Protoplasmic Incompatibility in *Podospira anserina*: Possible Involvement of the Plasma Membrane in the Trigger Mechanism

By DANIEL ASSELINEAU, JEAN BERNET AND
JACQUES LABARÈRE*

*Laboratoire de Génétique, Université de Bordeaux II, allée des Facultés, 33405 Talence,
France*

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Two mutations in the *modC* locus of *Podospira anserina*, which abolish the process of cell lysis resulting from the combination of the *R* and *V* non-allelic incompatibility genes, have previously been shown to produce complete suppression of a laccase exoenzyme. In the present study it was found that, in addition, the *modC* mutants show a marked increase in the activity of an amino-acid oxidase (an exo- and endoenzyme), most of the increase (some 9- or 15-fold) being in the extracellular fraction. The *modC* mutant strains are hypersensitive to cycloheximide in vivo, but biochemical and genetical investigations suggested that the hypersensitivity is not due to effects on the ribosome. The *modC(1)* strain also shows increased resistance to chlorate, a specific sensitivity to acid (6.5) and neutral (7.5) pH, and significant hypersensitivity to methylammonium and thiourea. *ModC* cells accumulate a higher amount of the urea analogue [¹⁴C]thiourea. The pH sensitivity associated with the *modC(1)* mutation is specifically suppressed by the combination of the *R* and *V* incompatibility genes, and the *RV modC(1)* strain is resistant to sorbose. The specific consequences of the *modC(1)* mutation and the suppression of some of its effects by the combination of the *R* and *V* incompatibility genes lead to the suggestion that protoplasmic incompatibility might be induced by action of the *R/V* initiating complex in association with the plasma membrane.

INTRODUCTION

In *Podospira anserina*, protoplasmic incompatibility is a phenomenon of cell destruction resulting from the association of allelic or non-allelic genes (Rizet & Esser, 1953; Bernet, 1967). Non-allelic incompatibility systems (*C/D*, *R/V*) involve the genes of nine loci (*c*, *d*, *r*, *v*, etc.). Biochemical investigations have shown that protoplasmic incompatibility essentially consists of either a marked increase in or *de novo* synthesis of specific enzymes, especially a proteolytic enzyme suspected of being responsible for cell lysis (Bégueret & Bernet, 1973; Boucherie *et al.*, 1976).

From the study of mutations of incompatibility genes (Bernet & Belcour, 1967; Bernet & Bégueret, 1967), it was suggested that incompatibility genes might be the structural genes for protein factors whose specific interaction was the basic mechanism initiating protoplasmic incompatibility. Micrurgical studies involving hyphal anastomoses between strains of different genotypes (Bernet, 1965; Labarère *et al.*, 1974) revealed that in each pair of interacting non-allelic factors one participant was in the form of a soluble cytoplasmic factor showing extensive diffusion in hyphae. Further investigations gave no information as to the location of the non-diffusible factor or on the subcellular component supporting the non-allelic interactions.

Mutations in the *modA* gene abolished cell lysis irrespective of the non-allelic system (*C/D*, *R/V*) involved in protoplasmic incompatibility. Indirect evidence, namely the sensitivity of the *modA* gene product to dihydrostreptomycin and its inhibitory action on translation, suggested that *modA* was a ribosomal gene and consequently that the ribosomes might be the site of non-allelic incompatibility gene interaction (Bernet *et al.*, 1973; Labarère *et al.*, 1974). However, no direct evidence in support of the idea that the *modA* gene product was really a ribosomal factor was obtained.

Mutations in the *modC* gene have been selected on the basis of the suppression of protoplasmic incompatibility resulting from the combination of the *R* and *V* non-allelic incompatibility genes (Labarère & Bernet, 1977). The results of the present study show that collateral resistances or hypersensitivities to various unrelated drugs are associated with the *modC* mutation. They suggest that the *modC* gene product might be an intrinsic constituent of the plasma membrane.

METHODS

Organism. *Podospora anserina* is a heterothallic ascomycete whose life cycle closely resembles that of *Neurospora crassa*. Its asci contain four binucleate spores that include the two products of a half tetrad. In about 5% of asci, one binucleate spore is replaced by two uninucleate spores. These give rise to the homokaryotic strains used in our experiments. For details see Esser (1974).

The *r* and *v* incompatibility loci. The original isolates of geographical races of *P. anserina* were *rV* or *RV1* (Bernet, 1967). In these strains the *R/V* non-allelic incompatibility system is present in addition to the *V/V1* allelic system. The ♀ *rV* × ♂ *RV1* cross was the source of the *RV* lethal strain which combines the *R* and *V* incompatibility genes. In the *RV* strain growth stops soon after spore germination and the cells lyse in response to the *R/V* interaction (Bernet, 1965).

Strains and mutations. The *rV* genotype was the standard genetic background of strains. Mutations in the *modC* gene—*modC(1)* and *modC(2)*—were selected on the basis of the suppression of the process of cell destruction resulting from the union of the *R* and *V* incompatibility genes (Labarère & Bernet, 1977). The mutations *cyR1* and *cyR2* were non-allelic ribosomal mutations selected for the ability to confer resistance to cycloheximide (Crouzet, 1978; Crouzet & Bégueret, 1978).

Amino-acid oxidase assay. Cell-free extracts from cultures grown on synthetic medium were prepared according to the procedure of Boucherie *et al.* (1976). The assay for amino-acid oxidase activity was that described by Rahe *et al.* (1970) for phenylalanine ammonia-lyase as modified by Boucherie & Bernet (1978). The reaction mixture contained 0.85 ml 0.1 M-borate buffer pH 8.8, 0.5 µCi L-[U-¹⁴C]phenylalanine (specific activity 436 mCi mmol⁻¹; 1 Ci = 37 GBq) in 0.05 ml buffer and 1 ml dialysed supernatant (1 mg protein ml⁻¹). After incubation at 37 °C for 10 min the reaction was stopped by adding 0.16 ml 12 M-HCl. For controls, the reaction was stopped at zero time. After centrifugation of the mixture at 10 000 g for 10 min the supernatant was collected and phenylpyruvate was extracted as an enol-borate complex with 2 ml diethyl ether. The radioactivity of the ether fraction was determined by liquid scintillation spectroscopy.

Thiourea uptake. Mycelium was grown in a synthetic alkaline (pH 8.5) liquid medium in Roux bottles for 12 h at 26 °C, and 1 ml fractions of the culture were then incubated with 10 µCi [¹⁴C]thiourea (specific activity 2 mCi mmol⁻¹). At appropriate intervals thiourea uptake was stopped by the addition of 1 ml ice-cold non-radioactive thiourea solution (1 mg ml⁻¹). The mycelium was collected on Whatman GF/C filters by suction and washed three times with 5 ml of the same arrest solution. The filters were transferred into 5 ml NCS scintillation fluid for radioactivity counting. For reference, the uptake and incorporation of a ¹⁴C-labelled amino acid mixture (specific activity 50 mCi mmol⁻¹ of each amino acid) was measured. The amount of label incorporated into proteins was determined by boiling each sample for 15 min with an equal volume of 10% (w/v) trichloroacetic acid (TCA), and collecting the precipitate on Whatman GF/C filters. Filters were rinsed three times with 5 ml 5% TCA and transferred to 5 ml NCS scintillation fluid for radioactivity counting. The [¹⁴C]thiourea and the ¹⁴C-labelled amino acid mixture were provided by CEA, Saclay, France.

Preparation of ribosomes and poly(U)-directed polyphenylalanine synthesis. Preparation of ribosomes and in vitro polypeptide synthesis were carried out according to Crouzet *et al.* (1978). For in vitro polypeptide synthesis, the reaction mixture (total volume 125 µl) contained 25 µl dialysed S140, 50 mM-Tris/HCl, pH 7.8, 10 mM-magnesium acetate, 25 mM-KCl, 16 mM-2-mercaptoethanol, 6 mM-phosphoenolpyruvate, 2 mM-ATP, 0.5 mM-GTP, 150 µg yeast tRNA, 3 units of pyruvate kinase, 20 µg poly(U), 0.125 µCi [¹⁴C]phenylalanine (450 mCi mmol⁻¹) and 2.5 A₂₆₀ units of ribosomes. After incubation at 37 °C for 30 min, 125 µl 10% TCA was added

and the tubes were heated for 15 min at 90 °C. After cooling, the precipitates were collected on Whatman GF/C filters. The filters were rinsed with 5% TCA and the radioactivity was assayed.

Preparation of polysomes and assay of their activity in vitro. This was done as described by Crouzet & Bégueret (1980).

RESULTS

Overproduction of amino-acid oxidase activity by the modC mutants

In wild-type strains, some amino-acid oxidase is found in cell extracts but the major part of the overall activity is associated with an exoenzyme (Boucherie & Bernet, 1978). In *modC(1)* and *modC(2)* strains the amino-acid oxidase activity in the culture medium was, respectively, 15 times and 9 times greater than that in the wild-type; by contrast, in the cell extracts the activity was only 1.2 times greater.

Sensitivity of modC strains to cycloheximide

In vivo resistance or hypersensitivity to cycloheximide generally provides a good indication of the presence of mutations affecting the ribosomes in eukaryotes (Pestka, 1971; Pongratz & Klingmüller, 1973; Rothschild *et al.*, 1975). Resistance to cycloheximide may be associated with different ribosomal proteins (Bégueret *et al.*, 1977; Crouzet *et al.*, 1978; Cooper *et al.*, 1967; Haugli & Dove, 1972). Furthermore, ribosomal mutations selected on a basis other than cycloheximide resistance were associated with in vivo and in vitro resistance or hypersensitivity to cycloheximide (Bayliss & Vinopal, 1971; Coppin-Raynal, 1977).

The results in Fig. 1 show that the *modC(1)* strain was hypersensitive to cycloheximide in vivo. This hypersensitivity was indicated by a significant increase of the initial lag time before growth renewal after inoculation, and by an approximately 40% reduction in growth yield (mycelial diameter). The *modC(2)* strain showed a lower sensitivity: the lag period was only 2 d and the reduction in growth yield was only 30%.

CyR1 and *cyR2* are mutations to cycloheximide resistance which alter two different proteins of the 60S subunit (Crouzet, 1978). None of the double mutants combining *modC(1)* or *modC(2)* with one of the *cyR* mutations showed a phenotypic trait, such as cold or heat sensitivity, which would suggest functional interdependence between the *modC* and *cyR* loci. Furthermore, the *modC cyR1 cyR2* triple mutant showed no significant difference from the *cyR1 cyR2* double mutant which is cold sensitive (Crouzet & Bégueret, 1978). Similar negative results were also obtained from studies of in vivo resistance to cycloheximide of the *modC cyR* double mutants. In particular, the presence of the *modC(1)* mutation reduced to the same extent (40%) the cycloheximide resistance conferred by the *cyR1* or by the *cyR2* mutation (Fig. 2). Thus, all these results failed to support the idea that the *modC* mutations might affect the ribosomes.

In vitro studies were thus the only means of determining whether or not *modC* sensitivity to cycloheximide in vivo was ribosomal. For each *modC* mutation [*modC(1)* and *modC(2)*] the following three *modC/modC⁺* genotype pairings were considered: *modC*/wild-type, *modC cyR1/cyR1* and *modC cyR2/cyR2*. Polysomes and ribosomes were extracted from strains of these nine genotypes. The effect of cycloheximide concentrations ranging from 5 to 200 µg ml⁻¹ on the elongation activity of the polysomes and on phenylalanine incorporation in ribosome preparations was determined. No difference was observed between two strains belonging to the same genotype pairing.

Hence, it is possible that the in vivo hypersensitivity to cycloheximide of the *modC* strains is the consequence of an increased permeability of the *modC* cells to the antibiotic. If *modC* strains were in fact affected in the cell envelopes it might be expected that they would also show hypersensitivity or increased resistance to various toxic drugs.

Fig. 1

Fig. 2

Fig. 1. Effect of cycloheximide ($2.5 \mu\text{g ml}^{-1}$) on growth of *modC(1)* (●) and wild-type (○) strains. Inocula were placed on the surface of the culture medium at zero time.

Fig. 2. Effect of cycloheximide ($20 \mu\text{g ml}^{-1}$) on growth of the cycloheximide-resistant strains *cyR1* (○) and *cyR2* (●), and the double mutants *modC(1) cyR1* (□) and *modC(1) cyR2* (■).

Fig. 3

Fig. 4

Fig. 3. Effect of sorbose on growth of *modC(1)* (□), *RV modC(1)* (○) and wild-type (●) strains. The diameters of the mycelia were measured 48 h after placing the inocula on the surface of the culture medium.

Fig. 4. Effect of thiourea on growth of *modC(1)* (□), *modC(2)* (●) and wild-type (○) strains. The diameters of the mycelia were measured 48 h after placing the inocula on the surface of the culture medium.

Resistance of the modC strains to L-sorbose and chlorate and their sensitivity to acid and neutral pH and to methylammonium and thiourea

The following analogues were used: 2-deoxyglucose, L-sorbose, methylammonium, chlorate, thiourea, 5-fluorouracil, *p*-fluorophenylalanine and ethionine. Significant differences between the *modC* strains and the wild-type were observed in media supplemented with L-sorbose, methylammonium, chlorate or thiourea. In fungi, the presence of L-sorbose in the culture medium results in the formation of slow-growing (colonial) mycelia. It can be seen from Fig. 3 that the *modC(1)* strain showed some resistance (growth yield 1.2 times that of the wild-type) at a low sorbose concentration (1.0 mg ml^{-1}) and was hypersensitive at concentrations of 2.5 mg ml^{-1} or more.

Fig. 5. [^{14}C]Thiourea uptake by *modC(1)* (○) and wild-type (●) strains and, as a control to indicate growth, the incorporation of ^{14}C -labelled amino acids into protein by *modC(1)* (□) and wild-type (■) strains. The strains were grown at 26 °C in a liquid medium maintained at pH 8.5; in this medium the *modC(1)* strain grows at the same rate as wild-type.

Fig. 6. Effect of pH on the increase in dry weight of *modC(1)* (●) and wild-type (○) strains. After 2 d culture in liquid medium at 26 °C, the pH of the medium was raised from 7.5 to 8.5.

Methylammonium is an ammonium analogue (Roon *et al.*, 1975). High concentrations of methylammonium were needed to obtain significant inhibition of the growth of *P. anserina* strains. At 20 mg ml⁻¹ the wild-type was completely unaffected: at the same concentration the growth yield of the *modC(1)* and *modC(2)* strains was inhibited by more than 40%.

The effect of chlorate on fungal growth is controversial. It has been postulated that it is transformed into a toxic chlorite by nitrate reductase (Aberg, 1947) or has, in addition, a side effect which indirectly results in nitrogen starvation (Cove, 1976). To obtain inhibition of the growth of *P. anserina* the experiments with chlorate had to be conducted in *Aspergillus* culture medium (Cove, 1976). At a chlorate concentration of 30 mg ml⁻¹ wild-type growth was reduced by about 50%, whereas that of the *modC(1)* strain was completely unaffected.

Thiourea is a urease inhibitor (Dunn & Patemann, 1972). The two *modC* strains, especially *modC(1)*, showed a greater sensitivity to thiourea than the wild-type (Fig. 4). The cause of this hypersensitivity was investigated by means of labelled thiourea. The results in Fig. 5 clearly show that the *modC(1)* strain accumulated more label than the wild-type following the addition of [^{14}C]thiourea. Under the conditions used, there was no difference in growth

between the wild-type and the *modC(1)* strain, as shown by incorporation into proteins of ^{14}C -labelled amino acids.

The *modC(1)* strain and wild-type were cultured in liquid standard media with the pH adjusted daily to 6.5, 7.5 or 8.5. The wild-type showed identical growth (dry weight per culture) at each pH. The *modC(1)* strain showed practically no growth (less than 5% of the wild-type yield) at pH 6.5, a growth yield of 70% of the wild-type at pH 7.5 and the equivalent of wild-type growth at pH 8.5. When the pH of the medium was increased from 7.5 to 8.5 during the growth of a *modC(1)* culture, the growth rate showed a marked increase (Fig. 6) indicating that the pH sensitivity of the *modC* cultures was not restricted to renewal of growth after inoculation.

Combination of the R and V incompatibility genes

The strains of *RV modC* genotypes [*modC(1)* or *modC(2)*] were compared with the *modC* and wild-type strains. The *RV modC* strains clearly showed four of the properties which distinguish *modC* from wild-type strains, namely chlorate resistance and hypersensitivity to thiourea, methylammonium and cycloheximide. In contrast, the *RV modC* strains differed significantly from the *modC* strains in pH dependence and from the *modC* and wild-type strains in sorbose sensitivity. As shown above, the *modC(1)* strain exhibited wild-type growth only at pH 8.5. The *RV modC(1)* strain showed the equivalent of wild-type growth at pH 7.5 and 6.5. Hence, it may be concluded that the combination of the *R* and *V* incompatibility genes suppresses the sensitivity to acid and neutral pH associated with the *modC(1)* mutation. In contrast to the *modC(1)* strain, the *RV modC(1)* strain was hyperresistant to sorbose at all concentrations of the sugar tested (Fig. 3).

Besides the *RV* combination, which is lethal in the absence of *modC* mutations, three genotypes were possible with the wild-type alleles *r* and *R*, *V* and *V1* of the *r* and *v* incompatibility loci (Labarère, 1973): *rV*, *rV1* and *RV1*. The genetic background of the *modC* strains investigated in the experiments described above was *rV*. Strains of *rV1 modC* and *RV1 modC* genotypes were constructed. These showed a pH dependence and a sorbose sensitivity identical to those of the *rV modC* strains. In consequence, it might be concluded that the suppression of the pH sensitivity associated with the *modC* mutation, which was observed in the *RV modC* strain, was not the result of the presence of the *R* or *V* genes but of their combination. In the same way, the sorbose resistance specific to the *RV modC* genotype is clearly the result of the three-gene combination, since any substitution in one of the three loci (*r*, *v* and *modC*) resulted in strains showing a nearly identical sensitivity to sorbose.

Additional support for this interpretation came from investigation of the *RV modC(1)* strain at 32 °C. It is known (Labarère, 1973) that the combination of the *R* and *V* incompatibility genes which is lethal at 26 °C (in the absence of the *modC* mutations) gives a wild-type phenotype at 32 °C. At the latter temperature the *RV modC(1)* strain showed the same pH dependence and sorbose resistance as the *modC(1)* strain that carried genes in the *r* and *v* loci whose association did not result in protoplasmic incompatibility.

DISCUSSION

Since the *modC(1)* cells incorporated more radioactive label than the wild-type following the addition of [^{14}C]thiourea (Fig. 5), it is possible that the *modC(1)* mutation results in an alteration of the plasma membrane or the cell wall. All the properties associated with the *modC* mutations (methylammonium sensitivity, chlorate resistance, non-ribosomal cycloheximide sensitivity) support this interpretation. Collateral hypersensitivity or resistance to the effects of unrelated inhibitors have been previously described in a variety of organisms (Eriksson-Greenberg *et al.*, 1971; Ling & Thompson, 1974; Sanderson *et al.*, 1974; Rank *et al.*, 1975); in all cases a lesion of the cell envelope was demonstrated or strongly suspected. In

view of the pH sensitivity of the *modC(I)* strain (Fig. 6), it may be inferred that this strain carries a lesion of the plasma membrane.

The *modC* strains are defective in laccase III activity (Labarère & Bernet, 1978), an exoenzyme whose synthesis is dependent on stable mRNA (Boucherie, 1979). The present results show that amino-acid oxidase, which also depends on a stable mRNA, was significantly increased in *modC* cultures, especially in the extracellular region. Mutant strains showing a deregulation of one or several exoenzymes (i.e. suppression, hyperproduction or a strong imbalance in the ratio of intracellular to extracellular enzyme activity) have been reported (Gratzner, 1972; Yoneda *et al.*, 1973; Murayama & Ishikawa, 1973; Kunst *et al.*, 1974); all these mutants were thought to be altered in their cell envelopes (plasma membrane or cell wall). The properties of *modC* strains thus suggest that the plasma membrane contains determinants which regulate the production of exoenzymes and, more generally, of proteins depending on stable mRNA.

It was previously postulated that the *modC* mutations might prevent the formation of a complex between the interacting *R* and *V* gene products whose presence is the basic mechanism initiating protoplasmic incompatibility (Labarère & Bernet, 1977). Suppression of the pH dependence associated with the *modC(I)* mutation by the combination of the *R* and *V* incompatibility genes and not by any of the other three non-lethal *rv* combinations (i.e. the *rV* and *RV1* wild-type genotypes and the recombinant *rV1*) suggests that the *R/V* complex is in fact formed in the *RV modC(I)* strain, but is non-functional because of the presence of the *modC* mutation. In the same way the sorbose resistance specifically associated with the *RV modC* strains (Fig. 3) is consistent with the formation of the *R/V* complex in association with the plasma membrane. The *V* gene product is in the form of a soluble factor spreading in hyphae whereas that of the *R* gene is a non-diffusible determinant (Labarère *et al.*, 1974). The present results thus suggest that the *R* gene product might be associated with the plasma membrane in a way similar to that of the *modC* gene.

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