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Search for ribosomal mutants in *Podospora anserina*: genetic analysis of cold-sensitive mutants

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SUMMARY

Twenty-four cold-sensitive (prototrophic) mutants were isolated after UV mutagenesis of protoplasts of the fungus *Podospora anserina*. Genetic analysis of these mutants was performed in order to detect those among them which were most likely to be impaired in translational fidelity. The 24 mutations belonged to 24 different genes. One half of the mutants were pleiotropic and displayed an altered phenotype: growth rate at the permissive temperature, germination of the spores, fertility and/or sporulation. Nine mutants differed from wild-type in their resistance levels to cycloheximide, trichodermin and/or paromomycin. Several mutations were linked to known ribosomal loci. Two mutations behaved like informational antisuppressors: one is allelic to the previously described As_3 gene and the other one defines a new antisuppressor gene, AS_6.

1. INTRODUCTION

In bacteria, genetic and biochemical analyses of ribosomal mutants have been of considerable value in the study of ribosome structure and function (see Jaskunas, Nomura & Davies, 1974; De Wilde *et al.* 1977; Piepersberg *et al.* 1980, for reviews). In eucaryotes, the genetic analysis of cytoplasmic ribosomes is still in an early stage because of the lack of suitable mutants, even in the genetically favourable yeast system (see McLaughlin, 1974; Begueret & Picard-Bennoun, 1979, for reviews).

In the ascomycete *Podospora anserina*, biochemical analysis of ribosomes was initiated by Begueret and co-workers, using mutants resistant to cycloheximide (Begueret, Perrot & Crouzet, 1977; Crouzet *et al.* 1978; Crouzet & Begueret, 1978). Our own investigations of ribosomes in *Podospora* are mainly focused on the control of translational fidelity. This problem is also currently being studied in *E. coli*. Ribosomal mutations (*ram*) which enhance translational ambiguity have been described: they act as informational suppressors. Mutations which antagonize the former ones are known as restrictive ribosomal mutations: they reduce the error level in translation and act as antisuppressors (see Gorini, 1974; Bollen *et al.* 1976; Piepersberg *et al.* 1980, for reviews). However, mutants displaying increased or

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decreased levels of errors in translation have not been reported in eucaryotes. Mutations that could be ribosomal suppressors and antisuppressors have been described in *Saccharomyces cerevisiae* (Hawthorne & Leupold, 1974; Gerlach, 1975; MacCready & Cox, 1973), in *Schizosaccharomyces pombe* (Kohli *et al.* 1980; Thuriaux *et al.* 1975) and more recently in *Aspergillus nidulans* (Roberts, Martinelli & Scazzocchio, 1979). Also, mutants resistant to the aminoglycoside hygromycin which is known to stimulate misreading in eucaryotic translation were described in yeast (Singh, Ursic & Davies, 1979). However, biochemical analyses which could have demonstrated a ribosomal alteration were not performed, to our knowledge, or else failed to show any alteration of ribosomes in the mutants tested (Bollen & Coppin-Raynal, personal communication for *S. pombe*).

In *Podospora*, we have obtained informational suppressors (symbol: *su*) which appeared to resemble the *ram* mutations of *E. coli* (Picard, 1973). Also informational antisuppressors (symbol: *AS*) which could be compared to restrictive mutations in bacteria were obtained (Picard-Bennoun, 1976). Biochemical analysis has supported these analogies quite well at least for the *su*1, AS_1 and AS_3 genes (Coppin-Raynal, 1977, 1978; Picard-Bennoun, in preparation). Finally, genetical and physiological observations strongly suggest that mutants resistant to the aminoglycoside paromomycin are impaired in cytoplasmic translation (Dequard *et al.* 1980).

For two reasons we thought that searching for cold-sensitive mutants would result in the identification of additional genes involved in the control of translational fidelity. The first reason was that in *E. coli*, cold-sensitive strains have proved to be a fruitful source of ribosomal mutants (Guthrie, Nashimoto & Nomura, 1969; Bryant & Sypherd, 1974). The same observation was made in eucaryotes (Hartwell, McLaughlin & Warner, 1970; Waldron & Roberts, 1974*b*, Schlitt & Russell, 1974; Bayliss & Ingraham, 1974; Falke & Wright, 1975; Ursic & Davies, 1979). The second reason was that most antisuppressors in *Podospora* are cold-sensitive (Picard-Bennoun, 1976). This paper reports the genetic analysis of 24 cold-sensitive mutants.

2. MATERIALS AND METHODS

The biology of and culture techniques for *Podospora anserina* were first described by Rizet & Engelmann (1949) and reviewed by Esser (1969).

The ascus of *Podospora* contains four spores. Each one develops around two non-sister nuclei of the postmeiotic mitosis, so that both spores of each half ascus are genetically identical and each spore contains the genetic information of half a tetrad. This property allows the spontaneous formation of spores which are heterocaryotic for one or several pairs of alleles in the appropriate crosses.

Crosses are routinely performed by confrontations between strains originated from uninucleate spores. However, reciprocal crosses (♀ wildtype × ♂ mutant and ♀ mutant × ♂ wild-type) can be carried out by spermatization, i.e. by spreading over a Petri dish culture of one parent (female) a filtered suspension of microconidia harvested from the male parent (Monnot, 1953).

Since conidia do not germinate, mutageneses were performed with protoplasts which were prepared with the method described by Belcour (1975). The protoplast suspension, first filtered in order to retain only the uninucleate ones, was irradiated with a UV lamp (General Electric Germicid Lamp G 15T8, 15 W) with a dose of 10000 ergs/mm^2 giving a survival rate of 10%. Protoplasts were allowed to regenerate at 27 °C. on corn meal medium supplemented with 200 g/l saccharose and 6 g/l sorbose. Colonies were transferred on synthetic medium supplemented with 2 g/l yeast extract. After 2 days at 27 °C, the colonies were replicated twice on synthetic medium and allowed to grow at 27 °C and 11 °C. The strains which displayed abnormal growth at 11 °C compared to 27 °C were retained.

Cold-sensitivity of the strains was ascertained by comparing the diameter of the thallus of each candidate on synthetic medium after 2 days at 27 °C and 8 days at 11 °C. The ratio diameter of the thallus after 8 days at 11 °C/diameter of the thallus after 2 days at 27 °C is 1 for the wild-type strain. All strains displaying a ratio lower than 0·5 were retained for further analysis.

To guard against cold-sensitivity corresponding to conditional auxotrophy, enriched media (corn meal medium or synthetic medium supplemented with 5 g/l yeast extract) were used. Only one out of 25 cold-sensitive strains was demonstrated by M. Crouzet to be auxotrophic for methionine.

Measures of resistance to antibiotics were performed in the same way by comparing the diameters of the thalli of mutant and wild-type strains on synthetic medium with or without the drug.

Cycloheximide was purchased from Sigma. Trichodermin was a kind gift of Dr Godtfredsen and Dr Kristensen of Leo Pharmaceutical Products, Ballerup, Denmark. Paromomycin was a kind gift of Parke Davis Laboratories.

3. RESULTS

(i) *All cold-sensitive mutations are nuclear*

Twenty-four cold-sensitive (prototrophic) strains were isolated in the course of three experiments in which a total of 20000 protoplasts were tested. Reciprocal crosses were performed between each cold-sensitive strain and the wild-type strain and the analysis of 15 asci for each cross showed that each of the 24 strains carries a single nuclear mutation. As described below, several cold-sensitive mutants displayed a pleiotropic phenotype. All the characters cosegregated in the crosses and for all these phenotypic properties, the mutant alleles were recessive with respect to the wild-type ones.

(ii) *Numerous cold-sensitive mutations are pleiotropic*

Ribosomal mutations are often pleiotropic. For instance, in *Podospora*, several ribosomal suppressors are female-sterile and display slow growth and poor germination of the spores (Picard, 1973); all of the strains carrying an AS_3 mutation (antisuppressor) are cold-sensitive and show poor germination of the spores and reduced fertility (Picard-Bennoun, 1976). Therefore, each cold-sensitive

mutant was carefully scored for growth rate, fertility and efficiency of spore germination. The 24 mutants are listed in Table 1 according to these three criteria. Eleven mutants differ from wild-type only in cold-sensitivity, while 13 differ in other characters.

Table 1. *Phenotypic characterization of the mutants*

Spore germination	Growth at 27 °C		Fructification and (or) sporulation
	Normal	Slow	
Normal	cs3, cs4*, cs5, cs6* cs7, cs8, cs9, cs13 cs16, cs21, cs22	cs25*	Normal
Normal	cs12,* cs15, cs18 cs19, cs23*	cs1, cs2, cs10*	Poor
Poor	cs17*	—	Normal
Poor	cs11,* cs24*	cs20	Poor

* Indicates that the mutant is also altered in its response to antibiotics (see the text and Table 2).

Among those which are impaired in fertility or sporulation, the following points can be made: *cs18* and *cs24* are completely female-sterile while *cs1* and *cs12* are defective in sporulation. The defect of *cs12* was carefully analysed with light and electron microscopy by Zickler & Simonet (1980). They found that the mutant shows normal meiotic and postmeiotic divisions. However, no vesicles allowing delimitation of the spores are formed. Then, the eight nuclei divide and after several rounds of mitoses, the asci are filled with a hundred or more nuclei. In contrast, in the wild-type strain, a single postmeiotic mitosis occurs before the ascospore delimitation. A second mitosis is observed only when the spores are completely delimited.

(iii) *The 24 cold-sensitive mutations define 24 different genes*

Complementation tests were performed either by constructing vegetative heterocaryons or by crossing the strains in order to obtain heterocaryotic spores. The results demonstrated that the 24 cold-sensitive mutations map in 24 different genes.

(iv) *Two mutations,* cs11 *and* cs24, *display an antisuppressor effect*

All cold-sensitive mutants were tested for suppressor and antisuppressor activities. To accomplish this each mutant strain was crossed with mutant *NG-193* which is a nonsense mutant impaired in spore pigmentation (Picard, 1971). This mutant allowed screening for ribosomal and tRNA-like suppressors (Picard, 1973). The test is very easy because suppression can be detected directly at the level of the spores in the crosses. A cross between NG-193 and a non-suppressor strain only gives asci of the type: two black spores – two unpigmented spores, while a cross between NG-193 and a suppressor-bearing strain gives two types of asci:

two black – two unpigmented spores *and* two black – two (more or less) pigmented spores. Suppression was not observed with any of the cold-sensitive mutations.

In order to test whether these mutations could act as antisuppressors, double mutant strains of the type *NG-193.cs* × . . . were constructed and crossed with other strains having the NG-193 mutation plus one of the known *su* mutations (*193 sul-1*, *193 su2-1*, *193 su4-1* and *193 su8-1*). *Su1* and *su2* are likely ribosomal suppressors while *su4* and *su8* are tRNA-like nonsense suppressors (Picard, 1973). These crosses allowed us to detect antisuppression at the level of spore pigmentation. *Cs11* and *cs24* acted as antisuppressors upon these four suppressors: like all known antisuppressors in *Podospora* (Picard-Bennoun, 1976), *cs11* and *cs24* decreased the efficiency of all informational suppressors and restored sporulation in strains homocaryotic for the *su4* mutation which are impaired in sporulation. Double-mutant strains of the type *cs-su* were isolated for most of the other *cs* mutations, but the *cs* mutations were never suppressed. All strains of genotype *cs-AS1* remained cold-sensitive but one such combination, $AS_1.cs24$, was lethal.

(v) *Several cold-sensitive mutants display an altered level of resistance to inhibitors of protein synthesis*

Most ribosomal mutants screened as suppressors and antisuppressors in *Podospora* were observed to differ from wild-type at the level of antibiotic resistance especially with paromomycin (Picard-Bennoun & Coppin-Raynal, in preparation)

Table 2. *Mutants displaying altered resistance levels towards cycloheximide* (*CHX*), *paromomycin* (*Pm*) *and* (*or*) *trichodermin* (*T*)

Mutants	Resistance to antibiotics
cs4, *cs10*	CHX^R
cs6, *cs23*, *cs24*	Pm^R
cs25	Pm^S
cs17	T^S
cs12	CHX^R, Pm^S
cs11	CHX^R, Pm^R, T^S

R and S indicate that the mutants are respectively more resistant or more sensitive than wild-type to the antibiotic.

and, to a lesser extent, with cycloheximide and trichodermin (Coppin-Raynal, 1977, 1978). Therefore, cold-sensitive mutants were analysed with respect to their resistance towards these three antibiotics. Cycloheximide was used at 2γ/ml, trichodermin at 4 γ/ml and paromomycin at 750γ/ml. At these concentrations, inhibition of wild-type growth is respectively 25 %, 10 % and 50 %. Comparison of growth rate inhibition allowed us to identify mutants that are more resistant to cycloheximide, more sensitive to trichodermin and more resistant or more sensitive to paromomycin than wild-type. These mutants are listed in Table 2. One can notice that the two antisuppressor strains (*cs11* and *cs24*) show increased resistance to paromomycin as is the case for most previously characterized antisuppressors.

(vi) *Mapping the cold-sensitive mutants*

20 out of the 24 cold-sensitive mutants were mapped. A revised genetic map of *Podospora* with 127 loci scattered on the 7 linkage groups will be published elsewhere (Simonet, Marcou, Picard-Bennoun, Masson, Le Coze & Piquepaille, in preparation), and so the precise localization of the cold-sensitive mutants is not reported here. However, we will briefly mention relevant points.

(*a*) It was observed that *cs11* displayed the same phenotypic properties as AS_3 mutations: antisuppressor action, cold-sensitivity, poor germination, strong pigmentation of the mycelium, reduced fertility, resistance to cycloheximide and paromomycin, hypersensitivity to trichodermin (Picard-Bennoun, 1976; Coppin-Raynal, 1977, 1978). Complementation tests revealed that *cs11* is an allele of the AS_3 gene.

(*b*) *Cs16* is strongly linked to the mating-type locus on linkage group I (no recombination among more than 200 asci analysed). Two genes which are likely to be ribosomal genes are strongly linked to the MT locus: AS_4 (Picard-Bennoun, 1976) and *Pm1* (Dequard *et al.* 1980). The former mutants were selected as antisuppressors: most of them are lethal when homozygous and the others are cold-sensitive. The latter ones were selected for their resistance to paromomycin: three out of the five mutants are cold-sensitive. Complementation was observed between *cs16*, *Pm1* and AS_4 cold-sensitive mutants but no recombinants were obtained in one hundred asci from each of two crosses: *cs16* × AS_4-*43* and *cs16* × *Pm1-3*. Therefore, three genes (*cs16*, AS_4 and *Pm1*) are clustered near the mating-type locus within less than one recombination unit. Two of these genes (AS_4 and *Pm1*) probably affect ribosomal function directly.

(*c*) *Cs23*, *Cs24* and *Cs5* are linked to *Pm2*, another gene for resistance to paromomycin (Dequard *et al.* 1980) on linkage group III. Although the linkage between these mutants is not very tight (four recombination units between *cs24* and *Pm2* which are likely to be the nearest in this area), *cs23*, *cs24*, and *Pm2* are all resistant to paromomycin. *Pm2* (Dequard *et al.* 1980) and *cs24* (Picard-Bennoun & Coppin-Raynal, in preparation) are probably ribosomal genes.

(*d*) Several cold-sensitive mutants are linked to assumed ribosomal suppressors. *Cs10* is linked to the ribosomal gene su_1 (3 centimorgan) on linkage group IV. *Cs9* and *cs17* are linked to su_2 and su_5 on linkage group VII (these four genes lie within a distance of five recombination units). *Cs1* and *cs12* are linked to su_7 on chromosome VII.

(*e*) *Cs7*, *cs15* and *cs19* are strongly linked being less than one centimorgan apart on linkage group VI.

4. DISCUSSION

Our aim in looking for cold-sensitive mutants in *Podospora* was to identify new ribosomal genes, especially those involved in the control of accuracy in translation. Since cold-sensitivity could result from mutations affecting practically any cellular process (see Waldron & Roberts, 1974*a*, *b*, as an example in another

filamentous fungus) we choose to analyse our cold-sensitive mutants in the following way. First we decided, in contrast to Waldron & Roberts, to keep all mutants which displayed an altered phenotype at the permissive temperature. Second, we eliminated auxotrophic mutants. Third, we tried to characterize the mutants with all possible genetical and physiological tools. For each mutant isolate we attempted to answer three pertinent questions (1) do these mutants act as informational suppressors?; (2) are they antisuppressors?; (3) do they display altered response to paromomycin? Positive answers to the first two questions would allow us to designate the mutant as similar to the ribosomal ambiguity and restrictive mutations described in *E. coli* and to our own fidelity mutations in *Podospora*. The third question is relevant because paromomycin is known to stimulate misreading in *Podospora* (Picard-Bennoun & Coppin-Raynal, 1977) as well as in other eucaryotes (Wilhelm, Pettitt & Jessop, 1978*a*, *b*,; Palmer & Wilhelm, 1978; Singh *et al.* 1979; Palmer, Wilhelm & Sherman, 1979). Furthermore, there is a strong correlation between resistance or hypersensitivity to aminoglycosides and the levels of misreading in mutants of *E. coli* (see Piepersberg *et al.* 1980 for review) and of *Podospora* (Picard-Bennoun & Coppin-Raynal, in preparation). Answers to the other questions we asked in this study were not so specific. However, alteration in the response to other inhibitors of protein synthesis such as cycloheximide and trichodermin, pleiotropy and tight linkage to ribosomal loci can be used as indications that the mutants might be ribosomal.

The results we obtained can be summarized as follows. As it was assumed, cold-sensitivity gives a very wide screen since the 24 mutants belong to 24 different genes. Half of these mutants are pleiotropic and nine display altered resistance level to cycloheximide, trichodermin and/or paromomycin. Four of them are more resistant and two more sensitive to paromomycin than is the wild-type strain. None of the 24 mutants behaves like an informational suppressor (but this test was carried out with only one nonsense mutant). However, two of the four paromomycin resistant mutants are antisuppressors. One of them (*cs11*) was demonstrated to be a new allele of AS_3 (now referred to as AS_{3-5}) while the other (*cs24*) defines a new AS gene which we now call AS_6. Some clustering of genes on the genetic map was observed, for example between *cs16*, AS_4, *Pm1* and the mating-type locus; between *cs9*, *cs17* and su_2; between *cs10* and su_1; between *cs24*, *cs23*, *cs5* and *Pm2*; between *cs15*, *cs19* and *cs7*; between *cs1*, *cs12* and su_7.

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