Genet. Res., Camb. (1982), **40**, *pp.* 149–164
With 1 *text-figure*
Printed in Great Britain

Mutations relieving hypersensitivity to paromomycin caused by ribosomal suppressors in *Podospora anserina*

BY EVELYNE COPPIN-RAYNAL AND DENISE LE COZE

Laboratoire de Génétique de l'Université de Paris-Sud, Bât. 400
(Laboratoire associé au CNRS no. 86)
91405 ORSAY Cedex, France

(Received 8 December 1981 and in revised form 5 April 1982)

SUMMARY

In the fungus *Podospora anserina*, mutations were selected which relieved the hypersensitivity to paromomycin caused by four suppressors assumed to be ribosomal ambiguity mutations (su_{1-31}, su_{1-49}, su_{1-60}, su_{2-5}). Our first purpose was to isolate new antisuppressor mutations and in fact a new antisuppressor gene, AS_7 was uncovered. The AS_{7-1} mutant displays a pleiotropic phenotype and particularly a sporulation defect. On the other hand, a new su_1 mutant was obtained which acts as a suppressor and also as an antisuppressor: it can specifically reduce the suppressor effect of certain su_2 mutations. This property of some su_1 and su_2 mutations was already known. Apart from these mutations probably involved in the control of translational fidelity, six mutations conferring cross-resistance to paromomycin and neomycin were isolated. While four of them are localized in the Pm_1 and Pm_2 loci previously identified, the two others define a new gene which controls paromomycin and neomycin resistance, Pm_3. Strains carrying the Pm_{3-1} allele are sensitive to temperature at the level of growth and sporulation. The three last mutations which were obtained confer no mutant phenotype when separated from the su_1 background. They are closely linked to the su_2 locus.

INTRODUCTION

Investigation of informational suppressors (*su*) and antisuppressors (*AS*) obtained in *Podospora anserina* (Picard, 1973; Picard-Bennoun, 1976) has shown that fidelity of protein synthesis can be changed in an eukaryote as in *E. coli* (for reviews see Gorini, 1974; Piepersberg *et al.* 1980). This situation, first suggested by genetic arguments, was confirmed (for at least some mutants) by biochemical evidence; translational fidelity was demonstrated to be decreased by *su* mutations (Coppin-Raynal, 1982 and unpublished data; Picard-Bennoun, personal communication) as it is in bacteria by ribosomal ambiguity (or *ram*) mutations (Rosset & Gorini, 1969). The opposite effect, namely an enhancement of fidelity, is brought about by *AS* mutations (Picard-Bennoun, 1981; Coppin-Raynal, 1982). These

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

mutations were selected to antagonize the effect of informational suppressors and should be similar to the restrictive mutations in *E. coli* (Strigini & Gorini, 1970). Although suppressors and antisuppressors were selected independently of paromomycin, an aminoglycoside able to promote mistranslation in eukaryotes (Singh, Ursic & Davies, 1979; Palmer, Wilhelm & Sherman, 1979), they were found to manifest altered susceptibility to this drug. When compared with the wild-type, most suppressors exhibited hypersensitivity while most antisuppressors exhibited increased resistance (Coppin-Raynal, 1981). This suggested that new ribosomal mutants, particularly restricted mutants, could be selected through their paromomycin phenotype. In fact, six antisuppressor genes have been identified, and since most of them are represented by few alleles (Picard-Bennoun, 1981) additional genes should be detectable by using another selection method. Two procedures were investigated: (1) direct selection for resistance to high concentrations of paromomycin, and (2) selection of paromomycin-resistant revertants of suppressor strains displaying hypersensitivity to this antibiotic. Genetic analysis of mutations obtained with the first method has been reported by Dequard *et al.* (1980). In this paper we describe the isolation and genetic characterization of 14 paromomycin-resistant revertants selected in strains carrying four different ribosomal suppressors.

MATERIALS AND METHODS

(i) *Organism*

Podospora anserina is a filamentous fungus. Its biology, and the genetic techniques used have been described elsewhere (Rizet & Engelmann, 1949; Esser, 1974). The ascus of *Podospora* contains four spores. Each one develops around two non sister nuclei of the postmeiotic mitosis. This property allows dominance and complementation tests *in situ* because of the spontaneous formation of heterocaryotic spores in the appropriate crosses (see Picard, 1971, fig. 1). A few asci (2–5 %) contain five spores among which two are smaller and uninucleated. Crosses were generally performed between homocaryotic strains obtained from the small spores.

(ii) *Strains*

All the strains used in the selection and genetic analysis of revertants are derived from the same wild-type of *Podospora* and are listed in Table 1. One su_2 mutation, su_{2-5}, and three su_1 mutations, su_{1-31}, su_{1-49}, su_{1-60}, were used to screen revertants. su_1 and su_2 are two genetically and physically independent loci (Marcou, Picard-Bennoun & Simonet, 1980). Biochemical evidence shows that su_{1-60} and su_{2-5} are ribosomal mutations that increase the translational ambiguity (Coppin-Raynal, 1977, 1982, and unpublished data; Dequard, 1980).

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

Table 1. *General characteristics of the strains used in this study*

	Strains	Characteristics	References
	193	White spores and mycelium	Picard (1971)
	leu_{1-1}	Auxotrophic for leucine	Kindly provided by Crouzet
Putative ribosomal suppressors (gene and allele unspecific)	su_{1-1}	None	Picard (1973)
	su_{1-31}	Pm^S, ♀ sterile	
	su_{1-49}	Pm^S	
	su_{1-60}	Pm^S	
	su_{2-5}	Pm^S, ♀ sterile	
	leu_{1-1} su_{2-18}	Prototrophic	Arnaise & Mignotte (Personal communication)
	leu_{1-1} su_{2-19}	Prototrophic	
Putative tRNA type suppressors (gene unspecific) (allele specific)	leu_{1-1} su_{4-4}	Prototrophic	
	leu_{1-1} su_{10-1}	Prototrophic	
	Pm_{1-3}	Pm^R, dark mycelium	Dequard *et al.* (1980)
	Pm_{2-2}	Pm^R, dark mycelium	
	mei_{1-1}	Blocked during meiosis	Simonet & Zickler (1978)
	mei_{3-1}	Blocked during meiosis	

Symbols are as follows: Pm^S and Pm^R respectively more sensitive and more resistant to paromomycin than the wild-type control.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

(iii) *Selection of paromomycin-resistant revertants*

The experimental procedure was as follows: small pieces of mycelium from each *su* strain were inoculated on 3 cm cellophane disks plated on solid minimal medium M 2. After growth for three days at 27 °C, the thalli were treated by u.v. irradiation at 250 or 500 erg/mm². They were then transferred onto M 2 containing 1 mM paromomycin sulphate (M 2 *Pm1*) on which *su* mutants could not grow (or only poorly). Revertants appeared as sectors able to grow on this medium. Sectors were picked which differed from each other in growth rate on the selective medium. In order to retain independant events only one sector was selected from each treated dish. The revertants are called rev (rev_1 to rev_{20}). rev_x means that the revertant was selected in a strain carrying the su_x mutation.

(iv) *Determination of the genotype of the revertants*

The *193* mutant, defective in spore pigmentation (see Table 1), was used to determine whether the revertants carried an antisuppressor mutation. The *193* mutation is suppressible by informational suppressors, and *193 su* strains provide more or less pigmented spores (light green to dark green depending on the efficiency of the suppressors). A cross *193* $su_x \times su_x$ only gives asci of the type: two black spores-two green spores, since *193* segregates at the first meiotic division and is always associated with su_x. If an antisuppressor (*AS*) is introduced in such a cross, unpigmented *193 su AS* spores are recovered in the progeny. Thus, antisuppression as well as suppression can be detected directly at the spore level in appropriate crosses. In *Podospora* a large number of asci may be observed after they have been ejected from the ripe perithecia.

To determine the genotype of the revertants, two crosses were performed: (1) $rev_x \times$ *193* su_x, which allowed direct detection of antisuppression; and (2) $rev_x \times$ *193*, which particularly allowed separation of the second mutation called *R* (if present) from the *su* background. Table 2 indicates the genotypes of the segregants issued from these crosses, and data expected according to the constitution of the revertants are given in Table 3. Then the *R* mutants were subjected to additional tests, and in particular those acting as antisuppressors were tested with another suppressible mutation, leu_{1-1}, in association with other *su* mutations. The prototrophic strain $leu_{1-1}su$ becomes auxotrophic when an *AS* mutation is introduced. Morever, the auxotrophy is relieved in the presence of paromomycin as previously reported (Coppin-Raynal, 1981*a*). Thus the strains carrying an antisuppressor *R* mutation were crossed with several double mutants: leu_{1-1} su_{4-4}, leu_{1-1} su_{10}, leu_{1-1} su_{2-18}, leu_{1-1} su_{2-19} previously obtained by selecting prototrophic revertants of leu_{1-1} (Arnaise and Mignotte, personal communication). The progeny was analysed on M 2 and M 2 supplemented with leucine (100 μg/ml) or paromomycin (1 mM).

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

Table 2. *Determination of genotypes of segregants from crosses* $rev_x \times 193$ su *and* $rev_x \times 193$

Genotype	Phenotype	
	Spore Pigmentation	Growth on M2 P1
Wild type	●	+
193 su$^+$ *R*$^+$	○	+
193$^+$ *su R*$^+$	●	−
193$^+$ *su R*	●	+
193 su R$^+$	⊕	−
193 su R (*R* ≠ *AS*)	⊕	+
193 su R (*R* = *AS*)	⊖ or ○[a]	+

Symbols are as follows: ●, black spores; ⊖, light green spores; ⊕, dark green spores; ○, unpigmented spores; +, growth; −, no growth or very poor growth.
[a] Depends on the antisuppressor efficiency.

(v) *Determination of resistance level to antibiotics, of cold-sensitivity and thermosensitivity*

This was achieved essentially as previously described (Coppin-Raynal, 1977). An estimate of the resistance level to antibiotics was obtained by comparing the diameters of thalli on M 2 with and without antibiotic after 4 days growth. Comparison of growth rates at 11, 26 (routine temperature) and 36 °C allows us to determine whether a strain is cold-sensitive or thermosensitive. In these tests mutant strains were always compared with wild-type strain.

(vi) *Chemical products*

Paromomycin sulphate was a gift from Substantia. Cycloheximide, emetin and neomycin sulphate were purchased from Sigma.

RESULTS

(i) *Selection and characterization of revertants*

It has been shown that, compared to the wild-type strain most *su* mutations cause decreased resistance to paromomycin (Coppin-Raynal, 1981). Four strains carrying the suppressors su_{1-31}, su_{1-49}, su_{1-60}, su_{2-5} and exhibiting no growth or poor growth on media containing 1 mM paromomycin were chosen to select paromomycin-resistant revertants. The experimental procedure and the origin and properties of the mutants used are described in Materials and Methods and Table 1. 14 u.v.-induced revertants were subjected to further analysis. They might correspond to a back mutation ($su \rightarrow su^+$) or to the appearance of a second mutation, designated as *R*, linked or unlinked to the *su* mutation and acting or not as an antisuppressor. In order to distinguish between these possibilities the

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

revertant strains were crossed with two testers as explained in Materials and Methods: *193 su* (the *su* mutation against which the revertant had been selected) and *193*. Furthermore the cross *rev* × *193* allowed separation of the *R* mutation from the suppressor background. A first classification was achieved by considering spore pigmentation in the progeny of the two crosses as summarized in Table 3. To confirm the conclusions drawn from spore pigmentation, to obtain further information, and to construct strains carrying the *R* mutation alone, sets of tetrads (15–20) were analysed on minimal media with and without paromomycin. The segregation of the *R* mutation can be followed through its interaction with the *su* mutation as shown in Table 2.

With respect to the first cross, two types of tetrad were generally observed: 2 *Pm*-resistant–2 *Pm*-sensitive, and 4 *Pm*-resistant. They corresponded respectively to prereduction and postreduction of the *R* mutation which segregated 2:2. Heterocaryotic strains ($su/su\ R^+/R$) from asci in which *R* segregated at the second division displayed resistance to paromomycin. This suggested dominance of the

Table 3. *Crosses performed to determine the genotype of the revertant strains (for more details see Materials and Methods)*

	A	B	C	A	B	C			Genotypes of the revertant strains
Types of Asci	● ● ⊕ ⊕	● ● ⊖ ⊖	● ● ○ ○	● ● ⊕ ⊕	● ● ⊖ ⊖	● ● ○ ○			
Controls	*WT* × *193su*$_x$			*WT* × *193*					
	+	+	+	−	−	+	▷	I[a]	
	su_x × *193su*$_x$			su_x × *193*					
	+	−	−	+	+	+	▷	II[a]	
	rev_x × *193su*$_x$			rev_x × *193*					
	+	−	−	+	+	+	▷	1 = II	*suR* . *R* ≠ *AS*
	+	+	+	−	−	+	▷	2 = I	su^+ (back mutation or second site mutation)
	+	+	−	−	+	+	▷	3	*suR* . *R* = second-site mutation[d]
	+	+	+[c] or −	+	+	+	▷	4[b]	*suR* . *R* = *AS* closely linked to *su*; *suR* . *R* = *AS* unlinked to *su*

Symbols are as follows: symbols for spore pigmentation are as in Table 2; + and −, presence and absence of the type of asci in the progeny.

[a] The *193* mutation is linked to the centromere of linkage group II (< 1 % postreduction) and unlinked to the suppressors used. The types of asci are in proportion which depends on the postreduction frequency of the suppressor (su_1: 40 %; su_2: 70 %) and on the dominance relationships between the mutant and wild-type alleles.

[b] The types of asci are in proportion which depends on the postreduction frequency of the *AS* mutation and the dominance relationships between the mutant and wild-type alleles. The frequency of asci A is lower than in control II.

[c] Presence or absence given the efficiency of the antisuppressor mutation.

[d] A strong suppressor providing dark green spores when associated with *193* can be transformed into a weak suppressor providing light green spores with *193* as previously described (Picard-Bennoun, 1976).

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

mutant allele with respect to suppression of paromomycin hypersensitivity conferred by the *su* mutation. When the R mutation did not alter the suppressor effect on *193* (case 1 in Table 3), it was not possible to deduce from the first cross whether it was linked or unlinked to the suppressor. Analysis of tetrads from the second cross made it possible to discriminate between the two situations. This could be done by considering five-spore asci of the type 2 black–3 dark-green spores (or vice versa). The small green spores had the constitution *193 su* and were tested for the presence of the R mutation. Recombinant spores *193 su* R^+ were always recovered, indicating that the *su* and R mutations were not closely linked. When R acted as an antisuppressor unlinked to *su* (case 4 in Table 3) the recombinant genotype could be inferred for dark green spores. Thus black spores with the genotype $193^+ su^+ R$ were available and gave rise to strains carrying the R mutation separately from the suppressor against which it had been isolated. The phenotype displayed by these strains in the presence of paromomycin indicated whether the R mutation alone could confer an altered susceptibility to paromomycin.

Fourteen revertants were subjected to genetic analysis. The revertants selected against su_{1-31} and su_{2-5} could abolish the female sterility. This property was necessary to perform the cross $\text{rev}_x \times 193\ su_x$ which facilitated the antisuppression detection. However, three more revertants which did not restore the fertility of su_{2-5} were analysed. Preliminary data indicated that they did not display any anti-suppressor effect and that they probably corresponded to second-site mutations; they were not studied further. Given the results of the two crosses $\text{rev}_x \times 193\ su_x$ and $\text{rev}_x \times 193$, revertants may be divided into several classes (Table 4).

(*a*) Presumed 'true' revertant (situation 2 in Table 3): rev_{12} and rev_{13} whose phenotype does not differ from wild-type.

(*b*) Revertants containing a second mutation named R: R_7 and R_{11} are unlinked antisuppressors (situation 4 in Table 3).

(*c*) R_{20} corresponds to situation 3 in Table 3. Since the $193\ su_{1-31} R_{20}$ spores are less pigmented than the $193\ su_{1-31} R_{20}^+$ spores, R_{20} may be a mutation in an antisuppressor gene closely linked to su_1 or a second-site mutation within the su_1 gene, transforming su_{1-31} into a weak suppressor as already reported (Picard-Bennoun, 1976).

(*d*) R_1, R_2, R_6, R_8, R_9 and R_{10} are mutations that confer increased resistance to paromomycin as compared with the wild type (situation 1 in Table 3).

(*e*) R_{16}, R_{18}, R_{19} do not exhibit any particular phenotype except suppression of female sterility and hypersensitivity to paromomycin of su_{1-31} (situation 1 in Table 3).

(ii) *Determination of antisuppression spectra*

The antisuppressor effect of the R_7 and R_{11} mutations was confirmed by using another suppressible mutation, leu_{1-1}, which confers a leucine requirement, in combination with different suppressor alleles. This was done as described in Materials and Methods and the data are summarized in Table 5. The R_7 mutation was found to manifest a broad antisuppression spectrum since it acted on all tested

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

Table 4. *General characteristics of the* R *mutations issued from the revertants*

Revertants			Characteristics of the *R* mutations when separated from the *SU* background					
	Doses (erg/mm²)	Genotypes	Antisuppression	Characteristics	Resistance* to antibiotics	Postreduction frequency	Linkage group	Nomemclature
rev1	250	su_{1-49} *R1*	.	Pigmented mycelium	Pm^R, N^R, Chx^R, Em^R	0·85	III	Pm_{2-6}
rev2	500	su_{1-49} *R2*	.	Pigmented mycelium	Pm^R, N^R, Chx^R, Em^R	0·85	III	Pm_{2-7}
rev6	500	su_{1-60} *R6*	.	None	Pm^R, N^R	0·98	I	Locus *Pml*†
rev7	500	su_{1-60} *R7*	+ unspecific	No sporulation	Pm^R	0	V	AS_{7-1}
rev8	500	su_{1-60} *R8*	.	Slow germination no sporulation thermosensitivity	Pm^R, N^R	0·65	VII	Pm_{3-1}
rev9	500	su_{1-60} *R9*	.	None	Pm^R, N^R	0·65	VII	Pm_{3-2}
rev10	250	su_{1-60} *R10*	.	Pigmented mycelium	Pm^R, N^R, Chx^R, Em^R	0·85	III	Pm_{2-8}
rev11	500	su_{2-5} *R11*	+ specific	Slow germination slight suppressor effect	Pm^S	0·40	IV	su_1
rev12	500	$su_{2-5}{}^+$	Presumed back-mutation					
rev13	250	$su_{2-5}{}^+$	Presumed back-mutation					
rev16	250	su_{1-31} *R16*	.	Wild-type phenotype		0·70	VII	Locus su_2 AS_2†
rev18	250	su_{1-31} *R18*	.	Wild-type phenotype				
rev19	250	su_{1-31} *R19*	.	Wild-type phenotype				
rev20	250	su_{1-31} *R20*	*R20* and su_{1-31} could not be dissociated					Locus su_1†

* *Pm*, Paromomycine; *N*, neomycin; *Chx*, cycloheximide; *Em*, Emetine; Pm^R and Pm^S, more resistant and more sensitive to the drug than the wild-type strain.

† Complementation test could not be performed to ascertain that the *R* mutation is an allele of the indicated gene.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

ribosomal ambiguity mutations and abolished their suppressor effect on leu_{1-1}. It can also inactivate a tRNA-type suppressor, su_{10-1}, but it is inefficient on the su_{4-4}. On the other hand, R_{11} was found to act specifically on some su_2 mutations e.g. su_{2-5} against which it was isolated and su_{2-18}. Moreover, the *193* R_{11} spores appeared to be slightly pigmented suggesting that R_{11} might manifest a very weak suppressor effect.

Table 5. *Determination of antisuppression spectrum of the* R_7 *and* R_{11} *mutations*

Tested-mutations		Growth on minimal medium in combination with the ested mutations	
		R_7	R_{11}
Ribosomal suppressors	leu_{1-1} su_{1-1}	−*	Not testable
	leu_{1-1} su_{1-60}	−	Not testable
	leu_{1-1} su_{2-18}	−	−
	leu_{1-1} su_{2-19}	−	+
tRNA like suppressors	leu_{1-1} su_{4-4}	+	Not tested
	leu_{1-1} su_{10-1}	−	+

* Indicates that the leu_{1-1} *su R* strain cannot grow on medium lacking leucine. Thus *R* is able to prevent the suppressor effect and the prototrophy can be restored by adding paromomycin (see Materials and Methods for more details).

\+ Indicates that *R* does not act as an antisuppressor in the tested genetic background.

As mentioned above, R_{20} could be a mutation of an antisuppressor gene closely linked to su_1 or a second-site mutation in su_1 leading to a weak suppressor phenotype. It was impossible to separate the site of the first mutation (*su*) and the site of the second one (*R*) by recombination. About 200 asci from the cross su_{1-31} $R_{20} \times su_1^+ R_{20}^+$ were analysed without recovering the suppressor phenotype (hypersensitivity to paromomycin). The leu_{1-1} su_{1-31} R_{20} strain was found to be auxotrophic for leucine. The auxotrophy could be alleviated by paromomycin. Weak suppressors unable to suppress the leu_{1-1} mutation had been previously identified but they had been found to be inefficient even in the presence of paromomycin. These observations suggest that R_{20} is an antisuppressor, but this has yet to be proved.

(iii) *Pleiotropic effects of the R mutations*

The *R* mutations, when separated from the *su* background in which they had been isolated, displayed different phenotypic properties. These phenotypes, which consistently segregated together with the *R* mutations, are listed in Table 4.

(*a*) *Spore germination, mycelium pigmentation and fertility.* The R_1 and R_2 mutations were found to confer a dark pigmentation to the mycelium. On the other hand, spores carrying R_{11} or R_8 exhibited a slowed germination so that the thalli were smaller than those from wild-type spores.

It was noticed that crosses heterozygous for R_7 or R_8 provided a high frequency

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

of asci exhibiting abnormal distribution of nuclei in the spores. Moreover, crosses homozygous for these mutations produced fructifications which did not contain any spores. su_{1-60} when homozygous suppressed the sporulation defect displayed by R_8 but was inefficient on R_7. In addition, crosses $su_{1-60}\ R_9 \times su_{1-60}\ R_9$ provided empty fructifications while crosses $su_1^+\ R_9 \times su_1^+\ R_9$ and $su_{1-60}\ R_9^+ \times su_{1-60}\ R_9^+$ displayed normal fertility.

(*b*) *Thermosensitivity and cold sensitivity*. These properties were determined as described in Materials and Methods. No R mutation displays cold sensitivity whereas one thermosensitive mutant was identified: in fact growth of the R_8 mutant stopped in a reversible way at 36 °C. Moreover sporulation in crosses homozygous for R_8 was restored at temperatures below the routine temperature (11 and 18 °C instead of 26 °C). On the other hand $R_7 \times R_7$ crosses were sterile even at low temperature.

(*c*) *Resistance to inhibitors of protein synthesis*. Most strains carrying an R mutation displayed increased resistance to paromomycin even in the absence of the suppressor (see Table 4). However, one mutation, R_{11}, was found to confer hypersensitivity to paromomycin. This means that two mutations, namely su_{2-5} and R_{11}, which caused hypersensitivity to paromomycin when separated failed to cause hypersensitivity when associated.

The mutants were further analysed with respect to resistance to three other antibiotics: neomycin (another aminoglycoside), cycloheximide (specific for the large ribosomal subunit) and emetine (specific for the small ribosomal subunit) (Pestka, 1977). Resistance levels of different strains carrying an R mutation are plotted in Fig. 1.

Given the results of this analysis, the R mutants may be divided into four groups:

(1) mutants which displayed cross resistance to all tested antibiotics: R_1, R_2, R_{10}.

(2) mutants which displayed cross resistance to only aminoglycosides: R_6, R_8, R_9.

(3) mutant R_7 which only displayed resistance to paromomycin.

(4) mutants which behaved like the wild-type strains.

(iv) *Dominance tests*

It has been previously mentioned that all R mutations are dominant with respect to suppression of paromomycin hypersensitivity of *su*. In fact the heterocaryotic strain $su/su\ R^+/R$ could grow on medium containing 1 mM paromomycin. Since its growth was generally slightly slower than $su/su\ R/R$ growth, the mutant allele was considered to be partially dominant. In addition, vegetative heterokaryons $R7/R7^+$ and $R8/R8^+$ were forced using two auxotrophic mutations leu_{1-1} and lys_{2-1}. They were found to exhibit good growth on media supplemented with paromomycin (1 mM) while the leu_{1-1}/lys$_{2-1}$ strain exhibited poor growth. This indicated dominance of R_7 and R_8 mutant alleles with respect to paromomycin resistance.

On the other hand, all the characteristics cosegregating with the resistance (e.g. mycelium colour for R_1, R_2, R_{10}, slow germination for R_{11}, and R_8, sporulation defect for R_7 and R_8) were recessive.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

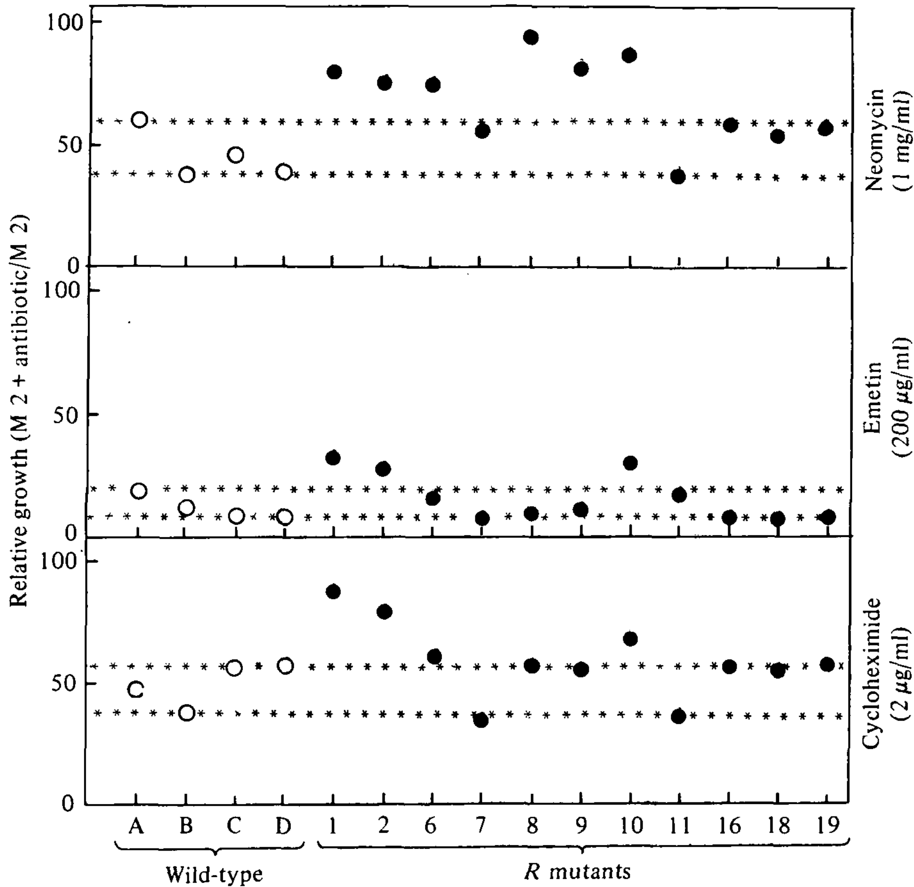

Fig. 1. Effect of cycloheximide, emetin and neomycin on growth of 4 wild-type strains and different R mutants. Resistance levels were expressed as the relative growth on M_2 plus antibiotic and M_2 as described in Materials and Methods.

Considering the antisuppression phenotype it could be determined whether a mutant allele was recessive or dominant by comparing the pigmentation of heterocaryotic spores with the following constitution

$$(a)\ \frac{193\ su_x R^+}{193\ su_x R^+}.\quad (b)\ \frac{193\ su_x R^+}{193\ su_x R}.\quad (c)\ \frac{193\ su_x R}{193\ su_x R}.$$

They were obtained in crosses $193\ su_x\ R \times 193\ su_x\ R^+$. For R_{11} we observed a gradient of pigmentation, spores (a) being the most pigmented. This indicated semi-dominance of the R_{11} mutant allele. On the other hand, spores (a) and (b) carrying R_{20} displayed the same pigmentation level, suggesting that R_{20} is recessive. We have no information concerning R_7. Since it always displays first division segregation spores (b) have never been observed.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

(v) *Mapping*

In a first step the R mutations were grouped in loci with the help of crosses such as $su_x R_1 \times su_x R_2$. 30 tetrads were analysed in each cross. The occurrence of recombinant spores ($su_x R_1^+ R_2^+$) displaying hypersensitivity to paromomycin showed that the mutations were not closely linked and therefore belonged to two different genes. If no recombinant was recovered the two mutations were considered to lie in the same locus. On this criterion several loci were identified: a locus with R_1, R_2, R_{10} a locus with R_8, R_9; a locus with R_{16}, R_{18}, R_{19}. The other R mutations define independent loci.

Then the mutations were localized on the different linkage groups. A revised genetic map of *Podospora* with more than one hundred loci scattered on the seven linkage groups has been published elsewhere (Marcou *et al.* 1980). Given their properties and their post-reduction frequency some R's could correspond to mutations in loci previously identified.

(*a*) R_6 was found to be closely linked to the mating-type locus. It displays cross-resistance to neomycin and paromomycin and may correspond to a mutation of *Pm1* characterized by Dequard *et al.* (1980).

(*b*) R_1, R_2 and R_{10} belong to the same locus and they exhibit cross-resistance to neomycin, paromomycin, cycloheximide and emetin. Moreover their mycelium is more pigmented than that of wild type. These properties and the high postreduction frequency suggested that they could be alleles of the *Pm2* gene (Dequard *et al.* 1980). Complementation tests were performed on the basis of mycelium pigmentation which was a recessive character. All the heterocaryotic spores, of the constitutions

$$\frac{R_1\,Pm_2^+}{R_1^+\,Pm_{2-2}}\quad \frac{R_2\,Pm_2^+}{R_2^+\,Pm_{2-2}}\quad \frac{R_{10}\,Pm_2^+}{R_{10}^+\,Pm_{2-2}},$$

exhibited mutant mycelium. Thus R_1, R_2 and R_{10} were allelic and belonged to the *Pm2* gene. They were designated as Pm_{2-6}, Pm_{2-7}, Pm_{2-8}.

(*c*) The R_8 and R_9 mutations were found to be linked to the su_4 gene on linkage group VII. The cross $su_{1-60}\,R_8 \times su_{1-60}\,R_9$ like the cross $su_{1-60}\,R_9 \times su_{1-60}\,R_9$, provided empty fructifications, while $su_{1-60}R_8 \times su_{1-60}$ and $su_{1-60}R_9 \times su_{1-60}$ had normal fertility. This suggested that R_8 and R_9 were alleles of the same gene, which we name *Pm3*. At the same locus a gene mei_3 involved in meiosis has been identified (Simonet & Zickler, 1978). The fact that R_8 and mei_3 do not complement each other indicates that mei_3 define a single gene.

(*d*) Since the R_7 mutation displayed a postreduction frequency different from that of all the antisuppressors previously characterized, it corresponded to a new antisuppressor gene, named AS_7. It was found to be linked to the *ap* gene on linkage group V. A meiosis gene mei_1 lies at this locus and allelism tests were carried out between R_7 and mei_{1-1}; both were unable to sporulate. The cross $R_7 \times mei_{1-1}$ is as fertile as controls $R_7 \times$ WT and $mei_{1-1} \times$ WT. Thus R_7 and mei_{1-1} complement each other, and define two distinct genes.

(*e*) R_{11} was found to act as an antisuppressor. Moreover it displayed a very weak

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

suppressor effect. It did not suppress the leu_{1-1} mutation but conferred a slight pigmentation to spores carrying the *193* mutation. Considering these properties such as its 40 % postreduction frequency and its hypersensitivity to paromomycin, it may be an allele of the su_1 gene. In fact su_1 and su_2 alleles can exhibit additivity or antagonistic action (Picard-Bennoun, 1976), and R_{11} was demonstrated to relieve the effect of only certain su_2 alleles. To check this hypothesis complementation tests were performed between R_{11}, su_{1-25} and su_{1-26} which manifested a germination phenotype. No complementation was observed. Thus R_{11} is an allele of the su_1 gene. Because of its phenotypic properties it differs from the su_1 mutations previously isolated.

(*f*) R_{16}, R_{18} and R_{19} were found to be linked to leu_{1-1}, indicating that they lie at the same locus as the su_2 and AS_2 mutations (Picard-Bennoun, 1976).

DISCUSSION

Hypersensitivity to paromomycin manifested by four ribosomal suppressors (su_{1-31}, su_{1-49}, su_{1-60} and su_{2-5}) was used to isolate paromomycin-resistant revertants. Our aim in selecting such revertants was to identify new ribosomal genes and in particular new antisuppressor genes. The genetic characterization allowed us to answer the most important questions: Do the revertants correspond to the appearance of a second-site mutation? Do these mutations act as informational antisuppressors? Do they display pleiotropic effects (cold sensitivity, thermosensitivity, sterility, altered response to inhibitors of protein synthesis)? Do they map at previously identified loci or at new loci? All this information is gathered in Table 4. It must be stressed that all characteristics displayed by an R mutation did cosegregate in all crosses.

Our first purpose had been achieved since a new antisuppressor locus, AS_7, has been uncovered. The AS_{7-1} mutation manifests a broad action spectrum: it acts on all ribosomal suppressors which have been tested and on some tRNA suppressors. Interestingly, strains homozygous for the AS_{7-1} mutation do not sporulate, while strains heterozygous for this mutation are fertile but provide a great proportion of asci displaying abnormal distribution of nuclei in the spores. Besides the AS_{7-1} mutation, a new allele of the gene su_1 has been obtained. It is efficient on only some su_2 mutations. Specific antisuppression by interaction between su_1 and su_2 suppressors was previously reported (Picard-Bennoun, 1976). In addition, R_{20} may correspond to a mutation at a new antisuppressor locus closely linked to the su_1 gene; but we cannot at present discriminate between this hypothesis and that of a second-site mutation within the su_1 gene.

Apart from these mutations probably involved in the control of translational fidelity, mutations conferring cross-resistance to paromomycin and neomycin were characterized: some of them were localized at the Pm_1 and Pm_2 loci previously identified (Dequard *et al.* 1980) but two others have been mapped in a new paromomycin-resistance gene named Pm_3. In fact this gene was already known and designated as mei_3 since it was related to meiosis. Interesting interactions between

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

R_9, R_9 and su_{1-60} at the level of sporulation have been pointed out. Although no evident explanation can be proposed for this phenomenon the data suggest that the ribosome may be involved.

Table 4 clearly shows that the types of mutations obtained in the selection procedure described in the paper depend on the suppressor against which they were isolated. In particular, only specific mutations in loci su_1 AS_8 or su_2 AS_2 are susceptible to suppress female sterility of the most efficient suppressors as previously reported (Picard-Bennoun, 1976) and even the use of paromomycin as a selection pressure does not lead to isolation of different mutations.

We have further remarks concerning the use of paromomycin in the isolation of antisuppressors. Direct selection for resistance to paromomycin only provided mutations at the Pm_1 and Pm_2 loci which, although supposed to act at the ribosomal level (Dequard, 1980), are neither antisuppressors nor suppressors. Specific genetic backgrounds containing a mutation displaying hypersensitivity to paromomycin seem to be required to obtain antisuppressors. The finding that two antisuppressor mutations (AS_{6-2} and AS_{7-2}) have been uncovered by selecting paromomycin-resistant revertants of another mutant hypersensitive to paromomycin and to low temperatures, cs_{12} (Picard-Bennoun & Le Coze, 1981), confirms this assumption (Picard-Bennoun, personal communication). Given that the AS_{6-2}, AS_{7-1} and AS_{7-2} antisuppressors exhibit a higher resistance level to paromomycin than the Pm_1 and Pm_2 mutants it is surprising that they were not isolated directly as paromomycin-resistant mutations. No clear explanation of this can yet be advanced. The fact that antisuppressors display specific resistance to paromomycin whereas the Pm_1, Pm_2 and Pm_3 mutations confer cross-resistance to paromomycin and neomycin and sometimes to other inhibitors of protein synthesis, nevertheless suggests that the mechanism leading to resistance is different in the *Pm* and *AS* mutants.

Until now we know that at least eight genes can be mutated in *Podospora anserina* which lead to a restrictive phenotype. All the antisuppressors were demonstrated to act on ribosomal and putative tRNA-type suppressors as well but their action spectra are different. Apart from these gene and allele-unspecific antisuppressors some su_1 alleles such as R_{11} behave as gene and allele-specific antisuppressors acting on certain su_2 mutations. On the other hand, other su_1 su_2 combinations display a cooperative effect (Picard, 1973). Ninety percent of the omnipotent suppressors (out of more than one hundred isolated so for) map at the su_1 and su_2 loci. These results all together suggest that the products of the su_1 and su_2 genes, probably ribosomal proteins, may play an important part in the ribosomal control of translational fidelity.

Putative ribosomal antisuppressors have been identified in yeasts. Unlike *Podospora's* antisuppressors they do not act on tRNA-like suppressors but only on ribosomal-like (or omnipotent) suppressors (Thuriaux *et al.* 1975; Liebman & Cavenagh, 1980). Up to now more biochemical evidence confirms that antisuppressors act at the ribosomal level. In the fission yeast alteration of a ribosomal protein was detected in cycloheximide-resistant mutants (Coddington & Fluri.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

1977) displaying an antisuppressor effect (Thuriaux *et al.* 1975). In *Podospora* we have tested the ribosomes of strains carrying different *AS* mutations in the poly (U)-dependent assay and in particular the ribosomes of the AS_{7-1} mutant described in the present paper. The degree of paromomycin-induced misreading by AS_{6-1} and AS_{7-1} ribosomes was found to be lower than that of the wild-type (Picard-Bennoun, 1981; Coppin-Raynal, 1982). It appears that an enhancement of the fidelity of protein synthesis leads to physiological perturbations since AS_{7-1}/AS_{7-1} strains are unable to sporulate while AS_{6-1} strains differentiate unfunctional female organs (Picard-Bennoun & Le Coze, 1980).

Models have been proposed to explain the control of translational fidelity particularly in prokaryotes (for a review see Kurland, 1979). The genetic approach has played an important part in the analysis of this phenomenon. *Podospora anserina*, in which isolation of mutations changing the translational accuracy is quite easy, appears to be a suitable organism for the study of this problem in eukaryotes.

We are grateful to J. F. Julien and P. Joyet for supplying the revertant strains. We thank Substantia Laboratories for their gift of paromomycin, M. Crouzet for his gift of the auxotrophic mutant and S. Arnaise and B. Mignotte for communicating unpublished data. We are also indebted to M. Bennoun for her encouragements and her critical reading of the manuscript. This work was supported by a DGRST grant (MRM/240), a NATO grant (no. 1637) and a grant from the Fondation pour la Recherche Médicale.

REFERENCES

Coddington, A. & Fluri, R. (1977). Characterization of ribosomal proteins from *Schizosaccharomyces pombe* by 2 dimensional polyacrylamide gel electrophoresis. Demonstration that a cycloheximide resistant strain cyh 1 has an altered 60S ribosomal protein. *Molecular and General Genetics* **158**, 93–100.

Coppin-Raynal, E. (1977). Ribosomal suppressors and antisuppressors in *Podospora anserina*: resistance to cycloheximide. *Journal of Bacteriology* **131**, 876–883.

Coppin-Raynal, E. (1981). Ribosomal suppressors and antisuppressors in *Podospora anserina*: altered susceptibility to paromomycin and relationships between genetic and phenotypic suppression. *Biochemical Genetics* **19**, 729–740.

Coppin-Raynal, E. (1982). Ribosomal control of translational fidelity in *Podospora anserina*: a suppressor and an antisuppressor affecting the paromomycin induced misreading *in vitro*. *Current Genetics* (in the Press).

Dequard, M. (1980). Thèse de 3ème Cycle, Université de Paris-Sud, Centre d'Orsay, France. Recherche de mutants ribosomiques chez le champignon *Podospora anserina*: analyse de mutants résistants et hypersensibles à la paromomycine.

Dequard, M., Couderc, J-L., Legrain, P., Belcour, L. & Picard-Bennoun, M. (1980). Search for ribosomal mutants in *Podospora anserina*: genetic analysis of mutants resistant to paromomycin. *Biochemical Genetics* **18**, 263–280.

Esser, K. (1974). *Podospora anserina*. In *Handbook of Genetics*, vol. 1 (ed. R. C. King), pp. 531–551. New York and London: Plenum.

Gorini, L. (1974). Streptomycin and misreading of the genetic code. In *Ribosomes* (ed. M. Nomura, A. Tissières and P. Lengyel), pp. 791–803. New York: Cold-Spring Harbor Laboratory.

Kurland, C. G. (1979). On the accuracy of elongation. In *Ribosomes, Structure, Function and Genetics* (ed. G. Chambliss, G. R. Craven, J. Davies, K. Davis, L. Kahan and M. Nomura), pp. 597–614. Baltimore: University Park Press.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press

LIEBMAN, S. W. & CAVENAGH, M. (1980). An antisuppressor that acts on omnipotent suppressors in yeast. *Genetics* **95**, 49–61.

MARCOU, D., PICARD-BENNOUN, M. & SIMONET, J. M. (1980). *Podospora anserina*. In *Genetic Maps* (ed. S. J. O'Brien) **1**, 151–159.

PALMER, E., WILHELM, J. & SHERMAN, F. (1979). Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature*, London **277**, 148–150.

PESTKA, S. A. (1977). Inhibitors of protein synthesis. In *Molecular Mechanisms of Protein Biosynthesis* (ed. H. Weissbach and S. Pestka), pp. 468–536. New York: Academic Press.

PICARD, M. (1971). Genetic evidence for a polycistronic unit of transcription in the complex locus '14' in *Podospora anserina*. I. Genetic and complementation maps. *Molecular and General Genetics* **111**, 35–50.

PICARD, M. (1973). Genetic evidence for a polycistronic unit of transcription in the complex locus '14' in *Podospora anserina*. II Genetic analysis of informational suppressors. *Genetical Research* **21**, 1–15.

PICARD-BENNOUN, M. (1976). Genetic evidence for ribosomal antisuppressors in *Podospora anserina*. *Molecular and General Genetics* **147**, 299–306.

PICARD-BENNOUN, M. (1981). Mutations affecting translational fidelity in the eukaryote *Podospora anserina*: characterization of two ribosomal restrictive mutations. *Molecular and General Genetics* **183**, 175–180.

PICARD-BENNOUN, M. & COPPIN-RAYNAL, E. (1977). Une fonction ribosomique essentielle: le contrôle de la fidélité de la traduction. *Physiologie Végétale*. **15**, 481–489.

PICARD-BENNOUN, M. & LE COZE, D. (1980). Search for ribosomal mutants in *Podospora anserina*: genetic analysis of cold-sensitive mutants. *Genetical Research* **36**, 289–297.

PIEPERSBERG, W., GEYL, D., HUMMEL, H. & BOCK, A. (1980). Physiology of ribosomal mutants. Physiology and biochemistry of bacterial ribosomal mutants. In *Genetics and Evolution of Transcriptional and translational apparatus*, pp. 359–377. Tokio: Kodansha Scientific.

RIZET, G. & ENGELMANN, C. (1949). Contribution à l'étude génétique d'un ascomycète tétra-sporé: *Podospora anserina*. *Revue de Cytologie et de Biologie Végétale* **11**, 201–304.

ROSSET, R. & GORINI, L. (1969). A ribosomal ambiguity mutant. *Journal of Molecular Biology* **39**, 95–112.

SIMONET, J. M. & ZICKLER, D. (1978). Genes involved in caryogamy and meiosis in *Podospora anserina*. *Molecular and General Genetics* **162**, 237–242.

SINGH, A., URSIC, D. & DAVIES, J. (1979). Phenotypic suppression and misreading in *Saccharomyces cerevisiae*. *Nature London* **277**, 146–148.

STRIGINI, P. & GORINI, L. (1970). Ribosomal mutations affecting efficiency of amber suppression. *Journal of Molecular Biology* **47**, 517–530.

THURIAUX, P., MINET, M., HOFER, F. & LEUPOLD, V. (1975). Genetic analysis of antisuppressor mutants in the fission yeast *Schizosaccharomyces pombe*. *Molecular and General Genetics* **142**, 251–261.

https://doi.org/10.1017/S0016672300019029 Published online by Cambridge University Press