarum polycephalum*[38].

The light sensitivity of *mbc*^{r} adds a further complexity because it represents the first clock mutant of *P. anserina* exhibiting light dependence. The light receptor of *Neurospora crassa* is situated in the hyphal membrane[39]. This might also be the case in *P. anserina*, since a light triggered growth rhythm is induced by K^+-ionophores[40], and this fungus also exhibits some light sensitivity when forming perithecia. Obviously, the same alteration as that leading to benomyl resistance activates the light receptor system, and in addition meiotic and mitotic aberrations, altered ion concentrations, reduced growth rate and light-dependent rhythmic growth. An explanation of how all these phenomena are related is still needed.

Acknowledgments. The authors thank Mrs S. Salam and C. Ehrlicher for technical assistance, Prof. M. Raudaskoski (Helsinki) for critical reading of our manuscript, and the DFG, Bonn/Bad Godesberg, for financial support.

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