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Isolation of the two allelic incompatibility genes *s* and *S* of the fungus *Podospora anserina*

Béatrice Turcq, Muriel Denayrolles* and Joël Begueret

Laboratoire de Génétique, UA CNRS 542, Avenue des Facultés, F-33405 Talence, France

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Summary. The two allelic genes *s* and *S* are responsible for heterogenic incompatibility between wild type strains of the fungus *Podospora anserina*. The *s* gene has been cloned by SIB selection and expression in a strain containing a neutral allele of this locus. The *S* gene was isolated from a genomic library using the DNA of the *s* locus as a probe. The physical map of the DNA fragments carrying the two genes are highly dissimilar and restriction polymorphism exists at the *s* locus between *s* and *S* strains. Nevertheless, homology between the two alleles was revealed by cross-hybridization at the DNA and RNA levels.

Key words: Fungus – Heterogenic incompatibility – SIB selection

Introduction

Heterokaryon incompatibility, also referred to as heterogenic incompatibility (Esser 1971), is wide spread in fungi. It has been described in *Aspergillus nidulans* (Grindle 1963), *Neurospora crassa* (Garnjobst and Wilson 1956), *Endothia parasitica* (Anagnostakis 1977) and *Podospora anserina* (Rizet 1952). It results in a failure to form heterokaryotic strains via hyphal fusion between different strains. In *P. anserina* the fusion between hyphae of incompatible strains leads to death of the heterokaryotic cells and results in the formation of a "barrage" between the incompatible strains when they are grown on agar medium (Fig. 1).

Genetic determinism of incompatibility has been well studied in *N. crassa* (Garnjobst and Wilson 1956) and *P. anserina* (Bernet 1965). In all species, incompatibility was found to result from genetic differences between strains. Allelic genes responsible for incompatibility have been identified in *N. crassa* and *P. anserina*. In this last species (Bernet 1965) and in *E. parasitica* (Anagnostakis 1977) non allelic incompatibility genes have also been described. The most studied allelic incompatibility locus in *P. anserina* is the *s* locus for which three different alleles have been identified in different geographical strains of this fungus. Two of them, *s* and *S*, determine heterokaryon incompatibility and barrage. The third one, s^x, is a neutral allele. Strains with s^x genotype are compatible with strains containing *s* or *S* alleles. Incompatibility is triggered by cytoplasmic proteins encoded by the *s* and *S* genes (Beisson-Schecroun 1962).

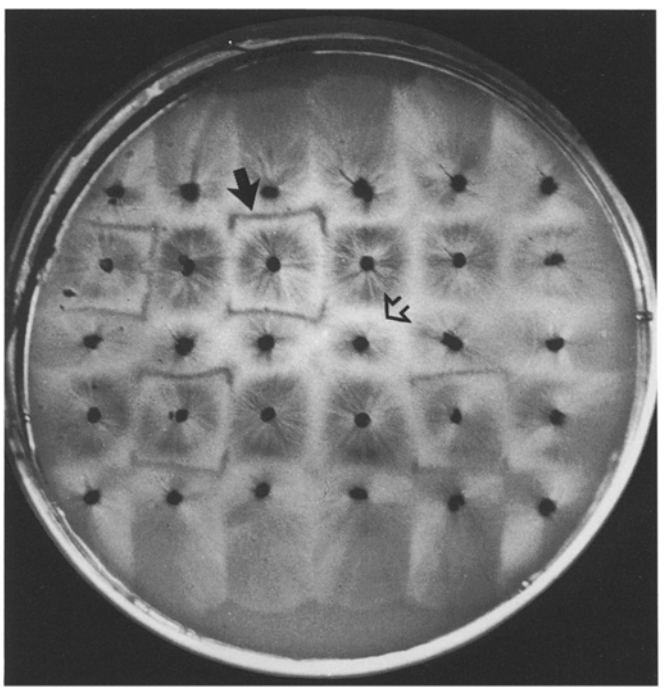

Fig. 1. Morphological aspect of incompatibility between strains of *P. anserina*. Strains were grown on solid medium. The *empty arrow* shows a normal contact between compatible strains and the *black arrow* a "barrage" between incompatible strains

Offprint requests to: M. Denayrolles

Other properties of the *s* and *S* genes have been reported besides incompatibility. In the offspring of $s \times S$ crosses, most of the strains with *s* genotype exhibit a new phenotype, named s^S, different of that of the *s* parent. Strains s^S are compatible with both *s* and *S* strains. The s^S phenotype is highly stable during the vegetative growth but it can revert to *s* phenotype by a protoplasmic contact with a *s* strain, i.e., by the presence of the *s* gene product. This was interpreted as a positive control of the expression of the *s* gene by its cytoplasmic product (Schecroun 1959). Strains with s^x can also be obtained through the formation of protoplasts from the mycelium of the *s* strain (Belcour 1975). Interaction between the two genes in $s \times S$ crosses can also lead to the abortion of spores with *S* genotype in the asci (Bernet 1965). So *s* and *S* genes exhibit some similarity with the distorter genes described in *Drosophila* (Sandler and Golic 1985).

The role of *S* and *s* cytoplasmic factors in incompatibility triggering, the control of expression of the *s* gene, and the distorter effect of the *s* gene on the *S* allele, could not be explored in the absence of molecular approaches. The availability of an efficient transformation system in *P. anserina* (Berges and Barreau 1989) has now allowed cloning of the *s* and *S* genes. The *s* gene was cloned by transformation of a s^x strain, with a cosmid library of genomic DNA of the *s* strain, and SIB selection. The *S* gene was cloned by probing a genomic library of the *S* strain with DNA fragments from the *s* locus. Comparison of the cloned fragments carrying the *s* and *S* genes, and probing of the genomic DNA of the two strains, revealed the presence of a high restriction polymorphism at this locus. However cross-hybridization at the DNA and RNA levels suggests that *s* and *S* genes are highly homologous.

Materials and methods

Strains. Podospora anserina is an Ascomycete whose life cycle and general methods for genetic analysis have been well described (Esser 1974). Strains *s* and *S* are geographical strains isolated and characterized by Rizet (1952). They were isogenized for 20 generations and only differ from each other for the allele present at the *s* locus. The s^x gene was identified by J. Bernet in the geographical strain *H* (unpublished results). Isolation and characterization of the *ura5-6* mutant deficient for OMPppase have been described previously (Razanamparany and Begueret 1986). The bacterial strain used for plasmid amplification was *Escherichia coli* BJ5183 (*endA, sbcB, recBC, galK, met, StR, thi1, bioT, hsdR*). Transformation of *E. coli* was performed as described by Hanahan (1983).

DNA and RNA analysis. The construction and characterization of the genomic DNA library of the *s* strain in pHC79-ura5 will be described elsewhere. The genomic library of the *S* strain in cosmid pHSU8 was constructed by R. Debuchy (Picard et al. 1987). Genomic DNA was isolated from *P. anserina* strains as described previously (Razanamparany and Begueret 1986). Restriction and DNA modification enzymes were used as specified by the manufacturers. PolyA$^+$ RNAs were isolated and analyzed as described previously (Turcq and Begueret 1987). General molecular methods were all as described (Maniatis et al. 1982).

Filter hybridization. DNA was digested with restriction enzymes, resolved on 0.8% agarose gels and transfered to nitrocellulose filters by the method of Southern (1975). Bacterial clones containing the recombinant cosmids were grown on solid LB medium containing ampicillin. Colonies were transfered to nitrocellulose filters and treated as described by Maniatis et al. (1982). Filters were prehybridized at 65°C for 3 h in 3×SSC [1×SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH7], 0,1× Denhardt solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA). The filters were hybridized with probes at 42°C for 16 h in a mixture containing 50% deionized formamide, 4×SSC, 50 mM Tris hydrochloride (pH 7.5), 100 µg of salmon sperm DNA per ml. After hybridization, filters were washed at 65°C for 2 h in 2×SSC, 0.1% sodium dodecyl sulfate. The probes were prepared by the random hexamer priming method of Feinberg and Vogelstein (1983). Probes with specific activities of about 6×10^8 cpm/µg were used at a concentration of 10^6 cpm/ml.

Transformation of P. anserina protoplasts. Protoplasts were prepared from the *ura5-6* mutant as described previously (Razanamparany and Begueret 1986). Protoplasts were incubated for 5 min at 48°C, then cosmid or plasmid DNA (10 µg per 10^8 protoplasts) was added. This procedure (Berges and Barreau 1989) gives about 200–500 transformants per µg of DNA for plasmids and about 20–40 transformants per µg of DNA for cosmids.

Results

Direct cloning of the incompatibility gene s

The existence of the neutral allele s^x at the incompatibility *s* locus makes possible the cloning of the *s* or *S* gene by transformation of a s^x strain by cosmid genomic libraries from *s* or *S* strains. Strains with the s^x genotype are compatible with *s* or *S* strains. The expression in a transformant of, for instance, the *s* gene would confer the *s* phenotype to the transformant, i.e., it would become incompatible with the *S* strain. Direct gene cloning by transformation and expression was possible in *P. anserina* due to the availability of an efficient transformation procedure (Berges and Barreau 1989). Cloning of the *s* gene was achieved by transformation and SIB selection (Akins and Lambowitz 1985) using a s^x strain containing the *ura5-6* auxotrophic mutation as recipient. This strain was transformed with a library of genomic DNA from the *s* strain made in the pHC79 cosmid in which the *ura5* gene was inserted as a selectable marker. Prototrophic transformants were recovered and used in a barrage test with the *S* strain. However, it was previously shown that the expression of the *s* phenotype requires the presence of the cytoplasmic factor *s* (Beisson-Schecroun 1962). As this factor is not present in the recipient strain, the prototrophic transformants were first confronted with the *s* strain to induce the expression of the *s* transforming gene, they were then tested with the *S* strain.

The s^x *ura5-6* strain was transformed with eight pools of cosmids, each containing about 250 recombinant cosmids of the genomic library of the *s* strain. About 4,500 prototrophic transformants were obtained and tested. Only six transformants from pool 6 showed the *s* phenotype (Fig. 2). Bacteria from this pool were distributed in 14 subpools, each containing 19 clones. Subpool 4 gave nine transformants exhibiting the *s* phenotype out of 402 prototrophic strains. Finally individual cosmids from subpool 4 were used to transform the s^x *ura5-6* strain.

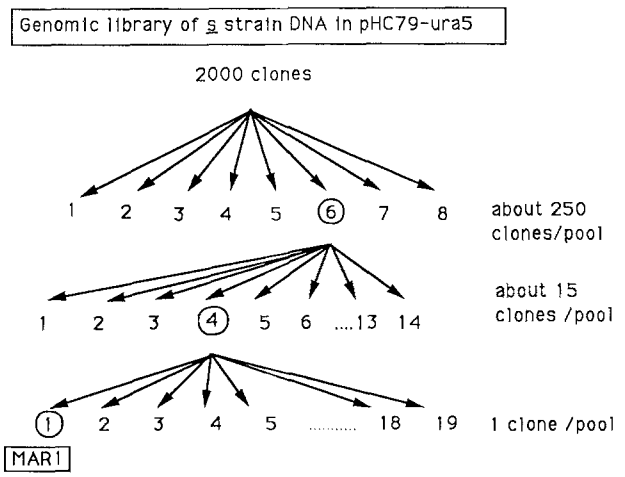

Fig. 2. Protocol for SIB selection of the cosmid MAR1 containing the *s* gene. See text for details

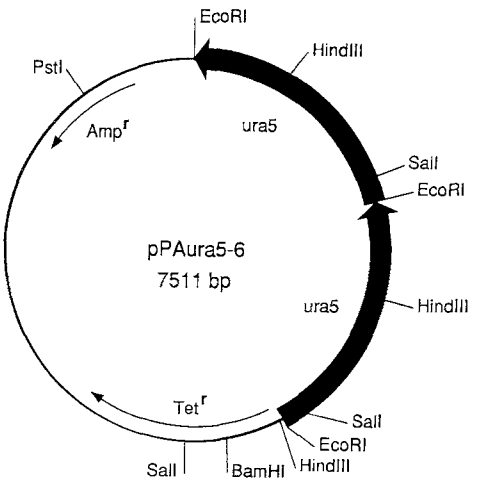

Fig. 3. Physical map of the plasmid pPAura5-6. The *thin line* represents pBR322 sequences and the *black thick arrows* the two copies of the *ura5* gene

Table 1. Effect of the digestion of the cosmid MAR1 and of the plasmid pMART8 by various restriction enzymes on their ability to confer the *s* phenotype to the s^x strain by transformation

Transforming vector	Enzymes used	Number of prototrophic transformants	Number of *s* transformants[a]	Percentage of *s* transformants
cosmid MAR1	*Bam*HI	84	7	8%
	*Pst*I	129	21	16%
	*Sph*I	127	25	20%
pMART8	*Bam*HI	156	4	3%
	*Bgl*II	117	47	40%
	*Eco*RI	117	2	2%
	*Hind*III	117	23	20%
	*Kpn*I	117	50	43%
	*Pvu*I	122	9	7%
	*Pvu*II	262	56	21%
	*Sal*I	117	0	0%
	*Xho*I	117	2	2%

[a] The prototrophic transformants were tested with the S strain to determine those which have gained the s phenotype

Only one cosmid gave transformants with an *s* phenotype. This cosmid, named MAR1, contained an insert of about 40 kb.

Transformants which exhibit the *s* phenotype were analyzed to localize the site of integration of the transforming cosmid. Five transformants were crossed with the s^x *ura5-6* strain and the segregation of *ura5* and *s* alleles was examined in the offspring. In these five transformants the cosmid was found to be integrated at the *s* locus. As it has been shown that cosmids preferentially integrate by homologous recombination (Picard et al. 1987), this result provided evidence that the genomic region cloned on the cosmid MAR1 is the *s* locus.

The *s* gene was subcloned from the MAR1 cosmid. This cosmid was digested to completion with either *Bam*HI, *Pst*I or *Sph*I. Each restriction mixture was used directly to transform protoplasts of the s^x *ura5-6* strain in the presence of the reporter vector pPAura5-6, containing two copies of the *ura5* gene (Fig. 3). The prototrophic transformants were tested as described above to estimate the number of transformants which can exhibit the *s* phenotype. The results are shown in Table 1. These results indicate that the entire *s* gene would be present on a *Pst*I or a *Sph*I fragment but that this gene should be inactivated by *Bam*HI. The different *Pst*I restriction fragments from cosmid MAR1 were cloned at the *Pst*I site of the plasmid pPAura5-6 and the individual recombinant plasmids were used to transform the s^x *ura5-6* strain. A recombinant plasmid which contains a 5.8 kb insert was found to transform the recipient strain to protophic transformants which exhibit the *s* phenotype. The physical map of the insert of this plasmid, termed pMART8, is shown in Fig. 4.

The position of the *s* gene on pMART8 was determined by digestion of the plasmid with various restriction endonucleases and transformation of the s^x *ura5-6* strain.

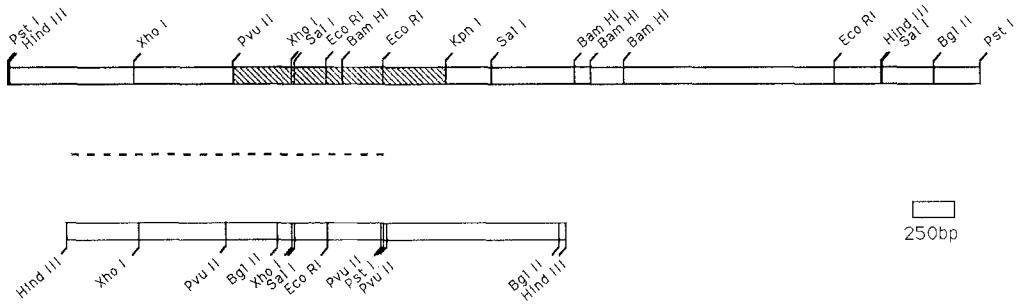

Fig. 4. Compared restriction maps of the DNA fragments carrying *s* (top) and *S* (bottom) genes. The *striped box* shows the localization of the *s* gene and the *dashed line* the regions of homology between the two fragments

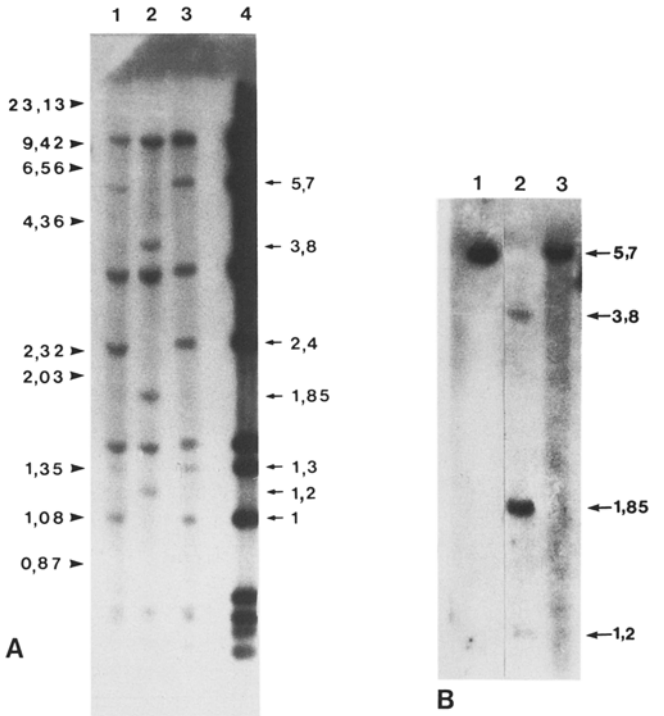

Fig. 5A, B. Genomic Southern analysis of the *s* locus. Genomic DNA from strains *s* (*lanes 1*), *S* (*lanes 2*), s^x (*lanes 3*) and from the cosmid MAR1 *lane 4* were digested with *Pst*I and probed with the nick-translated MAR1 DNA (*panel A*) or with the 5.8 kb fragment from pMART8 (*panel B*). The position of the length markers (kb) are shown on the left and the sizes of hybridizing fragments on the right

Transformation was performed in the presence of an equal amount of pPAura5-6 and the prototrophic transformants were tested for the ability to exhibit the *s* phenotype. As shown in Table 1, the restriction of pMART8 with some enzymes leads to a low frequency of prototrophic transformants which gain the *s* phenotype. We assumed that such enzymes should have restriction site(s) within the sequence of the *s* gene. These results allowed us to localize the *s* gene on a 1.4 kb *Pvu*II-*Kpn*I fragment (Fig. 4). This fragment was subcloned and we have shown that it confers the *s* phenotype to the s^x strain by transformation.

Cloning the S allele

The isolation of the *s* gene provides a probe to clone the *S* gene from a genomic DNA library of the *S* strain. Before probing this library, we analyzed the organisation of the *s* locus in different strains. This was achieved by probing genomic DNA digested by *Pst*I, with the MAR1 cosmid and the 5.8 kb *Pst*I fragment from the pMART8. As shown in Fig. 5, the nick-translated MAR1 hybridizes to *Pst*I fragments of *s* strain DNA which have the same size as the *Pst*I fragments of the cosmid. The same hybridization pattern was obtained with DNA of the s^x strain. In contrast, some *Pst*I fragments differ in the DNA of the *S* strain. Three *Pst*I fragments (5.8, 2.4 and 1.3 kb) present in the DNA of the *s* strain are missing in the DNA of the *S* strain. In this last strain, specific *Pst*I fragments (3.8, 1.85 and 1.2 kb) were revealed by this hybridization. The restriction fragment length polymorphism between *s* and *S* strains may involve the region carrying the *s* gene which was cloned as a 5.8 kb *Pst*I fragment in pMART8. This was confirmed by probing genomic DNA of *s*, *S* and s^x strains with this 5.8 kb fragment (Fig. 5). It hybridizes with a unique *Pst*I fragment which has the same size in the genomic DNA of *s* and s^x strains. For the DNA of the *S* strain three *Pst*I fragments (3.8, 1.85 and 1.2 kb) are revealed by this probe. The RFLP at the *s* locus reveals a sequence divergence between the *s* and *S* genes.

The *S* gene was cloned from a genomic DNA cosmid library of the *S* strain. This library was probed with the *Pst*I fragments from the cosmid MAR1 which were found to be common to *s* and *S* strains (deduced from the Southern analysis). Seven bacterial clones giving positive hybridization signals were identified. Each cosmid contained in these seven clones was used to transform the s^x strain. Only one of them, named G, confers the *S* phenotype to the s^x strain, i.e., an s^x strain transformed by cosmid G becomes incompatible with the *s* strain. Homology between genomic DNA sequences cloned in the cosmids G and MART1 was examined. They were digested with *Sph*I, the fragments were separated in agarose gel and probed with the nicked-translated G cosmid. Four fragments are identical in the two cosmids (data not shown) indicating that homologous regions of the genomic DNA of *s* and *S* strains have been cloned.

Table 2. Effect of the digestion of the cosmid G and of the plasmid pBTMD by various restriction enzymes on their ability to confer the *S* phenotype to the s^x strain by transformation

Transforming vector	Enzymes used	Number of prototrophic transformants	Number of *S* transformants[a]	Percentage of *S* transformants
cosmid G	*Hind*III	65	13	20%
	*Pst*I	65	3	5%
	*Sph*I	67	13	19%
pBTMD	*Bam*HI	60	33	55%
	*Bgl*II	62	0	0%
	*Cla*I	54	0	0%
	*Hind*III	101	60	60%
	*Pvu*I	83	0	0%
	*Pvu*II	98	1	1%
	*Sal*I	47	1	2%
	*Xho*I	86	0	0%

[a] The prototrophic transformants were tested with the s strain to determine those which have gained the S phenotype

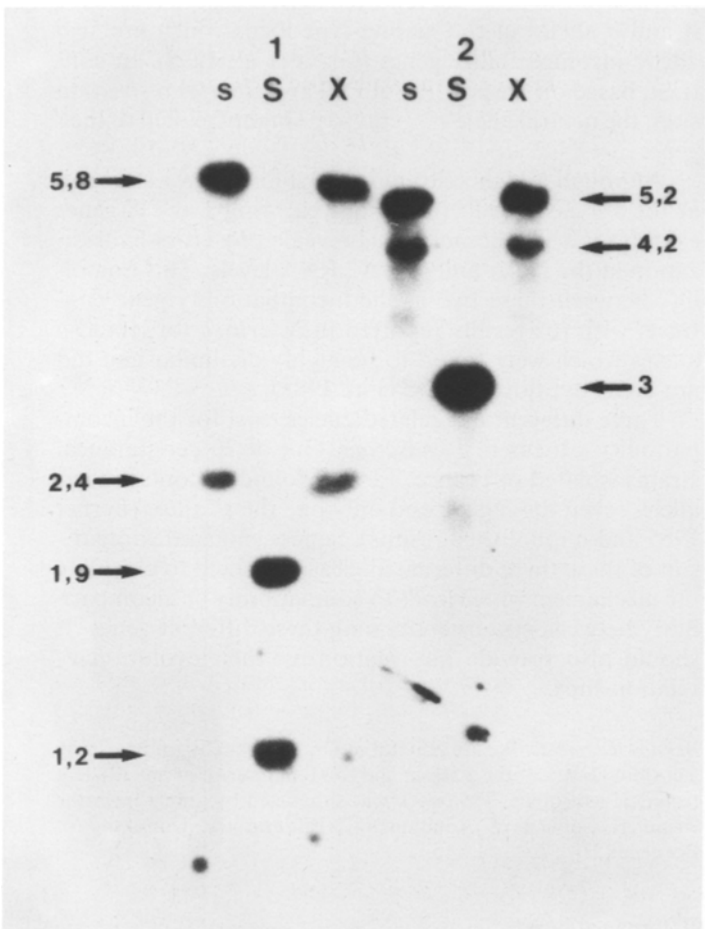

Fig. 6. Southern analysis of the genomic DNA of strains *s*, *S* and s^x. The DNAs were digested with *Pst*I (*panel 1*) or *Hind*III (*panel 2*) and probed with the 2.9 kb fragment of the pBTMD plasmid. The sizes of the hybridizing fragments are given in kb

Subcloning of the *S* gene from the cosmid G was performed as described for the *s* gene. The cosmid was digested by different restriction enzymes, the fragments were mixed with the pPAura5-6 plasmid and used to transform the recipient s^x *ura5-6* strain. The frequency of prototrophic transformants which exhibit the *S* phenotype is given in Table 2. From these results it could be inferred that the *S* gene should not contain any site for *Hind*III or *Sph*I. The *Hind*III fragments of the G cosmid were then cloned in pBR322 and the different recombinant plasmids were isolated. Each was used to cotransform the s^x *ura5-6* strain in the presence of pPAura5-6. This allowed us to isolate a recombinant pBR322, named pBTMD, which contains a 2.9 kb *Hind*III insert able to confer the *S* phenotype to the s^x strain.

The restriction map of this insert is given in Fig. 4. This physical map is quite different from that of the 5.8 kb *Pst*I fragment carrying the *s* gene. The precise localization of the *S* gene could not be determined by restriction cutting of this fragment as was possible for the *s* gene. In this case the only restriction enzyme which has at least one site in this fragment, and which does not inactivate the *S* gene, is *Hind*III (Table 2). However the *S* gene may overlap the central region of this DNA fragment where unique sites for *Pst*I, *Eco*RI and *Sal*I are present which inactivate the *S* gene. Localization of the *S* gene can now be determined by producing deletions from the two edges of the 2.9 kb fragment to identify the limits of the region which is necessary to confer the *S* phenotype to the s^x strain after transformation.

The 2.9 kb *Hind*III fragment carrying the *S* gene was used to probe the genomic DNA of *s*, *S* and s^x strains. As shown in Fig. 6, this probe detects restriction polymorphism for *Pst*I and *Hind*III sites between the DNA of *s* and *S* strains but the hybridization patterns are identical for the DNA of *s* and s^x strains. Furthermore, from this result and those reported in Fig. 5, it appears that there is cross-hybridization between the restriction fragments carrying the *s* and *S* genes. For instance, the 2.9 kb *Hind*III fragment carrying the *S* gene hybridizes even under high

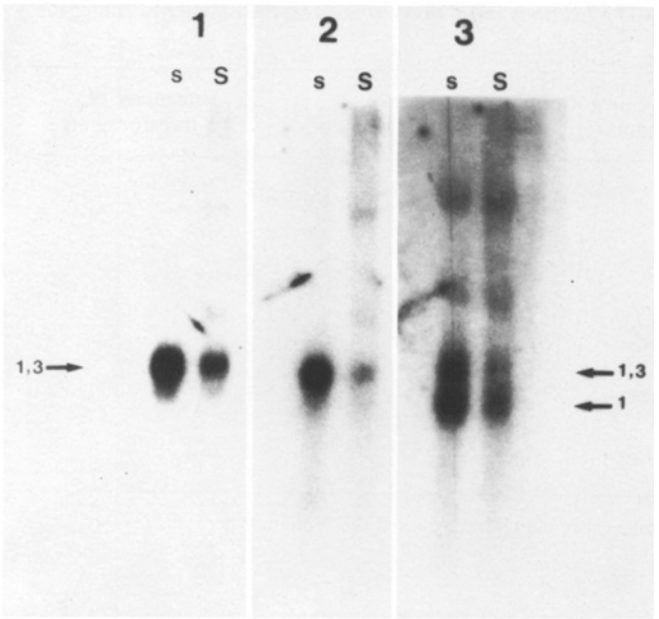

Fig. 7. Northern analysis of the polyA$^+$ RNA from *s* and *S* strains. PolyA$^+$ RNAs were purified and 20 µg fractionated in an agarose formaldehyde denaturing gel. After transfer onto nitrocellulose filters they were probed with the 1.4 kb *Pvu*II-*Kpn*I fragment carrying the *s* gene (*panel 1*). The filter was boiled in water and hybridized with the 2.9 kb *Hind*III fragment carrying the *S* gene (*panel 2*). In *panel 3* the same filter was again boiled in water and hybridized with a mixture of two probes containing the *s* gene and the *ura5* gene. This control shows that the weaker hybridization signals observed for *S* RNA were due to a weaker amount of polyA$^+$ RNA in *S* sample. The sizes of hybridizing RNA are given in kb. The 1 kb RNA is the RNA transcribed from the *ura5* gene (Turcq and Begueret 1984)

stringency (0.1 × SSC, 25°C) to the 5.8 kb *Pst*I fragment carrying the *s* gene.

To estimate the extent of homology between the two regions carrying the *s* and *S* genes, the cloned 5.8 kb *Pst*I and 2.9 kb *Hind*III fragments were digested with different restriction enzymes and then cross-hybridized. This analysis revealed that the two fragments carrying *s* and *S* genes contain a region of high homology which spans about 1.9 kbp and overlaps the position of the *s* gene on the 5.8 kb *Pst*I fragment (Fig. 4). Sequence homology between *s* and *S* genes was also examined at the RNA level. PolyA$^+$ RNA was purified from the *s* and *S* strains, fractionated in denaturing agarose gels and probed with the 1.4 kb *Pvu*II-*Kpn*I fragment carrying the *s* gene. As shown in Fig. 7 this probe hybridizes to a single RNA of 1.3 kb which is present in both strains. An identical hybridization pattern was observed when the same filter was probed with the 2.9 kb *Hind*III fragment carrying the *S* gene. Sequencing of *s* and *S* genes is now under progress to determine the exact level of homology between these two allelic genes.

Discussion

Incompatibility genes which prevent heterokaryosis have been identified in a wide range of fungal species. They have been studied using classical genetic methods but their physiological function remains unknown. The characterization of the function of such genes would be important at the evolutionary level because they limit outbreeding and thus would favour speciation. In the ascomycete *Podospora anserina* strains carrying mutations in genes involved in incompatibility can be selected and studied at the physiological level (Boucherie and Bernet 1974). From these studies, it has been proposed that some incompatibility genes are involved in morphogenetic pathways.

We were able to clone the incompatibility *s* gene from a cosmid library directly by SIB selection and expression in a strain containing a neutral allele. The *s* gene is involved in an allelic incompatibility system, being incompatible with the *S* allele. This latter was isolated from a genomic library from the *S* strain, by probing this library with DNA fragments of the *s* cloned locus.

The physical maps of the restriction fragments carrying the *s* and *S* genes are highly dissimilar and restriction polymorphism at the *s* locus was observed between the genomic DNA of strains containing *s* or *S* genes. Such a polymorphism has been reported in *Neurospora crassa* for *A* and *a* alleles of the mating-type locus which are also allelic incompatibility genes (Glass et al. 1988). In contrast, based on the position of *Pst*I and *Hind*III restriction sites, the neutral allele s^x seems to be highly related to the *s* gene.

Although a high restriction polymorphism was found at the *s* locus, the DNA regions carrying *s* and *S* genes exhibit sequence homology as revealed by cross-hybridization at the DNA and polyA$^+$ RNA levels. This homology between these two allelic incompatibility gene contrasts with the results reported in *N. crassa* for *A* and *a* alleles which were found to be highly dissimilar and did not cross-hybridize (Glass et al. 1988).

Three different but related alleles exist for the incompatibility *s* locus in *P. anserina*. Out of 20 geographical strains isolated in France, 12 were found to contain the *s* allele, seven the *S* gene and only one the s^x allele (Bernet 1965 and unpublished results). Sequencing and comparison of these three different alleles could help to elucidate the mechanism which leads to compatibility or incompatibility between strains containing these different genes. It should also provide information on their evolutionary relationships.

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