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Identification of two genes controlling kasugamycin resistance in the filamentous fungus *Podospora anserina*

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Summary

We have investigated the effect of the ribosome-targeted antibiotic kasugamycin (ksg) in *Podospora anserina*. While ksg inhibits both growth and sporulation, it has a stronger inhibitory effect on the sporulation process. It was previously reported that sporulation of *Podospora* could be impaired when ribosomes translate with a too high accuracy, and since ksg was demonstrated to increase the ribosomal accuracy in *E. coli*, we wondered whether it would act similarly in *Podospora*. As a first approach we have isolated two mutations at different loci, *Ks1* and *Ks2*, that increase the resistance to ksg at the level of both growth and sporulation. Interestingly *Ks1-1* also confers a decreased resistance to paromomycin, which is a mistranslation inducer. Characterization of *Ks1-1* and *Ks2-1* mutants suggests that they could be ribosomal mutants.

Introduction

Kasugamycin (ksg) is a ribosome-directed aminoglycoside widely used as a fungicide in agriculture (Umezawa *et al.* 1966). This antibiotic also inhibits bacterial growth and its mode of action has been principally investigated in procaryotes (for a review see Van Knippenberg, 1986). *In vitro* studies have shown that ksg inhibits initiation of protein biosynthesis and, more recently, that it decreases translational misreading (Van Buul *et al.* 1984). This last property is specific to ksg since most aminoglycosides such as streptomycin or paromomycin induce the opposite effect – they increase translational errors (Gorini 1974; Palmer and Wilhelm, 1978). The resistance to ksg manifested by *ksg A* mutants in *E. coli* was demonstrated to be caused by the lack of methylation of two adjacent adenosines at the 3′ end of 16S rRNA (Helser *et al.* 1971). This defect seems to alter the decoding properties of the ribosomes. In fact an increase of the leakiness of certain nonsense and frameshift mutations was observed *in vivo* in the *ksgA* mutant suggesting a stimulation of the ribosomal misreading (Van Buul *et al.* 1984). The 3′ end is a very conserved part of the RNA of the small ribosomal subunits. The two modified adenosines are located in the loop of an universal hairpin structure and only a few exceptions are known they are non-methylated (Van Knippenberg *et al.* 1984). The 3′ terminal domain is associated with many functions such as tRNA decoding, subunit association, control of translational fidelity and initiation of translation (for a review see Noller *et al.* 1986). The *ksg* mutants indicate the functional importance of dimethylation of the adenosines in this domain.

Additional information could be provided by extending the study to eucaryotes. A filamentous fungus, *Podospora anserina*, seemed to be particularly suitable for this study. Genetic analysis is easy in that organism and we have already selected and characterized a large number of ribosomal mutants, in particular mutants with altered translational accuracy (Picard-Bennoun *et al.* 1983; Dequard-Chablat *et al.* 1986). Most of the mutations altering translational accuracy were isolated by screening for informational suppressors (*su*) and antisuppressors (*AS*). Biochemical analyses confirmed that most *su* and *AS* mutations truly act on ribosomes by changing their misreading (see Dequard-Chablat and Coppin-Raynal, 1984 for an example). Ribosomes from the *su* mutants misread more than wild-type ribosomes and *su* can be called low accuracy mutations. Conversely ribosomes from the *AS* mutants misread less than wild-type, and *AS* can be named high accuracy mutations. Moreover 2 *su* genes and 3 *AS* genes were shown to code for a protein of the small ribosomal subunit (Dequard-Chablet *et al.* 1986). The phenotypic analysis of that collection of mutants has indicated that development of the fungus is often impaired when translational accuracy is changed

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(Picard-Bennoun, 1982). For example a too high accuracy has been shown to cause a sporulation deficiency (Dequard-Chablat and Coppin-Raynal, 1984). By studying the phenotypic effect of ksg on the wild-type strain and ribosomal *su* and *AS* mutants of *Podospora anserina*, and by characterizing mutants resistant to this drug, we hoped to better understand the mechanism of action of ksg in eucaryotes and to determine whether it could influence translational accuracy.

The present paper reports the different effects of ksg on *Podospora anserina* and the selection and genetical analysis of two mutants exhibiting an increased resistance to ksg as compared to the wild-type. It has been shown that the resistance appears at the level of cytoplasmic protein synthesis, suggesting that the ribosomes are involved. Moreover, indirect evidence suggests that ksg increases translational accuracy in *Podospora* in the same way as in *E. coli*.

Materials and methods

(i) *Organism and strains*

Podospora anserina is an ascomycete filamentous fungus. Its main characteristics were first described by Rizet and Engelmann (1949) and have been reviewed by Esser (1974) *Podospora* was routinely grown on synthetic minimal agar medium (M2) at 26 °C. Mycelium for preparation of protoplasts and assays of protein synthesis was grown in Roux flasks in liquid minimal medium supplemented with yeast extract (5 g/l). Protoplasts were prepared as described by Belcour (1975) and modified by Brygoo and Debuchy (1986).

The *AS3-1* mutation has been shown to alter the properties of ribosomes (Coppin-Raynal, 1977) and their structure (Dequard-Chablat *et al.* 1986). 193 is a UGA mutation which affects mycelium and spore pigmentation. *Su4-1* is a UGA suppressor mapping in the gene for a $tRNA^{ser}$ (Brygoo and Debuchy, 1985).

(ii) *Selection and genetic analysis of revertant strains of AS3-1*

Protoplasts of the *AS3-1* mutant were spread on agar regeneration medium containing 2 mg/ml of kasugamycin (20 plates with about 10^6 protoplasts). Both the regeneration and growth of protoplasts were strongly inhibited on this selective medium. Revertants appeared as thicker areas of mycelium after a two week incubation. A small section was isolated from each of these areas and tested for its growth on M2 medium with 2 mg/ml of kasugamycin. Two revertants which grew better than the *AS3-1* original strain were retained and submitted to further genetic analysis. The segregation of *AS3-1* was scored directly by its bad germination and dark mycelial pigmentation. The ksg phenotypes were scored on M2 medium containing 2 mg/ml ksg after a 4 days incubation.

(iii) *Assays of in vivo protein synthesis*

Mycelium grown 2 days in a Roux flask was fragmented in 10 ml of liquid synthetic medium for 15 min in a MSE homogenizer. After a 5 min centrifugation at 5000 g the pellet was resuspended in 50 ml of fresh liquid medium. Assays were performed in 3 ml fractions. Fractions were preincubated with the antibiotic for 4 h before adding [^{14}C]leucine [0·2 μCi/ml; 10 mCi/mmole]. At various times 0·5 ml aliquots were taken and precipitated with 0·5 ml of cold 10% trichloroacetic acid. The precipitate was collected on a glass fibre filter and digested in a mixture of Luma Solv-Lipo Luma-water (1:10:0·2, v/v) and counted.

(iv) *Chemical products*

Kasugamycin sulfate was purchased from Boehringer. Paromomycin sulphate was a gift from Substantia. Luma Solv and Lipo Luma were from Lumac (Netherlands).

Results

(i) *Effects of ksg on the wild-type strain*

(a) *Effect on vegetative growth*

Ksg was demonstrated to slow down the growth of *Podospora* but the concentrations required were fairly high (1–3 mg/ml are needed for 50% inhibition as shown in Fig. 1). The inhibition is never complete even at higher concentrations (5 mg/ml). It was shown that sensitivity of *Podospora* to ksg was not influenced by the pH of the medium in contrast to the fungus *Pyricularia oryzae*, in which there is a stronger inhibitory effect of ksg in an acidic than in a neutral medium (Umezawa *et al.* 1965).

(b) *Effect on cytoplasmic protein synthesis*

This was determined by measuring the incorporation of labelled leucine into proteins in whole cells with and without the drug. Suspensions of fragmented mycelium were prepared and preincubated with the

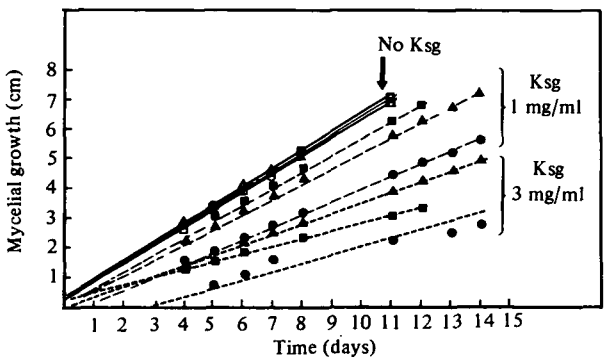

Fig. 1. Growth curves of *Ks1-1* (△, ▲), *Ks2-1* (□, ■) and the wild-type (○, ●) on synthetic medium with and without ksg. (filled and opened symbols respectively).

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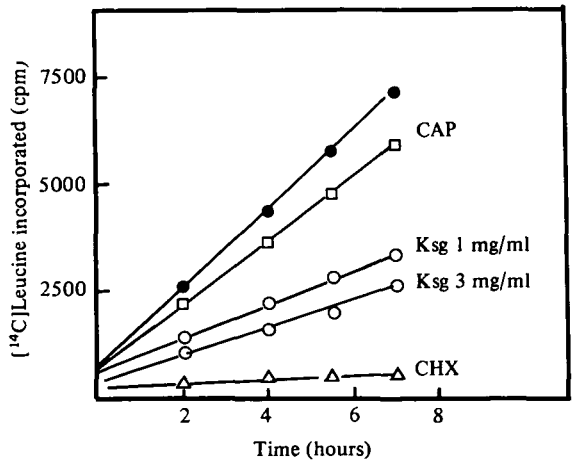

Fig. 2. *In vivo* effect of ksg, cycloheximide (CHX) and chloramphenicol (CAP) on [^{14}C]leucine incorporation in the TCA precipitable material.

antibiotic before adding the [^{14}C]leucine as described in Materials and methods. Cycloheximide and chloramphenicol were used as controls for the inhibition of cytoplasmic and mitochondrial protein synthesis respectively. The result of one representative experiment is presented in Fig. 2. The finding that [^{14}C] leucine incorporation was completely inhibited by cycloheximide but only slightly reduced by chloramphenicol indicated that the cytoplasmic translation was principally measured in that system. The decrease of leucine incorporation observed in the presence of ksg suggested that the drug inhibited cytoplasmic protein synthesis. Ksg at 1–3 mg/ml was found to cause an inhibition of cytoplasmic protein synthesis similar to its inhibition on growth (50–70% inhibition).

(c) *Effect on fertility*

This was tested by performing crosses on media containing different amounts of ksg. High concentrations of ksg (> 1 mg/ml) were found to block the development of female organs. Such a phenotype is often observed when the growth is impaired. More interesting is the finding that low concentrations of ksg ($\geqslant$ 50 μg/ml) specifically inhibited the sporulation process without preventing the development of fruiting bodies. A cytological analysis similar to that carried out in the sporulation deficient mutants (Zickler and Simonet, 1980) would be necessary to determine precisely the stage subsequent to fertilization which is sensitive to the ksg action (meiosis, postmeiotic mitosis or ascospore formation).

(ii) *Effects of Ksg on mutants with altered translational accuracy*

A correlation has been found between the effect of *su* and *AS* mutations on accuracy and their susceptibility to paromomycin, a drug which increases translational

Table 1. *General characteristics of the ribosomal mutants analyzed for their ksg phenotype. For further details see Picard-Bennoun et al. 1983; Dequard-Chablat et al. 1986*

	Designation	Kasugamycine phenotype[a]	Paromomycine phenotype[b]	Other phenotypes	
Low accuracy ribosomal mutants	sul-1	O	O	–	
	sul-25	R	S	–	
	sul-26	R	S	Sterile	
	sul-31	O	S	Sterile	
	sul-51	R	R	Slow germination	
	su2-1	O	O	–	
	su2-5	O	S	Sterile	
High accuracy ribosomal mutants	AS1-1	R	S	–	
	AS1-2	R	O	–	
	AS3-1	S	R	Cold sensitive	AS1 codes for r-protein S12
	AS3-2	S	R	Cold sensitive	
	AS4-4	D	R	Poor growth	
	AS4-43	O	R	–	
	AS6-1	R	R	Cold sensitive	AS6 codes for r-protein S19
	AS6-4	O	R	No sporulation	
	AS7-1	O	R	No sporulation	

[a] The ksg phenotype was determined by comparing rates on media with ksg (0·75 and/or 1 mg/ml) and without ksg. Inhibition was about 50% for the wild-type strain. When the difference between the mutant and the wild-type strain was less than 10% these were considered as similar (symbol: O) when difference was more than 20% these were considered as different. Mutants could be hypersensitive (S), resistant (R) or dependent (D: better growth on ksg medium).

[b] The Pm phenotypes were determined previously (Coppin-Raynal, 1981).

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misreading: most suppressors are more sensitive to paromomycin than the wild-type strain whereas most antisuppressors exhibit resistance to this antibiotic (Coppin-Raynal, 1981 and Table 1). Ksg is assumed to display the opposite effect to paromomycin on translational accuracy. Therefore, we could expect to find the converse situation with respect to ksg phenotype. It can be seen in Table 1 that although several *su* and *AS* mutations change the susceptibility to ksg, there is no particular rule. The cytoplasmic protein synthesis measured by labelled leucine incorporation into proteins was as sensitive to ksg as the wild-type in the *AS*1 and *AS*3 mutants (data not shown). This result suggests that the resistance and hypersensitivity to ksg displayed by those ribosomal mutants (Table 1) do not appear at the level of ribosomes. Other mechanisms such as permeation could be involved.

(iii) *Selection of mutants with altered ksg susceptibility*

Selection of ksg resistant mutants can be made at the level of growth and at the level of sporulation. Both procedures were utilized. A search for mutants able to sporulate in the presence of ksg (60 μg/ml) gave negative results; no such mutants were found. On the other hand, selection for growth in the presence of ksg enabled us to obtain two mutants. They were revertants screened from protoplasts of the *AS3-1* mutant which displays a decreased resistance to ksg as compared with the wild-type (see Table 1). They regenerated better on ksg medium (2 mg/ml) and they exhibited a better growth than *AS3-1* when transferred to ksg minimal medium (2 mg/ml) (see Materials and methods for more details).

(iv) *Genetic analysis of revertants*

The two revertants were crossed with the wild-type strain in order to analyze the segregation of the ksg phenotype. Analysis of 20 tetrads from each cross indicated that both revertants carried a second external mutation which segregated 2:2. These mutations exhibited different second division segregation (SDS) frequencies which indicated that they mapped at two different loci, which we have named *Ks1* and *Ks*2. They reversed the ksg sensitivity caused by *AS3-1*: *AS3-1 Ks1-1* and *AS3-1 Ks2-1* segregants could grow on medium supplemented with ksg at 3 mg/ml whereas growth of *AS3-1 Ks*$^+$ segregants was inhibited. Moreover, when separated from *AS3-1, Ks1-1* and *Ks2-1* conferred an increased resistance level to ksg. In fact *AS3*$^+$ *Ks1-1* and *AS3*$^+$ *Ks2-2* segregants displayed a better growth than *AS3*$^+$ *Ks*$^+$ on ksg medium. This phenotype was confirmed by analysis of subsequent crosses: *AS3*$^+$ *Ks1-1* × *AS3*$^+$ *Ks*$^+$ and *AS3*$^+$ *Ks2-1* × *AS3*$^+$ *Ks*$^+$. This demonstrated that *Ks1-1* displayed 60% SDS frequency whereas *Ks2-1* never segregated at the second divison. *Ks2-1* mapped close to the centromere of chromosome II, a locus which did not correspond to a previously identified *su*, *AS* or any other ribosomal locus. Localization of *Ks1-1* has not been determined but it is not linked to any known *su* or *AS* mutations displaying a 60% SDS frequency. Cosegregation of a slow germination character with the ksg phenotype was observed in all crosses involving the *Ks1-1* mutant. This suggested that the *Ks1-1* mutation might be responsible for both the two phenotypes although no direct evidence allowed us to rule out the presence of two closely linked mutations.

(v) *Phenotypic analysis of Ks1-1 and Ks2-1 mutants*

The resistance to ksg was ascertained by comparing the growth of the two mutants and the wild-type strain on media supplemented with ksg. Growth curves presented in Fig. 1 confirm that *Ks1-1* and *Ks2-1* mutants are more resistant to ksg than the wild-type strain. We further compared the effect of ksg on [^{14}C] leucine incorporation into proteins. The data summarized in Table 2 suggest that the resistance to ksg manifests itself at the level of cytoplasmic protein synthesis. Then the effect of ksg on sporulation was examined in the two mutants. It can be seen in Table 3 that *Ks1-1* and *Ks2-1* mutants can sporulate on media containing 250 μg/ml and 120 μg/ml of ksg, respectively, while sporulation is already inhibited at

Table 2. *Inhibition of* [^{14}C]*leucine incorporated in vivo by ksg in wild-type Ks1-1 and Ks2-1 strains*

	ksg concentration (mg/ml)	
Strains	1	3
WT	79%	86%
KS1-1	7%	30%
Ks2-1	27%	29%

Kinetics were performed as in Fig. 2. The percentage of inhibition was determined from the ratio of the incorporation rates with and without ksg.

Table 3. *Sporulation of Ks1-1 and Ks2-1 strains on ksg supplemented media*

	ksg concentration (μg/ml)								
Crosses	0	40	60	80	100	120	150	200	250
Ks$^+$ × Ks$^+$	+	+	−	−	−	−	−	−	−
Ks1-1 × Ks1-1	+	+	+	+	+	+	+	+	+
Ks2-1 × Ks2-1	+	+	+	+	+	+	−	−	−
Ks1-1 × Ks$^+$	+								
Ks2-1 × Ks$^+$	+		+						

Only qualitative data are indicated with the following symbols: + spores; − no spore differentiated in the fruiting bodies.

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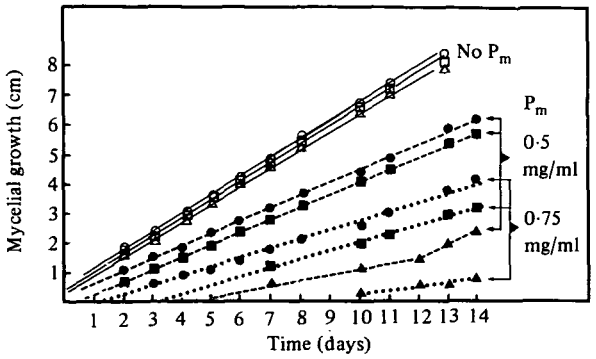

Fig. 3. Growth curves of *Ks1-1* (△, ▲), *Ks2-1* (□, ■) and the wild-type (○, ●) on synthetic medium with and without paromomycin (filled and open symbols respectively).

50 μg/ml in the wild-type strain. Moreover sporulation occurred on medium containing ksg at 60 μg/ml in crosses heterozygous for *Ks2-1*, whereas it was inhibited in crosses heterozygous for *Ks1-1*. Thus *Ks2-1* and *Ks1-1* were respectively dominant and recessive for this character. These data show that *Ks1-1* and *Ks2-1* confer increased resistance to ksg at the levels of both growth and sporulation. Do they alter sensitivity to other ribosome-targeted antibiotics? The two mutants were found to have the same susceptibility to cycloheximide as the wild-type. However, mutant *Ks1-1* was more sensitive to paromomycin than the wild-type whereas mutant *Ks2-1* displayed more or less the same sensitivity as wild-type (see Fig. 3). Since the *Ksg*A mutation increases misreading in *E. coli* (Van Buul *et al.* 1984), an alteration of the translational accuracy could be expected in *Ks1-1* and *Ks2-1* mutants. The fact that *Ks1-1* led to paromomycin hypersensitivity as did the suppressors (see Table 1) could be considered as a further argument. Therefore, we examined the ability of *Ks1-1* and *Ks2-1* to suppress the *193* nonsense mutation, commonly used in our suppression tests (see Picard-Bennoun *et al.* 1983). *Ks1-1* and *Ks2-1* did not lead to suppressor activity. Moreover retention of the same suppressor activity in *193 su4-1 Ks1-1* and *193 su4-1 Ks2-1* as in *193 su4-1 Ks*$^+$ indicated that they neither act as antisuppressors nor as allosuppressors.

Discussion

The results presented in this paper show that the antibiotic ksg inhibits cytoplasmic protein synthesis in *Podospora anserina*. This effect may account for the reduction of growth rate. High concentrations of ksg (1–3 mg/ml) are required for the inhibition of growth and cytoplasmic protein synthesis (which even then is not completely inhibited). Much lower concentrations (about 50 μg/ml) were found to inhibit the sporulation process. No other inhibitor of protein synthesis has been found to cause a sporulation deficiency. In fact, spores develop on media containing cycloheximide or emetin which act on the elongation step of translation, as well as on media containing aurintricarboxylic acid, an inhibitor of initiation. That comparison suggests that the effect of ksg on sporulation is probably not mediated through an inhibition of cytoplasmic protein synthesis. The presumed effect of ksg in increasing translational accuracy may account for the inhibition of sporulation in *Podospora*. We can compare the sporulation deficiency caused by ksg to the sporulation deficiency caused by the ribosomal mutations, *AS6* and *AS7*. Characterization of those mutations clearly indicated that an increase of translational accuracy blocked sporulation of the fungus whereas vegetative growth was not impaired (Dequard-Chablat and Coppin-Raynal, 1984). Thus, it appears that an improved accuracy in translation can have destructive consequences (for a discussion of this notion see Picard-Bennoun, 1982, and Kurland 1987). In conclusion, we can assume that low concentrations of ksg could enhance the translational accuracy and specifically affect the sporulation process, similarly to the *AS6* and *AS7* mutations. Alternative but not exclusive hypotheses rely on the assumption that ksg might exert differential effects at different stages of development. In *E. coli* it was reported that ksg was more inhibitory to the overall biosynthesis of cytoplasmic proteins than to that of envelope proteins (Hirashima *et al.* 1973). These authors suggested the existence of two distinct biosynthetic systems which would differ in some parameters (ribosomes, factors and messengers). Similarly we can postulate that the translational apparatuses might be different during vegetative growth and sexual reproduction, as has been already proposed in *Podospora anserina* (Picard-Bennoun, 1983).

The experimental evidence does not allow us to discriminate between these hypotheses, since the mode of action of ksg is not fully known in eucaryotes. In order to obtain more information on its mechanism of action, two resistant mutants were selected and characterized. They define two new genetic loci, *Ks1* and *Ks2*. In *Pyricularia oryzae* three resistant mutants were found to carry mutations at three different loci (Taga *et al.* 1979). New resistance loci could probably be revealed by further selection of mutants in the two fungi. In *Podospora* the resistance is expressed at the levels of both growth and sporulation. Interestingly, the *Ks1-1* mutation was found to decrease the resistance of the strain to paromomycin. Paromomycin and ksg display antagonistic effects on ribosomal misreading at least in *E. coli*. In *Podospora* paromomycin hypersensitivity is a property manifested by most low accuracy mutants (Picard-Bennoun *et al.* 1983) which suggests that the *Ks1-1* mutant could be a ribosomal ambiguity mutant. The paromomycin phenotype displayed by the *Ks1-1* mutant is a further argument suggesting that ksg might increase the ribosomal accuracy in *Podospora* as in *E. coli*. Biochemical analysis of ribosomes from the *Ks1-1* mutant may allow us to confirm this hypothesis.

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