Current Genetics (1985) 9:361–368

Current Genetics

Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amdS* gene

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Summary. Conidial protoplasts of an *A. nidulans amdS* deletion strain (MH1277) have been transformed to the AmdS$^+$ phenotype with a plasmid carrying the wild type gene (p3SR2). Optimalisation of transformation and plating conditions now has resulted in frequencies of 300–400 transformants per μg of DNA.

Analysis of DNA from AmdS$^+$ transformants of MH1277 showed that transformation had occurred by integration of vector DNA sequences into the genome. In virtually all these transformants multiple copies of the vector were present in a tandemly repeated fashion, not preferentially at the resident, partially deleted *amdS* gene. It is suggested that the observed integration phenomena are dependent on the genetic background of the *A. nidulans* strain, used for transformation. A model to explain the tandem type of integration is proposed.

Key words: Transformation of Aspergillus – Conidial protoplasts – Multicopy/tandem integration – Gene amplification

Introduction

Fungi play an important role in chemical industry. They are used in fermentation processes as well as for the production of antibiotics and different metabolites. Several of the biotechnologically important species belong to the genus *Aspergillus*. Well known examples are *A. niger* and *A. oryzae* which are used for the production of organic acids and enzymes (Berry et al. 1977; Jakubowska 1977; Barbesgaard 1977). The development of methods for in vitro genetic manipulations might be exploited for a more direct and efficient improvement of Aspergilli. For the cloning and expression of eukaryotic genes in *Aspergillus* a suitable DNA vector system has to be developed. In this context we have studied the aspects of transformation, using the genetically well marked, but industrially unimportant species *A. nidulans* as a model system.

For this organism effective methods have been developed in our laboratory for the production of large quantities of protoplasts from both hyphae and conidiospores (van den Broek et al. 1979; Bos and Slakhorst 1981). Especially the conidial protoplasts were thought to be a very useful starting material for transformation experiments.

A. nidulans is insensitive to most antibiotics. Even the aminoglycoside G418 and hygromycin B, which is applicable to yeast and higher eukaryotes (Jimenez and Davies 1980; Colbère-Garapin et al. 1981; Gritz and Davies 1983) does not sufficiently inhibit growth of *A. nidulans*. We therefore were not able to use resistance genes of bacterial origin for the selection of transformants. Instead we used the *A. nidulans amdS* gene as a selection marker in our transformation studies. The *amdS* gene has been studied by Hynes and coworkers (Hynes and Pateman, 1970; Hynes 1982) and recently the gene has been isolated, cloned and characterised (Hynes et al. 1983). The successful use of this gene as a selection marker in the transformation of mycelial protoplasts, was recently reported by Tilburn et al. (1983).

In this paper we describe the transformation of conidial protoplasts of *amdS* deletion strains using various plasmids with the cloned wild type *amdS* gene. A model explaining the results is presented.

Abbrevations: bp, base pairs; kb, 1,000 bp; EtBr, ethidiumbromide; PEG, polyethyleneglycol; r-DNA, ribosomal DNA; c.f.u., colony forming units

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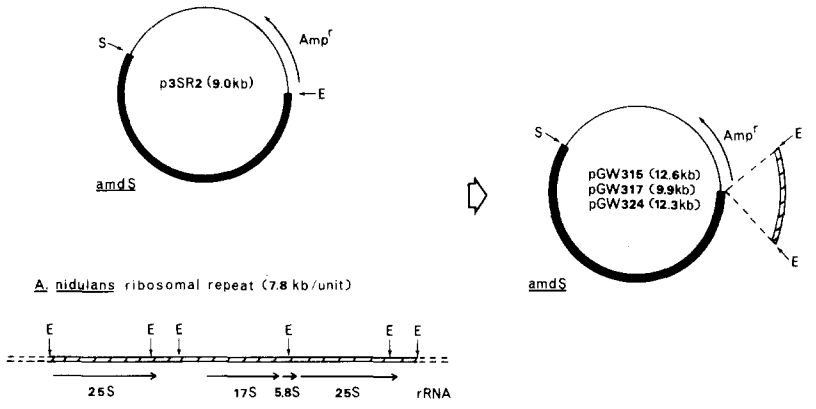

Fig. 1. Schematic drawing of the plasmids p3SR2, pGW315, pGW317, pGW324 and the *A. nidulans* ribosomal repeat unit. Plasmid p3SR2 is a pBR322 derivative (Bolivar et al. 1977) in which the smaller *Eco*RI-*Sal*I fragment has been replaced by a 5.2 kb *A. nidulans* DNA fragment, containing the complete wild type *amdS* gene (Hynes et al. 1983). Plasmids pGW315, pGW317 and pGW324 were constructed by inserting three *Eco*RI fragments (3.6, 0.9 and 3.3 kb respectively), constituting the *A. nidulans* ribosomal repeat unit, into the *Eco*RI restriction site of p3SR2. These r-DNA fragments were derived from plasmid pMN1 (Borsuk et al. 1982). *E* = *Eco*RI restriction site, *S* = *Sal*I restriction site

Materials and methods

Chemicals and enzymes. Acetamide and L-proline were purchased from Sigma, acrylamide (gel electrophoresis quality) from Serva. Polyethyleneglycol (PEG) was from Merck, KochLight and BDH. For selective medium Oxoid purified agar was washed with cold, distilled water followed by washing in 96% ethanol and subsequent drying at 65 °C before use. [α-^{32}P]dATP was from New England Nuclear. Blots were made on Schleicher & Schüll BA85 nitrocellulose. Restriction endonucleases were purchased from Boehringer Mannheim, T4-DNA ligase and DNA polymerase from New England Biolabs. All other chemicals used were of analytical grade.

Strains. As recipients for transformation the *A. nidulans* strains MH1277 (*biA*1, *amdS*320, *amdI*18, *amdA*7, *niiA*4; Hynes et al. 1983), WG290 (a *yA*2, *pantoB*100-derivative of MH1277; Tilburn et al. 1983) and MH1354 (*biA*1, *amdS*368, *amdA*7, *niiA*4; Hynes et al. 1983) were used.

Plasmids were propagated in *E. coli* K12 strain JA221.

Media and growth conditions. For the preparation of conidiospores, the *A. nidulans* recipients were grown on agar-solidified complete medium (Pontecorvo et al. 1953) without nitrate, containing 3.7 g NH_4 Cl/l as nitrogen source, supplemented with 15 nM D(+)biotin and 8 μM pantothenic acid. Plates were inoculated with approximately 1×10^3 conidiospores and incubated for 3 days at 37 °C.

AmdS$^+$ transformants were selected on minimal medium (Pontecorve et al. 1953), in which nitrate was replaced by 10 mM acetamide as sole nitrogen source, 15 mM CsCl, 1.0 M sucrose, 15 nM D(+)biotin and 8 μM pantothenic acid, solidified with 1.2% agar. In non-selective medium the acetamide/CsCl was replaced by 10 mM L-proline. Transformants were propagated on the selective medium described above, in which 1.0 M sucrose was replaced by 50 mM glucose.

For the preparation of mycelium *A. nidulans* strains were grown in non-selective minimal medium containing 50 mM glucose. One liter flasks, containing 250 ml of medium were inoculated with 10^6 conidiospores/ml and incubated for 18 h in a New Brunswick G25 orbital shaker at 37 °C (300 rpm). Mycelium was harvested by filtration, washed with distilled water, blotted dry and frozen immediately in liquid nitrogen. It was either used directly for DNA isolation or stored at −80 °C until use.

E. coli was grown in L-broth medium supplemented with the appropriate antibiotics.

Plasmids. Plasmid p3SR2 is described in Fig. 1. The plasmids pGW315, pGW317 and pGW324 were constructed as explained in the legend of Fig. 1.

Plasmid DNA was extracted from *E. coli* cells using standard procedures (Birnboim and Doly 1979) and purified by two successive isopycnic centrifugations in CsCl/ethidiumbromide followed by Sepharose 6B column chromatography.

Preparation of protoplasts. For the preparation of conidial protoplasts the procedure described by Bos and Slakhorst (1981) was scaled up to convert 2×10^9 conidiospores into $1-1.5 \times 10^9$ viable protoplasts; the residual intact conidiospores (1–30%) were not removed. The conidial protoplast suspension was stored overnight in the lytic mixture at 4 °C.

Mycelial protoplasts were prepared according to van den Broek et al. (1979). Cellular debris was removed by centrifugation for 1 min at 1,000 x g. These protoplasts were used for transformation immediately after preparation.

Protoplasts were collected by centrifugation, washed twice with 1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5) and resuspended in the same buffer at a density of $0.5-1.0 \times 10^9$ protoplasts/ml.

Transformation of A. nidulans protoplasts. Protoplasts were transformed essentially as described by Tilburn et al. (1983) with minor modifications. A 100 μl aliquot of the protoplast suspension was mixed with an equal volume of the same buffer containing the vector DNA. Immediately thereafter the suspension was thoroughly mixed with 1 ml of a solution containing 60% (w/v) PEG 6000, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5). After 20 min incubation at room temperature the protoplasts were collected by centrifugation (5 min, 12,000 x g) and resuspended in 400 μl 1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris (pH 7.5). Aliquots of 100 μl were plated on selective medium using a 2 ml 0.25% agar overlay. The viability of the protoplasts was determined by plating appropriate dilutions on non-selective medium. Transformant colonies appeared after 24–36 h of incubation at 37 °C.

Isolation of A. nidulans DNA. DNA was isolated from 0.5–1.0 g liquid N_2 frozen mycelium as described by Yelton et al. (1984).

DNA manipulations. DNA was digested with restriction endonucleases according to the manufacturers instructions. Ligations were performed with T4-DNA ligase using standard procedures. DNA was fractionated on 0.6% agarose gels and transferred from the gels onto nitrocellulose according to Southern (1975).

Specific DNA fragments were isolated from gels using a glass powder-binding procedure (Vogelstein and Gillespie 1979).

Hybridisation probes were labelled by nick translation (Rigby et al. 1977) and hybridised with the blots at 68 °C for at least 24 h as described by Maniatis et al. (1982). After washing the blots were autoradiographed on Sakura X-ray film at −80 °C using Tungsten intensifying screens.

Results

Properties of AmdS$^+$ transformants

Transformants appearing on selective medium after transformation of MH1277 with p3SR2 show a wide variation in growth rate. Two classes of colonies can be distinguished:

- Type I, showing continuous growth and normal sporulation on acetamide,
- Type II, which are extremely small, never sporulate and do not show further development upon prolonged incubation.

In control experiments, in which no transforming DNA is added, colonies are never found. The Type II transformants cannot be considered to be abortive, as they can be rescued by conversion into Type I. This may be achieved by transferring Type II colonies to non-selective medium, followed by replating of conidiospores on selective medium. With at least 50% of the Type II colonies this results in the appearance of Type I, whereas Type II is no longer found. Although the Type I colonies, found after transformation, have a fairly homogeneous appearance (no sectors), initially a large variation is found in the fraction of conidiospores, possessing the AmdS$^+$ property; values ranging from 0.1% to virtually 100% are found. However, after this first subculturing step colonies are all homogeneous in the AmdS$^+$ property.

The individual Type I transformants differ greatly in growth rate on medium containing the non-inducing substrate acrylamide as sole nitrogen source and several (about 0.1%) of the AmdS$^+$ transformants have a colony morphology which differs from the parental type suggesting that transformation to the AmdS$^+$ phenotype is not an identical event in all individual transformants.

The stability of the AmdS$^+$ property through mitosis in two p3SR2-derived transformants of MH1277, ht6 and ht7, was investigated during subculturing for five successive growth cycles on nonselective medium. From each cycle none of the 400 colonies tested had lost the AmdS$^+$ property, suggesting that these p3SR2-derived transformants were mitotically stable. The stability of the same two transformants through meiosis was investigated by analysing selfed fruiting bodies. A relatively low viability of the ascospores was observed for both transformants and MH1277 (about 10% compared to a wild type strain). From both transformants 350 individual colonies, arisen from ascospores, were tested; all still contained the AmdS$^+$ property.

Transformation frequency

With 10 μg of p3SR2 in general 10–100 transformants of Type I and 10–500 of Type II were obtained, using conidial protoplasts. This frequency (1–50/μg) is comparable to that reported for *S. cerevisiae* or *N. crassa* using integrating vectors (Hinnen et al. 1978; Case et al. 1979). In the system used, the viability of the protoplasts after transformation is relatively high (40–50%). Both the transformation frequency and the ratio of Type I/Type II colonies varies between individual experiments. However, with aliquots of one batch of protoplasts always reproducible results have been obtained.

In contrast to the results for *S. cerevisiae* with linearised vectors (Orr-Weaver et al. 1981), linearisation of the vector p3SR2, either within or outside the *amdS* coding sequence, does not alter the transformation frequency.

Since the possibilities for direct cloning and selection of genes in *Aspergillus* depends on a high transformation frequency, many parameters which might affect this frequency (e.g. carrier DNA, incubation period, incubation temperature, PEG molecular weight, PEG concentration, addition of cations, purity of transforming DNA) have been studied. Only the purity of the vector DNA was a factor of great importance for efficient transformation. Best results were obtained with DNA, extensively purified by two cycles of CsCl/ethidiumbromide centrifugation, followed by Sepharose 6B column chromatography.

A non-linear relationship was observed between the number of transformants and the amount of p3SR2 used for transformation. A minimum of 2 μg of vector DNA was needed to obtain any transformant at all. The number of transformants increased almost exponentially with an increasing amount of p3SR2 DNA (up to 10 μg) and at higher concentrations this relation became linear. With more than 50 μg the frequency dropped, possibly caused by impurities in the DNA preparations (results not shown).

For our transformation system recently a correlation was found between transformation frequency and density of plating. In the standard procedure 10^8 protoplasts are transformed and plated at a density of 2.5×10^7 c.f.u./plate, normally resulting in a frequency of 50–70 transformants/μg of DNA. When 10^7 protoplasts are used for transformation and plated at 2.5×10^6 c.f.u./plate, the frequency drops about one order of magnitude. If, however, 2.5×10^6 incubated protoplasts are plated in the presence of 2.5×10^7 untreated conidiospores from the *amdS* deletion strain, the transformation frequency is at least 5 times higher than under the stan-

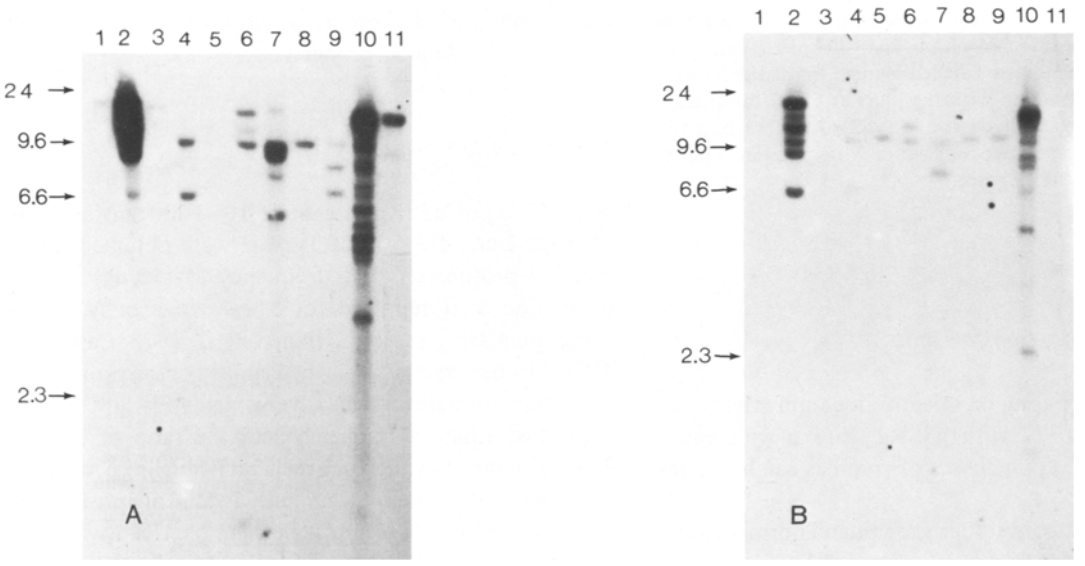

Fig. 2A, B. Analysis of DNA from AmdS$^+$ transformants obtained with conidial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with *Eco*RI, fractionated on an agarose gel and transferred to nitrocellulose as described in Materials and methods. Blots were probed with ^{32}P-labelled *amdS*-fragment (i.e. the large *Eco*RI-*Sal*I fragment of p3SR2) (panel **A**) or ^{32}P-labelled plasmid pBR322 (panel **B**). *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4*, DNA from ct1–3 and *lanes 6–10*, DNA from ct4–8. *Lane 5* contains linearised p3SR2. *Arrows* indicate the position of molecular weight markers (kb)

dard condition. Under these optimised conditions frequencies of 300–400 transformants/μg of DNA can routinely be obtained.

Vector modifications

For *S. cerevisiae* it has been reported that an increased homology between the integrating vector and the chromosome results in an increase in transformation frequency (Szostak and Wu 1979). To increase homology of the *amdS* containing vector and the *A. nidulans* genome the three *Eco*RI fragments, together constituting the ribosomal repeat unit of *A. nidulans,* were cloned in p3SR2 (Fig. 1). The resulting plasmids were then used to transform *A. nidulans.* Irrespective the r-DNA fragment inserted, the transformation frequency was similar to that obtained with p3SR2. Also for these modified vectors, linearisation did not influence the frequency of transformation.

Biochemical analysis of AmdS$^+$ transformants

To investigate the state and location of the vector sequences used for transformation, DNA of a number of AmdS$^+$ transformants, obtained with p3SR2, has been analysed by Southern blotting and hybridisation with specific ^{32}P-labelled probes.

When Southern blots of undigested DNA from different transformants were probed with ^{32}P-labelled p3SR2, strong hybridisation signals were found in the region of the chromosomal DNA (results not shown). Even under conditions where 1 copy/10 *A. nidulans* nuclei could be detected, no indications for the presence of free plasmid DNA sequences were obtained from these experiments. This indicates that transformation probably has occurred by integration of the vector DNA into the *A. nidulans* genome.

To analyse the integrated vector sequences, DNA of eight AmdS$^+$ transformants (ct1–ct8) obtained with conidial MH1277 protoplasts and p3SR2, was digested with *Eco*RI prior to gel electrophoresis and blotting. The autoradiographs after hybridisation with the ^{32}P-labelled *amdS* fragment (i.e. the large *Eco*RI-*Sal*I fragment of p3SR2; Fig. 1) or pBR322 are shown in Fig. 2. The hybridisation patterns observed are, in general, very complex, with up to 21 distinct bands (ct8) hybridising to pBR322, the *amdS* fragment or both. Furthermore each individual transformant shows a different pattern. This may indicate multiple insertions of vector DNA into the genome. From this experiment it could not be established whether or not integration had occurred at the site of the resident, partially deleted, *amdS* gene since the *Eco*RI fragments from the wild type and the deletion strain, hybridising to the *amdS* probe, migrate very similarly on the gel (Fig. 3A, lanes 1 and 11).

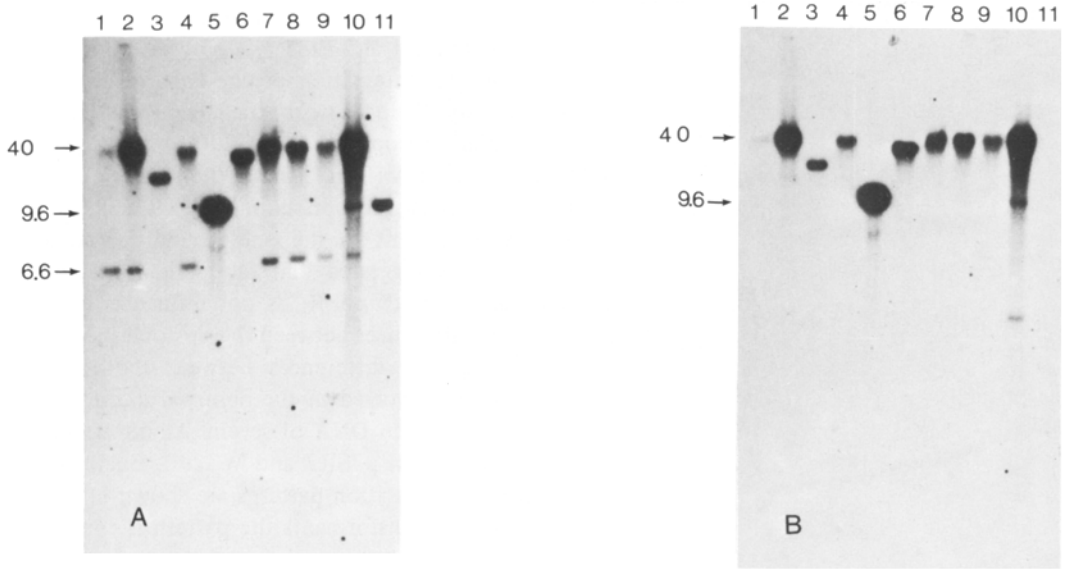

Fig. 3A, B. Analysis of DNA from AmdS$^+$ transformants obtained with conidial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with *Xho*1, fractionated by agarose gel electrophoresis and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment (panel **A**) or ^{32}P-labelled plasmid pBR322 (panel **B**). *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4,* DNA from ct1–3 and *lanes 6–10,* DNA from ct4–8. *Lane 5* contains linearised p3SR2. *Arrows* indicate the position of molecular weight markers (kb)

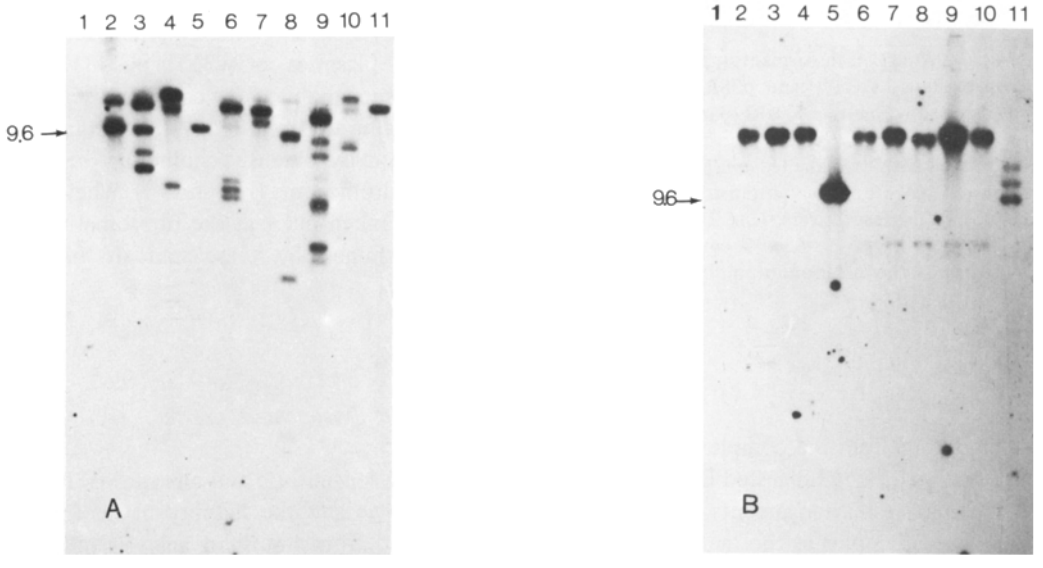

Fig. 4A, B. Analysis of DNA from AmdS$^+$ transformants obtained with mycelial protoplasts of MH1277 and plasmid p3SR2. *A. nidulans* DNA (2–5 μg) was digested with either *Eco*RI (panel **A**) or *Xho*1 (panel **B**), fractionated on an agarose gel and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment. *Lanes 1* and *11* contain DNA from the untransformed MH1277 strain and a wild type strain respectively; *lanes 2–4,* DNA from ht1–3 and *lanes 6–10,* DNA from ht4–8. *Lane 5* contains linearised p3SR2. *Arrow* indicates the position of molecular weight maker (kb)

Blots of *Xho*I digested DNA of the same set of transformants showed much simpler hybridisation patterns with both pBR322 and the *amdS* fragment (Fig. 3B). In all transformants, except ct2, a fragment of at least 40 kb can be observed, hybridising strongly to both probes. Since no *Xho*I restriction site is present in p3SR2, this result suggests that the integration of the vector in most transformants must have occurred in a tandemly repeated fashion. However, the possibility of a free multimeric form of vector DNA cannot be completely excluded (see

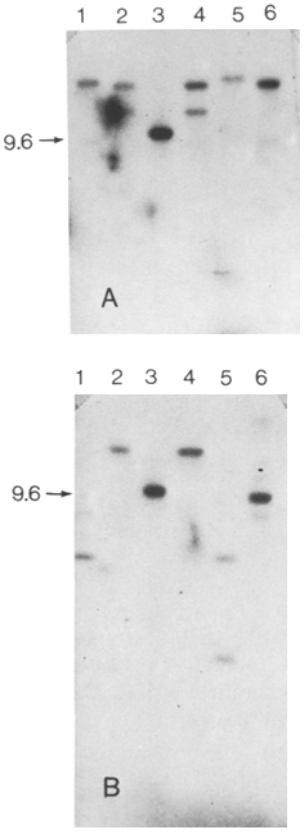

Fig. 5A, B. Analysis of DNA from AmdS$^+$ transformants (yt1–3) obtained with conidial protoplasts of WG290 and p3SR2. *A. nidulans* DNA (2–5 μg) was digested with either *Eco*RI (panel **A**) or *Xho*1 (panel **B**), fractionated on an agarose gel and transferred to nitrocellulose. Blots were probed with ^{32}P-labelled *amdS* fragment. *Lanes 1* and *6* contain DNA from the untransformed WG290 strain and a wild type strain respectively; *lane 2* DNA from yt1 and *lanes 4–5*, DNA from yt2–3. *Lane 3* contains linearised p3SR2. *Arrow* indicates the position of molecular weight marker (kb)

Discussion). Based on this observation, the complex hybridisation patterns, obtained with *Eco*RI digested DNA, can only be explained if sequence rearrangements have taken place within the individual copies in the tandem. In addition to the large *Xho*I fragment in most transformants a 7 kb fragment, hybridising only with the *amdS* probe and corresponding to the *amdS* deletion fragment in MH1277, is present. If integration of the vector has occurred, in most transformants this has not taken place at the resident *amdS* locus.

Over 30 conidial p3SR2-derived transformants of MH1277 have been analysed as described before. In only one case (ct2) a hybridisation pattern was found which could be explained as being the result of integrative recombination of one single copy of p3SR2 at the resident *amdS* locus.

These results are completely different from those reported by Tilburn et al. (1983), who observed predominantly, as in ct2, integration of one copy of p3SR2 at the resident *amdS* locus with transformants obtained from mycelial protoplasts of WG290, a *yA2*, *pantoB100*-derivative of MH1277. Since the results obtained by us with mycelial protoplasts of MH1277 (Fig. 4) are completely equivalent to the ones found for conidial protoplasts, it is suggested that the tandem integration of multiple copies of p3SR2 is not influenced by the physiological differences between the protoplasts.

Considering that differences between the acceptor strains might be involved in the observed discrepancies we then analysed the DNA of several AmdS$^+$ transformants obtained with p3SR2 and WG290. Examples of the resulting hybridisation patterns are shown in Fig. 5. In most WG290 transformants the pattern is consistent with the integration of one copy of the vector at the partially deleted *amdS* gene (yt1 and yt2). Although in very few cases different hybridisation patterns were observed (yt3), tandemly repeated, multiple copy integrations were never found. These results strongly suggest that the type of integration is dependent on the strain used for transformation.

The influence of additional *A. nidulans* ribosomal DNA sequences in the vector on the hybridisation pattern, was examined by analysing MH1277 transformants, obtained with the plasmids pGW315, pGW317 and pGW324. The results invariably show complex hybridisation patterns indicating that multicopy tandem integration is not influenced by extensive homology between the vector and the chromosome (not shown). Whether or not integration had taken place at the ribosomal repeat could not be determined by these standard blotting procedures.

Stability of the tandemly repeated inserts

The correlation between mitotic and meiotic stability of the AmdS$^+$ phenotype and the stability of the integration patterns derived from Southern analysis has been investigated with transformants ht6 and ht7. The hybridisation patterns of *Eco*RI digested DNA from the extensively subcultured transformants remained completely unchanged. This indicates that not only the AmdS property, but also the integration of the tandemly arranged vector DNA sequences is inherited in a stable manner during mitosis.

Meiotic stability was investigated with DNA from colonies, arisen from ascospores of selfed cleistothecia from the same set of transformants. The results (Fig. 6) show that in some cases alterations in the hybridisation patterns have occurred. This indicates that in spite of the

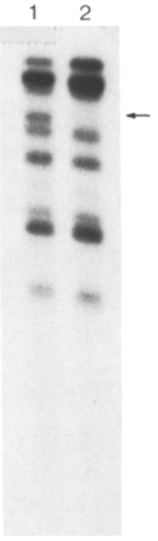

Fig. 6. Meiotic stability of the hybridisation pattern of AmdS$^+$ transformants. DNA from two progeny colonies of selfed cleistothecia of ht7 was digested with *Eco*RI and probed with ^{32}P-labelled *amdS* fragment. The *arrow* indicates the difference between the two hybridisation patterns. *Lane 2* is identical to the parental pattern

apparent phenotypic stability of the AmdS$^+$ property, the tandemly repeated inserts are not always meiotically stable.

Discussion

Transformation of *A. nidulans* mycelial protoplasts has been reported now by a few research groups using different selection markers (Ballance et al. 1983; Tilburn et al. 1983; Yelton et al. 1984; J. L. Johnstone, University of Glasgow, pers. comm.). In general, one copy of vector DNA becomes integrated into the genome at the homologous site.

In this paper we show that also conidial protoplasts of *A. nidulans* can be used for transformation. Conidial protoplasts have the advantage that they can be prepared and purified easily in large amounts (Bos and Slakhorst 1981) and give viability values after handling, which are much higher than those reported for mycelial protoplasts (Yelton et al. 1984). Using the conidial protoplasts, transformation of *amdS* deletion strains results in the formation of growing colonies (Type I), but also in a large fraction of transformant colonies, which fail to develop into mature ones (Type II). Others (Tilburn et al. 1983; Yelton et al. 1984) have also observed such type of colonies, which they called "abortives". Our observations support the assumption that this description is not adequate since the majority still contains the vector DNA sequences, as indicated by their capacity to be converted into well growing (Type I) transformants. We would like to speculate that, once the vector DNA has entered the cell, integration into the genome is limited in time. The Type II colonies would then have stopped growing by lack of sufficient nitrogen, before integration has taken place. Upon transfer to non-selective medium, growth is resumed, thus providing new opportunities for the vector to integrate. The large variation in the fraction of AmdS$^+$ conidiospores from the initial Type I transformants might reflect the same time limitation: an early integration event will lead to a large fraction of AmdS$^+$ conidiospores, whereas later events will result in an increased number of cells which do not contain the AmdS$^+$ property. That such colonies nevertheless grow and sporulate on selective medium can be explained by cross-feeding of untransformed cells by AmdS$^+$ cells.

In contrast to other organisms, homology between the vector and the genome of the acceptor does not seem to have great impact on the transformation frequency. Neither an increased homology by adding r-DNA fragments to the vector, nor a decreased homology by using acceptor strains, in which all *amdS* sequences are deleted (e.g. MH1354, unpublished results) has an effect on transformation frequency.

Under optimised transformation conditions we now are able to obtain 300–400 transformants/μg DNA. However, since this frequency is still rather low for direct selection of cloned genes in *A. nidulans* we continue our search for elements and conditions which will improve the transformation frequency.

Southern blotting experiments with DNA from transformants of MH1277 revealed a type of gene amplification: multiple copies of complete and incomplete vectors arranged in a tandemly repeated fashion. Although the results shown here do not fully exclude the possibility of free multimeric forms of the vector DNA (cf. Grant et al. 1984), results on the genetic analysis of these transformants (Wernars et al. to be published) unambiguously indicate chromosome-linked inheritance of the AmdS$^+$ property, thus strongly favouring the idea of integrated vector DNA copies. In *A. nidulans* occasionally integratin of more than one copy of the vector has been observed (Tilburn et al. 1983; Yelton et al. 1984), but amplified, integrated vector DNA as a result of transformation is very unusually and may even be unique. Amplification of integrated sequences in eukaryotes has been reported, but only as the result of the application of selective pressure (see for review: Stark and Wahl 1984).

The hybridisation results with the *A. nidulans* strains MH1277 and WG290 strongly suggest that the type of integration is strain dependent. It can be speculated that a cryptic mutation, present in MH1277 but not in WG290 is responsible for this. In fact, strains with different *amdS* deletions, e.g. MH1354 (Hynes et al. 1983) but sharing the same genetic background as MH1277 exhibit similar integration phenomena upon transformation (unpublished results). At present we are trying to map the putative mutation on the *A. nidulans* genome by genetic analysis, in order to exploit it also in combination with

other selection markers. The blotting data anyhow clearly indicate that the integration is not preferentially at the resident, partially deleted, *amdS* locus. Together with the occurrence of *A. nidulans* transformants, which show altered colony morphology this could signify that integration of transforming sequences might lead to gene disruption. If integration was to be completely at random, this would provide novel means of isolating *A. nidulans* genes. Detailed analysis of a number of MH1277 transformants should elucidate whether integration shows any site specificity.

To explain the observed multicopy type of integration we propose the following model. An efficient non-homologous recombination system is assumed, which is repressed in WG290 but derepressed in MH1277, due to the cryptic mutation. This system efficiently recombines a number of plasmid molecules, which have entered the protoplast, in a non-homologous fashion. The resulting "scrambled" cointegrate subsequently is integrated into the genome. Since apart from this non-homologous recombination system, homologous recombination still occurs, integration into the genome can take place either at the resident, partially deleted *amdS* locus or at random locations. The presence of a "scrambled" cointegrate as an intermediate in the integration process not only explains the formation of tandem vector repeats, but also the sequence rearrangements observed within these tandems.

The transformation system described here seems to be a promising one; it combines fairly high transformation frequencies with high stability and amplifications of the integrated vector DNA sequences.

Acknowledgements. We thank Dr. Michael Hynes for providing plasmid p3SR2 and *amdS* deletion strains; Dr. Ewa Bartnik for plasmid pMN1. We are grateful to Joan Tilburn and Dr. Claudio Scazzocchio for the *A. nidulans* strain WG290 and their help in our first transformation experiments; Piet Madern for the reproduction of figures and autoradiographs; Trees Makkes, Aafke Sieswerda and Elly van Liempt for typing of the manuscript.

The investigations were supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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Communicated by K. Esser

Received January 29, 1985