Interactions of Ribosomal Antibiotics and Informational Suppressors of *Aspergillus nidulans*

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Strains of *Aspergillus nidulans* containing informational suppressors were grown on medium containing antibiotics known to affect protein synthesis at the ribosomal level. These strains reacted in the anticipated manner: presumed ribosomal suppressors *suaA101*, *suaA105*, *suaC109* and *sua-115* were sensitive or even hypersensitive to aminoglycoside antibiotics, whereas presumed tRNA-like suppressors *suaB111*, *suaD103* and *D108* were only slightly sensitive or wild-type in response. Hygromycin and paromomycin were the most useful antibiotics. All the antibiotics reduced the colony radial growth rate, K_r , increased the lag phase and produced wrinkled morphology. Hygromycin was the most toxic. Resistant sectors were produced on paromomycin and hygromycin. The selective action of 'misreading' antibiotics on *suaA* and *suaC* strains is further evidence that these are ribosomal suppressors, whereas *suaB* and *suaD* may code for altered tRNA molecules. The results imply that hygromycin or paromomycin could be used for isolating ribosomal suppressors.

INTRODUCTION

Suppressors of alleles in four unrelated genes (*alX*, *sB*, *alcA* and *niaD*) have been isolated in *Aspergillus nidulans* by coreversion (Roberts *et al.*, 1979, 1983). These suppressors can be distinguished from physiological suppressors by the fact that they are specific for alleles but not for genes. Also they restore allantoinase enzyme activity in strains containing the *alX4* allele (Roberts *et al.*, 1979) and nitrate reductase in *niaD500* strains (Roberts *et al.*, 1983). They are thus translational or informational suppressors. They are presumed to be nonsense suppressors since all the suppressible alleles have chain termination properties. Two classes of suppressor have been characterized: *suaB111*, *suaD103* and *D108* are semidominant in diploids, suppressing only *alX4* and *sB43*, and do not alter growth; *suaA101*, *suaA105*, *suaC109* and *sua-115* are recessive, have altered morphology and slow growth, and all but *suaA105* suppress at least five alleles. The two classes are thought to be due to alterations in tRNA and ribosomal proteins respectively.

Ribosomal mutants, with altered rRNA or ribosomal proteins, can be obtained by selecting for many kinds of phenotypic alteration. When mutants selected by one means have been examined for other alterations, they have frequently exhibited pleiotropy. Amongst eukaryotes, examples come from work on cold sensitive mutants of *A. nidulans* (Waldron & Roberts, 1974), and suppressor and antisuppressor mutants (Coppin-Raynal, 1977) and cold sensitive mutants (Picard-Bennoun & Le Coze, 1980) of *Podospora anserina* which can all have altered susceptibility to cycloheximide. Strains of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* selected for antibiotic resistance can be cold sensitive (Berry *et al.*, 1978; Singh *et al.*,

Abbreviations: CM, complete medium; GEN, gentamycin; HYG, hygromycin; KAN, kanamycin; NEO, neomycin; PAR, paromomycin.

1978, respectively). *SUP46*, a ribosomal suppressor in *Sacch. cerevisiae* is hypersensitive to paromomycin (Masarekar *et al.*, 1981). Similarly in *P. anserina*, antisuppressors can confer resistance and suppressors can confer hypersensitivity to paromomycin (Coppin-Raynal, 1981), whereas paromomycin resistant mutations can weakly suppress some other mutations (Dequard *et al.*, 1980). Suppressor mutations of *A. nidulans* can also make strains cold sensitive for growth (Roberts *et al.*, 1979; S. D. Martinelli, unpublished results).

Similar examples in *Escherichia coli* confirm the link between the suppressor activity of *ram* mutants and antibiotic sensitivity and between antibiotic resistance and antisuppressor activity (Bollen *et al.*, 1975; Cabezon *et al.*, 1976; Ozaki *et al.*, 1969; Zimmermann *et al.*, 1971).

Since there is a correlation between drug sensitivity or resistance and informational suppressors which act at the level of the ribosome, I tested inhibitors of the 40S ribosomal subunit against various informational suppressors of *A. nidulans*. It was hoped that these tests would confirm the ribosomal nature of some suppressors and prove to be a general method for classifying unknown suppressors.

METHODS

Cultural and genetical techniques were derived from Pontecorvo *et al.* (1953).

Strains. All numbers are those of Birkbeck stock cultures of *Aspergillus nidulans* regardless of the origin of the strains. Most strains contain the mutations *fwA1* (fawn conidia) and *pabaA1* (*p*-amino benzoic acid requirement). Dr C. Scazzocchio of Essex University supplied strain 77, with *alX4*, from which the following strains were derived in stepwise mutation events by T. J. Roberts of Birkbeck College: 390 also has *SB43*, 391 has the latter plus *alcR125*; strains 374, 371, 377, 380, 383 and 384 are derived from 390 and bear suppressor mutations (see Table 4); strain 370 with *suaA101* was isolated from strain 391. Roberts *et al.* (1979) should be consulted for details of all suppressors except *sua-115*, which has recently been characterized (unpublished results) and has identical properties to *suaC109* even to being located on linkage group VII. Crosses between strains containing suppressors *sua-115* and *suaC109* are sterile, hence the affinities of *sua-115* are unknown. In a previous paper (Roberts *et al.*, 1979) *sua-115* was incorrectly located on linkage group III. *alcA125* (Roberts *et al.*, 1979) has been renamed *alcR125* by Pateman *et al.* (1983), since it is now thought to be a mutation in a gene which codes for a regulatory protein.

Medium. The complete medium (CM) used is as described by Cove (1966). Filter sterilized aqueous stock solutions of antibiotics and solid antibiotics were stored at -18°C . They were added to molten medium at 50°C and 10 ml was poured immediately into 5 cm plates on a levelled board. Plates were always inoculated on the day of preparation. The antibiotic concentrations mentioned in the text refer to the final ones in agar medium. Small plates are more economic and overcome the problem of colony interaction found with multiply inoculated large plates (see below).

Hygromycin B was a gift from Eli Lilly, Indianapolis, Ind., USA and paromomycin was a gift from Parke-Davies, Pontypool, UK. Other antibiotics were purchased from Sigma.

Growth measurement. Conidial suspensions were stab inoculated at the centres of 5 cm plates. At least two, normally three, and up to ten replicate plates were made for each strain in each experiment. Plates were incubated at 37°C .

Colony diameter was measured with a ruler two or three times per day on an illuminated colony counter with a $2\times$ magnification. The increase in mean radius against time was plotted. The colony radial growth rate (K_r) was calculated (wherever possible) and the lag period noted. The lag period used in this work refers to the period before the occurrence of colony growth visible to the naked eye. Several strains have a lower intrinsic growth rate than the controls, owing to the presence of suppressor mutations. This was compensated for as follows. For each strain a K_r ratio was calculated, i.e. K_r from growth on antibiotic : K_r from CM. This gave a numerical value for resistance. This value (expressed as a percentage in Tables 1 and 2) could then be compared with a similar K_r ratio calculated for the control strain, to assess the relative resistance of each strain.

RESULTS

Growth on aminoglycoside antibiotics

Three control strains were used: 390, 391 and 77, but mainly 390. The mean colony lag phase of strain 390 on CM was 11 h and the mean K_r was $254 \pm 26 \mu\text{m h}^{-1}$ (Table 1). Since response to the incorporation of antibiotics in the medium could involve a change in either the lag phase or K_r , or both, it was necessary to record both types of measurement.

In all antibiotic tests, strains were also inoculated and measured on CM without antibiotic

Table 1. *Radial growth rate and length of lag phase on complete medium*

Strain*	n†	K_r ($\mu\text{m h}^{-1}$)		Lag (h)‡	
		$\bar{x}$	$6n - 1$	$\bar{x}$	$6n - 1$
390	14	264	26.2	10.2	2.85
391	6	253	22.5	—	—
77	9	246	30.4	11.9	1.55
380	19	243	35.5	14.2	3.9
384	17	241	29.7	13.3	3.2
370	9	192	17.6	11.4	3.9
374	11	239	30.4	13.0	3.6
371	11	292	37.3	10.8	3.1
377	13	293	40.0	11.2	3.3
383	12	289	32.0	10.6	3.3

* Full genotypes of each strain are given in Methods and relevant mutations in Table 4.

† n , Number of growth experiments with the strain on complete medium; at least two, usually three, and at most ten replicates were made per strain per experiment. $\bar{x}$, Mean; $6n - 1$, SD.

‡ Lag refers to the extrapolation of the initial growth rate from colony appearance to give germination time. This will only be approximate since no microscopic observations were made.

since suppressor-containing strains have different intrinsic growth rates from the wild-type (Table 1). Lag phases varied over a range of 4 h, which is not significant since the maximum standard deviation was nearly 4 h. The mean deviation in K_r was $31 \mu\text{m h}^{-1}$. Strains which differed by more than this from the controls were considered to have a different K_r . Therefore strains 383, 377 and 371 with tRNA-like suppressors were faster growing than the control, and strain 370 with ribosomal suppressor *suaA101* was slower growing. In every experiment, strains 380, 384 and 374 grew more slowly than the control although appearing within the limit of variation here.

Five antibiotics known to affect the function of 30S ribosomal units were used in growth media. Kanamycin (KAN), neomycin (NEO), gentamycin (GEN) and paromomycin (PAR) had little effect on the lag or K_r of control strain 390 at concentrations of $600 \mu\text{M}$ and below. Hygromycin (HYG), by contrast, was quite toxic at low concentrations. HYG and PAR were chosen for the majority of tests on mutant strains. Initial tests with PAR and HYG, using multiply inoculated 9 cm plates, gave misleading results. For example, strain 374 appeared to be sensitive at low HYG concentrations but resistant at high concentrations compared to controls, and not sensitive to low PAR concentrations. These results conflicted with those obtained using singly inoculated plates. All the following results are from strains inoculated singly on to 5 cm plates of medium.

Using concentrations of PAR up to $600 \mu\text{M}$, the K_r dropped only slightly with little difference between cultures except that strains 380, 384 and 374 now had a long initial phase of slow growth. This was followed by a phase of accelerated growth before a linear growth rate was achieved. Strains 390 and 370 sometimes adopted this initial non-linear mode of growth but other strains always grew at a linear rate. On 1 mM -PAR the strains gave three different responses. Strains 380, 384 and 374 had their K_r reduced from $225 \mu\text{m h}^{-1}$ (CM) to about $60 \mu\text{m h}^{-1}$. Strain 370 grew slightly faster than these, with a K_r of $138 \mu\text{m h}^{-1}$, after a long phase of slower growth. Strains 383, 371 and 377 were barely affected and neither was the control strain 390. The same groups were obvious on 2 mM -PAR with strains 380, 374 and 384 being hypersensitive, 370 sensitive and the rest relatively resistant like the control (Table 2). For the first 40 h on PAR strains 380, 370 and 374 grew very slowly with nonlinear kinetics (K_r about $50 \mu\text{m h}^{-1}$) but after that a linear growth rate was seen owing to the production of resistant sectors. All resistant strains had lag phases approximately 4 h longer than normal whereas sensitive strains had an extra 20 h lag before starting to grow. Overall the resistance of the strains can be summarized: $383 = 371 = 377 \sim 390 \geq 370 \geq 380 > 374 = 384$, with the most resistant strain first.

Table 2. *Comparative growth of suppressor strains on paromomycin*

These results come from one experiment with one batch of paromomycin. Colonies were also grown on 600 μM and 1 mM-PAR with similar but less extreme results. Strain genotypes are given in Methods and Table 4.

Strain	$\frac{K_r \text{ PAR}^*}{K_r \text{ CM}^\dagger} \times 100$	K_r ratio as percentage of control ‡
390	77	100
380	20	26
384	22	29
370	39	51
374	28	36
371	78	101
377	83	107
383	69	90

* 2 mM-Paromomycin.

† *Aspergillus* complete medium.

‡ Strain 390.

Table 3. *Comparative growth of suppressor strains on hygromycin*

These results come from one growth experiment only, although many others were performed (see Results). Strains 390, 371, 377 and 383 also grew on 100 μM - and 400 μM -HYG in this experiment. Strains 370 and 374 both grew slightly at 100 μM - but not at 400 μM -HYG and are therefore more resistant than strains 380 and 384, which did not grow at all. See Tables 1 and 4 for other details.

Strain	$\frac{K_r \text{ HYG}^*}{K_r \text{ CM}^\dagger} \times 100$	K_r ratio as percentage of control ‡
390	77	100
380	22	29
384	16	21
370	33	43
374	55	77
371	71	92
377	85	110
383	77	100

* 50 μM -Hygromycin.† *Aspergillus* complete medium.

‡ Strain 390.

At all concentrations of HYG strains 380, 384, 370 and 374 were sensitive in their growth, i.e. K_r and lag (Table 3), compared with strains 390, 383, 371 and 377. Since both HYG solutions and powder appear to lose potency at -18°C and three different batches of HYG were used, the molarities given can only be a guide. For example, at 50 μM , one of the lower concentrations used, K_r was always reduced and the lag phases increased in strains 380, 384, 370 and 374, whereas 100 μM -HYG prepared from a new batch of drug was normally lethal to these strains. At 50 μM other strains were completely normal or, with a fresh batch of compound, K_r was reduced slightly. At all concentrations of HYG used, lags were increased by more than 4 h and, if the increase was more than 10 h, this was standard for all strains able to grow on this medium. Whatever the batch of drug used, 500 μM was always lethal. Strains 377, 371 and 383 behaved in a virtually identical manner, being little affected by low concentrations of HYG but more so at concentrations above 200 μM when they were actually more resistant than the control strain 390. The sensitivity rating for strains, taking into account data not presented for growth on higher concentrations, was $380 = 384 > 370 > 374 > 390 \sim 377 = 383 = 371$, with the most resistant strains last. For classifying suppressors 50 μM was found useful because it invariably allowed a distinction to be made between the response of a wild-type or control strain and that of a sensitive suppressor-containing strain. As on PAR, hypersensitive strains frequently produced resistant sectors which usually coincided with a change in growth kinetics.

NEO and GEN at 600 μ M and below reduced the K_r of strains 383, 371 and 377 slightly like that of 390. Lag phases were also increased but not significantly. Strains 370, 374, 380 and 384 were generally more affected by these antibiotics but the results were not so clear as those produced by PAR and HYG and are not given. KAN had a negligible effect on growth.

All antibiotics except KAN reduced the K_r of strains 370, 374, 380 and 384 more than those of strains 383, 371, 377 and the control 390, thus distinguishing two groups of strains. Strains in the first group all contain the suspected ribosomal suppressors *suaA101*, *suaA105*, *suaC109* and *sua-115*. Strains in the second group, apart from the control, contain the three tRNA-like suppressors *suaD103*, *D108* and *suaB111*.

Morphological effects of ribosomal antibiotics

Morphology was not examined in detail microscopically but trends in the gross appearance of colonies were recorded. The most obvious drug-induced alteration was the occurrence of radial wrinkles. These wrinkles started near the colony centre and extended to various lengths along the radii. The number and length of these wrinkles were increased by increasing antibiotic concentrations. In extreme cases the radial lengths were 7/8 of the colony radius. All the antibiotics produced this effect and all the strains were susceptible. The effects were most marked in strains containing suppressors *suaA*, *suaC* and *sua-115* and were produced by HYG and PAR. As a corollary, it was interesting to observe that strains with known ribosomal mutations, i.e. *arp* (altered ribosomal profile), and known cycloheximide resistant strains exhibited wrinkled morphology even on CM (unpublished results)

Appearance of resistant sectors during growth on antibiotics

All strains gave rise to resistant sectors but at different rates. As a rule, the more toxic the antibiotic and the more susceptible the strain, the more sectors that were produced. In extreme cases a fast outgrowth occurred so soon after germination that this sector almost completely surrounded the more inhibited central inoculum and probably in some cases obscured it. Strains 380, 384, 370 and 374 were particularly prone to producing sectors on HYG and PAR. Some sectors were aconidial and other more velvet looking in these extreme cases.

DISCUSSION

Aminoglycoside antibiotics have a specially important role in studies on informational suppression. They cause phenotypic suppression of mutations in bacteria (reviewed by Gorini, 1974), in *Sacch. cerevisiae* (Singh *et al.*, 1979; Palmer *et al.*, 1979) and in *A. nidulans* (Roberts *et al.*, 1979; Martinelli & Roberts, 1983). These antibiotics also affect the rate and accuracy of protein synthesis *in vitro* using prokaryotic (Davies *et al.*, 1965) or eukaryotic ribosomes (Palmer & Wilhelm, 1978; Wilhelm *et al.*, 1978*a, b*), and *in vivo* in *E. coli* (Edelmann & Gallant, 1977).

Bacterial mutants resistant to aminoglycosides can have alterations in ribosomal proteins which confer resistance to the 'misreading' effect of the antibiotics, e.g. *strA* coding for S12 and *neaA* for S17 (Ozaki *et al.*, 1969; Bollen *et al.*, 1975, respectively). These mutations give rise to ribosomes which are more faithful in their reading of mRNA, allowing fewer errors in reading bases 1 and 2 of the codon (Gorini, 1974) and thus changing the intrinsic error frequency in translation. Conversely, *ram* mutants (ribosomal ambiguity) result in alterations in ribosomal proteins S4 or S5 (Zimmermann *et al.*, 1971; Cabezon *et al.*, 1976) which permits a higher error level. This can lead to suppression of all types of point mutations. In addition, *ramA1* and *ramC319* are hypersensitive to streptomycin (Rosset & Gorini, 1969; Cabezon *et al.*, 1976). The basis for this hypersensitivity could be the additive effects of two agencies, mutation and aminoglycoside, which increase error levels during protein synthesis to a lethal amount. Thus it is not surprising that all of the putative ribosomal suppressors of *Aspergillus* are hypersensitive to HYG and PAR and that tRNA-like suppressors behave like wild-type control strains (Table 4). These results alone make *suaA101*, *suaA105*, *suaC109* and *sua-115* very strong candidates for *ram*-type suppressors. Confirmation was obtained recently by Harvey & Martinelli (1983) who found that *suaA101* and *suaC109* have altered ribosomal profiles on ion-exchange columns and

Table 4. Summary of the properties of suppressor mutations

Strain	Relevant mutation	Proposed suppressor class	Suppression <i>in vivo</i> * of:				Semi-dominance in diploids	Cold sensitivity	Morphological alteration†	Resistance‡ to antibiotics		Linkage group
			<i>alX4</i>	<i>sB43</i>	<i>alcR125</i>	<i>niaD500</i> ‡				HYG	PAR	
390	wild-type§											
380	<i>suaC109</i>	ribosomal	+	+	+	+	×	✓	✓	SS	W	VII
384	<i>sua-115</i>	ribosomal	+	+	±	+	×	✓	✓	SS	SS	VII
370	<i>suaA101</i>	ribosomal	+±	+	+	+	×	✓	✓	S	S	III
374	<i>suaA105</i>	ribosomal	±	±	-	-	×	×	✓	S	SS	III
371	<i>suaD103</i>	tRNA	++	+	-	-	✓	×	×	W	W	VII
377	<i>suaD108</i>	tRNA	++	+	-	-	✓	×	×	W	W	VII
383	<i>suaB111</i>	tRNA	++	+	-	-	✓	×	×	W	W	III

* Growth is expressed as maximum (+ +) to minimum (-) on various selective media designed to test for suppression of particular alleles.
† The slow growth and altered morphology accompanying the suppressor mutations *suaC109*, *sua-115*, *suaA101* and *A105* was one of the reasons for distinguishing them as 'ribosomal-type' originally. Similarly, *suaD103*, *D108* and *B111* are thought to be alterations in tRNA genes since these mutations disturb the phenotype very little and are semidominant.

‡ Resistance is expressed by K, only. W, wild-type response whether sensitive or resistant compared with other controls; S, more sensitive than wild-type; SS, hypersensitive.

§ Strain 391 is the wild-type control for 370, but has identical properties in these tests.

are different from one another. The ribosomal nature of other fungal suppressors hypersensitive to PAR has been proved by two-dimensional electrophoresis of ribosomal proteins (Ishiguro *et al.*, 1981) and by *in vitro* protein synthesis (Coppin-Raynal, 1982; Masurekar *et al.*, 1981). The action of PAR and HYG on *suaA101* and *suaC109* strains also indicates that in *Aspergillus* these antibiotics act directly on ribosomes.

The less dramatic results obtained with GEN and NEO are probably due to the low concentrations used. In phenotypic suppression tests, along with many other aminoglycosides, both these antibiotics produced zones of growth inhibition in lawns of *Aspergillus* around discs seeded with 0.6 mg antibiotics, (Martinelli & Roberts, 1983). Probably 2 mM-NEO would have a marked effect on *Aspergillus*, as it does with *P. anserina* (Dequard *et al.*, 1980; Coppin-Raynal, 1981) which is also inhibited by 2 mM-PAR. However, other more toxic antibiotics tested by Martinelli & Roberts (1983) should prove useful if they become available, i.e. G418 and tobramycin.

Colony radial growth rate (K_r) measurement is thought to be unsuitable as a means of comparing the specific growth rates of two species of fungi (Trinci, 1969) or of two strains of the same species (Bainbridge & Trinci, 1969). However, since the ratio for a strain grown on antibiotic to that on normal medium (K_r ratio) has been calculated, these measurements should permit an assessment of resistance. Since alterations in growth rate and lag did not change concomitantly in all cases, it is particularly important that in growth tests of this kind an end-point diameter is not measured after a fixed period of time for all strains. This conclusion was also reached by Gull & Trinci (1970), who recommended that K_r should be used to determine the effect of inhibitors on fungal growth.

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