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

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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, *su*2, *su*3, *su*4). Our first purpose was to isolate new antisuppressor mutations and in fact a new antisuppressor gene, *AS*, was uncovered. The *AS*-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 *Pm1* and *Pm2* loci previously identified, the two others define a new gene which controls paromomycin and neomycin resistance, *Pm3*. Strains carrying the *Pm3-1* 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evidence; translational fidelity was demonstrated to be decreased by *su* mutations (Coppin-Raynal, 1982 and unpublished data; Picard-Bennoun, personal communication) as it is in bacteria by ribosomal ambiguity (or *ram*) mutations (Rosset & Gorini, 1969).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
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 su2 mutation, su2-5, and three su1 mutations, su1-31, su1-49, su1-60, were used to screen revertants. su1 and su2 are two genetically and physically independent loci (Marcou, Picard-Bennoun & Simonet, 1980). Biochemical evidence shows that su1-60 and su2-5 are ribosomal mutations that increase the translational ambiguity (Coppin-Raynal, 1977, 1982, and unpublished data; Dequard, 1980).
### Table 1. General characteristics of the strains used in this study

| Strains | Characteristics | References |
|---------|----------------|------------|
| 193     | White spores and mycelium | Picard (1971) |
| leu<sub>1</sub>-1 | Auxotrophic for leucine | Kindly provided by Crouzet |
| su<sub>1</sub>-1 | None | Picard (1973) |
| su<sub>4</sub>-31 | Pm<sup>5</sup>, ♀ sterile | Arnaise & Mignotte (Personal communication) |
| su<sub>4</sub>-49 | Pm<sup>s</sup> | |
| su<sub>1</sub>-60 | Pm<sup>s</sup>, ♀ sterile | |
| su<sub>2</sub>-5 | Prototrophic | |
| leu<sub>1</sub>-1 su<sub>2</sub>-18 | Prototrophic | |
| leu<sub>1</sub>-1 su<sub>2</sub>-19 | Prototrophic | |
| leu<sub>1</sub>-1 su<sub>4</sub>-4 | Prototrophic | |
| leu<sub>1</sub>-1 su<sub>10</sub>-1 | Prototrophic | |
| Pm<sub>1</sub>-3 | Pm<sup>s</sup>, dark mycelium | Dequard et al. (1980) |
| Pm<sub>2</sub>-5 | Pm<sup>s</sup>, dark mycelium | |
| mei<sub>1</sub>-1 | Blocked during meiosis | Simonet & Zickler (1978) |
| mei<sub>2</sub>-1 | Blocked during meiosis | |

Symbols are as follows: Pm<sup>s</sup> and Pm<sup>R</sup> respectively more sensitive and more resistant to paromomycin than the wild-type control.
(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 Pml) 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 × 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 × 193 su x, which allowed direct detection of antisuppression; and (2) rev x × 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 x x, in association with other su mutations. The prototrophic strain leu x x su becomes auxotrophic when an AS mutation is introduced. Moreover, the auxotrophy is relieved in the presence of paromomycin as previously reported (Coppin-Raynal, 1981a). Thus the strains carrying an antisuppressor R mutation were crossed with several double mutants: leu x x su 4-4, leu x x su 1o, leu x x su 2-18, leu x x su 2-19 previously obtained by selecting prototrophic revertants of leu x x (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).
Table 2. Determination of genotypes of segregants from crosses \( rev_x \times 193 su \) and \( rev_x \times 193

| Genotype             | Spore Pigmentation | Growth on M2 P1 |
|----------------------|--------------------|-----------------|
| Wild type            | ⬗                   | +               |
| 193 su\(^+\) R\(^+\) | ○                   | +               |
| 193\(^+\) su R\(^+\) | ⬗                   | −               |
| 193 su R\(^+\)      | ©                   | +               |
| 193 su R \((R \neq AS)\) | © 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 M2 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
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

| Types of Asci | Controls | Genotypes of the revertant strains |
|---------------|----------|-----------------------------------|
|               |          | WT × 193su_x                     |
|               |          | + + + +  +                       |
|               |          | − − − −  +                       |
|               |          | rev × 193su_x                    |
|               |          | + − − +  +                       |
|               |          | + + + +  +                       |
|               |          | + + + c | +                       |
|               |          | or                             |

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).
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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 $rev_x \times 193\ su_x$ 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 antisuppressor effect and that they probably corresponded to second-site mutations; they were not studied further. Given the results of the two crosses $rev_x \times 193\ su_x$ and $rev_x \times 193$, 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) $R_1$, $R_2$, $R_6$, $R_8$, and $R_{10}$ are mutations that confer increased resistance to paromomycin as compared with the wild type (situation 1 in Table 3).

(e) $R_{16}$, $R_{18}$, and $R_{19}$ 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
### 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 | Nomenclature |
| rev1       | 250 su₁₋₄₉ R₁              | .              | Pigmented mycelium | Pm₉, N, Chx, Em | 0.85 | III | Pm₂₋₆ |
| rev2       | 500 su₁₋₄₉ R₂              | .              | Pigmented mycelium | Pm₉, N, Chx, Em | 0.85 | III | Pm₂₋₇ |
| rev6       | 500 su₁₋₅₀ R₆              | .              | None              | Pm₉, N | 0.98 | I | loc₅ Pmt† |
| rev7       | 500 su₁₋₄₉ R₇              | + unspecific   | No sporulation    | Pm₉ | 0   | V | AS₂₋₁ |
| rev8       | 500 su₁₋₅₀ R₈              | .              | Slow germination  | Pm₉, N | 0.65 | VII | Pm₂₋₁ |
| rev9       | 500 su₁₋₅₀ R₉              | .              | None              | Pm₉, N | 0.65 | VII | Pm₂₋₂ |
| rev10      | 250 su₁₋₄₉ R₁₀             | .              | Pigmented mycelium | Pm₉, N | 0.85 | III | Pm₂₋₈ |
| rev11      | 500 su₁₋₅₀ R₁₁             | + specific     | Slow germination  | Pm₈ | 0.40 | IV | su₁ |
| rev12      | 500 su₂₋₅ ²/²              | Presumed back- |                     |                | 0.70 | VII | Locus su₁ AS₂ † |
| rev13      | 250 su₂₋₅ ²/²              | mutation       |                     |                |                |                |                |
| rev16      | 250 su₁₋₃₁ R₁₆             | .              | Wild-type phenotype |                | 0.70 | VII | Locus su₁ AS₂ † |
| rev18      | 250 su₁₋₃₁ R₁₈             | .              | Wild-type phenotype |                | 0.70 | VII | Locus su₁ AS₂ † |
| rev19      | 250 su₁₋₃₁ R₁₉             | .              | Wild-type phenotype |                | 0.70 | VII | Locus su₁ AS₂ † |
| rev20      | 250 su₁₋₃₁ R₂₀             | R₂₀ and su₁₋₃₁ could not be dissociated | Locus su₁ † |

* Pm, Paromomycine; N, neomycin; Chx, cycloheximide; Em, Emetine; Pm₉ and Pm₈, 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.
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Ribosomal ambiguity mutations and abolished their suppressor effect on \( \text{leu}_{1-1} \). It can also inactivate a tRNA-type suppressor, \( \text{su}_{10-1} \), but it is inefficient on the \( \text{su}_{4-4} \). On the other hand, \( R_{11} \) was found to act specifically on some \( \text{su}_{2} \) mutations e.g. \( \text{su}_{2-5} \) against which it was isolated and \( \text{su}_{2-18} \). 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 tested mutations |
|------------------|---------------------------------------------------------------|
| Ribosomal suppressors | \( R_{7} \) | \( R_{11} \) |
| \( \text{leu}_{1-1} \ \text{su}_{1-1} \) | — | — |
| \( \text{leu}_{1-1} \ \text{su}_{1-40} \) | — | — |
| \( \text{leu}_{1-1} \ \text{su}_{2-18} \) | — | + |
| \( \text{leu}_{1-1} \ \text{su}_{2-19} \) | — | + |
| tRNA like suppressors | \( \text{leu}_{1-1} \ \text{su}_{4-4} \) | + | — |
| \( \text{leu}_{1-1} \ \text{su}_{10-1} \) | — | + |

* Indicates that the \( \text{leu}_{1-1} \ \text{su} \ \text{R} \) 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 \( \text{su}_{4} \) or a second-site mutation in \( \text{su}_{4} \) leading to a weak suppressor phenotype. It was impossible to separate the site of the first mutation (\( \text{su} \)) and the site of the second one (\( R \)) by recombination. About 200 asci from the cross \( \text{su}_{1-31} \ \text{R}_{20} \times \text{su}_{4}^{+} \ \text{R}_{20}^{+} \) were analysed without recovering the suppressor phenotype (hyper-sensitivity to paromomycin). The \( \text{leu}_{1-1} \ \text{su}_{1-31} \ \text{R}_{20} \) strain was found to be auxotrophic for leucine. The auxotrophy could be alleviated by paromomycin. Weak suppressors unable to suppress the \( \text{leu}_{1-1} \) 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 \( \text{su} \) 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
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 $R$ but was inefficient on $R$. In addition, crosses $su_{1-60} R_g \times su_{1-60} R_g$ provided empty fructifications while crosses $su_R^+ R_g \times su_R^+ R_g$ and $su_{1-60} R_g^+ \times su_{1-60} R_g^+$ 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 $R$ mutant stopped in a reversible way at 36 °C. Moreover sporulation in crosses homozygous for $R_g$ 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: $R_g$, $R_8$, $R_9$.
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 $R_7/R_7^+$ and $R_8/R_8^+$ were forced using two auxotrophic mutations $leu_1$ and $lys_2$. They were found to exhibit good growth on media supplemented with paromomycin (1 mM) while the $leu_1$/$lys_2$ 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.
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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 M2 plus antibiotic and M2 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:

\[
\begin{align*}
(a) & \quad \frac{193 \text{su}_x R^+}{193 \text{su}_x R^{+*}}; \\
(b) & \quad \frac{193 \text{su}_x R^+}{193 \text{su}_x R}; \\
(c) & \quad \frac{193 \text{su}_x R}{193 \text{su}_x R^{*}}.
\end{align*}
\]

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.
(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 $Pm_{2-4}, Pm_{2-7}, Pm_{2-8}$.

(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* involved in meiosis has been identified (Simonet & Zickler, 1978). The fact that $R_8$ and *mei* do not complement each other indicates that *mei* 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 *AS7*. It was found to be linked to the *ap* gene on linkage group V. A meiosis gene *mei* lies at this locus and allelism tests were carried out between $R_7$ and *mei*; both were unable to sporulate. The cross $R_7 \times *mei_1$ is as fertile as controls $R_7 \times WT$ and *mei_1* × WT. Thus $R_7$ and *mei_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
Paromomycin-resistant revertants in Podospora

It did not suppress the \textit{leu_{1-1}} mutation but conferred a slight pigmentation to spores carrying the \textit{193} mutation. Considering these properties such as its 40\% postreduction frequency and its hypersensitivity to paromomycin, it may be an allele of the \textit{su_4} gene. In fact \textit{su_4} and \textit{su_2} alleles can exhibit additivity or antagonistic action (Picard-Bennoun, 1976), and \textit{R_{11}} was demonstrated to relieve the effect of only certain \textit{su_2} alleles. To check this hypothesis complementation tests were performed between \textit{R_{11}}, \textit{su_{1-25}} and \textit{su_{1-26}} which manifested a germination phenotype. No complementation was observed. Thus \textit{R_{11}} is an allele of the \textit{su_4} gene. Because of its phenotypic properties it differs from the \textit{su_1} mutations previously isolated.

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

**DISCUSSION**

Hypersensitivity to paromomycin manifested by four ribosomal suppressors (\textit{su_{1-31}}, \textit{su_{1-49}}, \textit{su_{1-60}} and \textit{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 \textit{R} mutation did cosegregate in all crosses.

Our first purpose had been achieved since a new antisuppressor locus, \textit{AS_7}, has been uncovered. The \textit{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 \textit{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 \textit{AS_{7-1}} mutation, a new allele of the gene \textit{su_1} has been obtained. It is efficient on only some \textit{su_2} mutations. Specific antisuppression by interaction between \textit{su_1} and \textit{su_2} suppressors was previously reported (Picard-Bennoun, 1976). In addition, \textit{R_{29}} may correspond to a mutation at a new antisuppressor locus closely linked to the \textit{su_1} gene; but we cannot at present discriminate between this hypothesis and that of a second-site mutation within the \textit{su_4} 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 \textit{Pm_1} and \textit{Pm_2} loci previously identified (Dequard et al. 1980) but two others have been mapped in a new paromomycin-resistance gene named \textit{Pm_3}. In fact this gene was already known and designated as \textit{mei_3} since it was related to meiosis. Interesting interactions between

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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 far) 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.
Paromomycin-resistant revertants in Podospora 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 AS7-1 mutant described in the present paper. The degree of paromomycin-induced misreading by AS6-1 and AS7-1 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 AS7-1/AS7-1 strains are unable to sporulate while AS6-1 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.

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