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ORIGINAL PAPER

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Autonomously replicating plasmids carrying the *AMA1* region in *Penicillium chrysogenum*

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Abstract Plasmid vectors containing the *AMA1* sequence transformed with high efficiency and replicated autonomously in *Penicillium chrysogenum*. The efficiency of transformation of *P. chrysogenum* was related to the length of the *AMA1* fragment used for constructing the different autonomously replicating plasmids. One of the two palindromic inverted repeats of *AMA1* (the 2.2-kb *Sal*I-*Hin*dIII fragment) is sufficient to confer autonomous replication and a high transformation efficiency. Deletion of the 0.6-kb central fragment located between the inverted repeats did not affect either the ability of the plasmids to replicate autonomously or the efficiency of transformation, but did alter the mitotic stability and the plasmid copy number. Deletion of any fragment of the 2.2-kb repeat caused the loss of the ability to replicate autonomously and reduced the transformation efficiency. Most of the transformants retained the original plasmid configuration, as multimers and without reorganization, after several rounds of autonomous replication. The *AMA1* region works as an origin of replication in *P. chrysogenum* and *A. nidulans* but not apparently in *Acremonium chrysogenum*.

Key words Filamentous fungi · Origins of replication · Stability · Plasmid multimers

Introduction

Several vectors have been developed to transform *Penicillium chrysogenum* (Cantoral et al. 1987; Bull et al. 1988; Kolar et al. 1988; Gouka et al. 1993). Some of them, using the *pyrG* gene as marker, give high efficiency of transformation but all of them resulted in integrative transformants (Cantoral et al. 1987).

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Many attempts have been made to find autonomously replicating plasmids in filamentous fungi but with limited success. Plasmid vectors which are able to replicate autonomously have been constructed for use in *Saccharomyces cerevisiae* employing either the replication origin of the 2 μm plasmid (Beggs 1978; Som et al. 1988) or the autonomous replicating sequence (ARS) of *S. cerevisiae* chromosomes (Struhl et al. 1979; Williamson 1985). Yeast plasmids are not functional in filamentous fungi.

Chimeric plasmids that replicate autonomously in both *Escherichia coli* and *Neurospora crassa* were reported by Hughes et al. (1983). An ARS sequence that allows autonomous replication was isolated from the zygomycete *Mucor circinelloides* (Van Heeswicjck and Roncero 1984). Other "ARS-type" sequences were cloned from genomic libraries of *N. crassa* (Case 1982) and *Aspergillus nidulans* (Tilburn et al. 1983); these sequences do not allow autonomous replication in filamentous fungi. Further attempts to obtain autonomously replicating vectors by recombination of DNA-fragments with conventional plasmids have been made in *Ustilago violacea* (Perlin et al. 1990; Bej and Perlin 1991), *Phanerochaete chrysosporium* (Randall et al. 1989; 1991), and *Pleorotus ostreatus* (Peng et al. 1992, 1993).

Gems et al. (1991) isolated a DNA fragment, named *AMA1*, from unstable colonies of *A. nidulans* transformed with a plasmid genomic library of the same microorganism. The *AMA1* sequence consists of a central region flanked by two palindromic sequences (inverted repeats) with a total size of 6.1 kb (see Fig. 1). A plasmid (ARP1) containing the *AMA1* region was shown to replicate autonomously; this plasmid increased the transformation efficiency of *A. nidulans* 250-fold and was mitotically unstable. Because of the industrial relevance of *P. chrysogenum* and *Acremonium chrysogenum* it was of interest to determine whether the *AMA1* sequences replicate autonomously in these fungi. As shown in this article, plasmids containing the *AMA1* sequence transform and replicate very efficiently in *P. chrysogenum* but apparently not in *A. chrysogenum*.

Materials and methods

Microorganisms. *P. chrysogenum npe5 pyrG* and *npe6 pyrG* are uridine auxotrophs blocked in penicillin biosynthesis and derived from *P. chrysogenum* Wis 54-1255 (Cantoral et al. 1993). *A. chrysogenum* C10 is a moderately high cephalosporin producer released by Panlabs (Demain 1983; Gutiérrez et al. 1991a). *E. coli* DH5α (Clontech, Palo Alto, California) was used as a host with high transformation efficiency to obtain the plasmids employed in the present paper.

Plasmid vectors. Plasmid pHELP (Gems et al. 1991) was provided by J. Clutterbuck. pBCKS+ was obtained from Stratagene (La Jolla, California). pGPH1 was constructed by B. Díez, J. L. Barredo and S. Gutiérrez (unpublished) and pULFR40 was made by B. Díez (Gutiérrez et al. 1991b). The *P. chrysogenum pyrG* gene (Cantoral et al. 1988) was subcloned from pULG, and the *ble* gene (phleomycin resistance gene of *Streptoalloteichus hindustanus*) was isolated from pULJL43 (Gutiérrez et al. 1991b). The different plasmids employed were constructed using standard DNA techniques.

Transformation of P. chrysogenum and A. chrysogenum. Protoplasts of *P. chrysogenum* were obtained as described by Fierro et al. (1993) and transformed according to the procedures of Cantoral et al. (1987) and Díez et al. (1987). Transformation of *A. chrysogenum* protoplasts was performed as described by Gutiérrez et al. (1991b, 1992).

Extraction and purification of total DNA from transformants. Mycelia of the different *P. chrysogenum* transformants grown in MPPY (Montenegro et al. 1992; Fierro et al. 1993) and of *A. chrysogenum* transformants grown in MMC (Gutiérrez et al. 1991b) were collected by filtration. When small amounts of DNA were required, mycelia were lyophilized and triturated. Then 300 µl of triturated lyophilized mycelia were suspended in 0.18 M Tris, 4.5 mM EDTA, 1% SDS pH 8.2 in a final volume of 1 ml. Phenol (1 ml) was added and the mixture was kept for 30 min at 50 °C with frequent agitation. The upper phase was collected and extracted nine times with phenol-CIA (chloroform: isoamyl alcohol 24:1) and once more with CIA, precipitated with 0.3 M sodium acetate and 2.5 vol of ethanol, and resuspended in 50 µl of TE buffer (F. J. Fernández, personal communication). When large amounts of DNA were required, it was obtained by a procedure modified from Kolar et al. (1988).

Southern blotting and hybridization. Total DNA (3–4 µg) of the different strains was digested with restriction endonucleases, electrophoresed in 0.7% agarose and blotted by standard techniques (Sambrook et al. 1989). Undigested total DNAs were electrophoresed in 0.5% agarose. Probes were labelled by nick translation with [α^{32}P]-dCTP and hybridized by standard methods.

Plasmid recovery in E. coli. Total DNA (1 µg) of each *P. chrysogenum* transformant was used to transform competent *E. coli* DH5α cells obtained according to Hanahan (1986) and transformed clones were selected by standard procedures.

Plasmid stability studies. Spores of the *P. chrysogenum* transformants were collected from plates of Power medium (Fierro et al. 1995) by filtration through Nytal filters (pore size 25 µm) to obtain isolated spores. The suspension was counted under the microscope and diluted to a final concentration of 2×10^3 spores per ml. Then 0.1 ml of this suspension was plated in Czapek minimal medium with or without uridine. After 7 days of incubation at 25 °C, the colonies that had lost the plasmid were determined by their inability to grow in Czapek medium without uridine.

Determination of the number of copies of each plasmid. To calculate the number of copies of each plasmid we compared the density of the hybridizing bands [with the pBCKS(+) *pyrG* probe] corresponding to the endogenous *pyrG* gene with those belonging to the plasmid. A correction factor was applied since the length of the probe hybridizing with the endogenous *pyrG* is shorter than that hybridizing with the plasmid sequences. The quantifications were made with a scanner (Discovery Series, PBI) using the Diversity One 1.0 Software (PBI, Huntington Station, New York).

Results

Construction of a set of plasmids containing the *AMA1* sequence

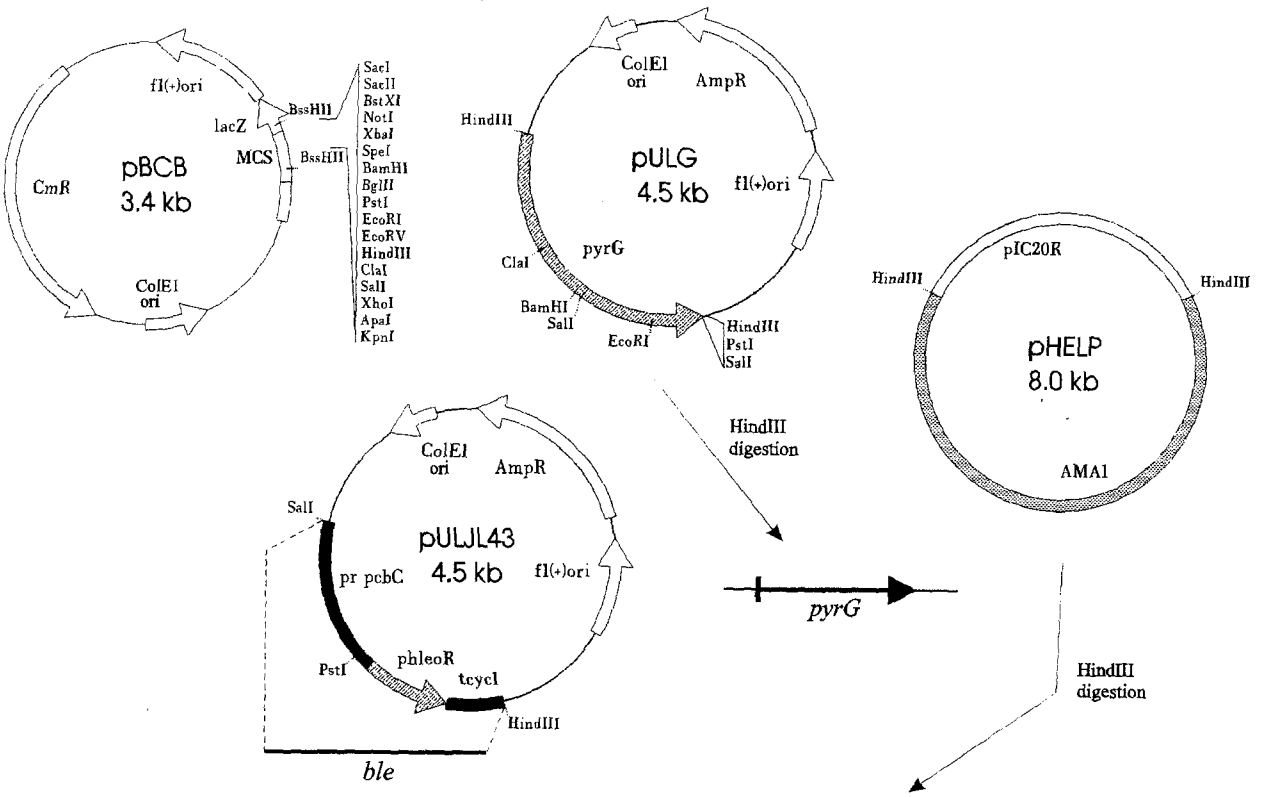
A set of plasmids, named pAMPF2 to pAMPF10 and pAMPF21, was constructed using different fragments of *AMA1* (Fig. 1). Plasmids pAMPF2 and pAMPF21 carry the largest (5.0-kb *Hin*dIII) fragment of *AMA1* together with the *pyrG* or the *ble* (phleomycin resistance) gene, respectively, as a selective marker for filamentous fungi in a modified pBCKS+ vector (containing a *Bgl*II site in the polylinker) (Fig. 1). In plasmids pAMPF7and pAMPF8 the central fragment of the *AMA1* was replaced by the *ble* gene. Removal of the central fragment of the *AMA1* region by linking directly the two repeats gave constructions which could not be isolated from *E. coli.* Plasmids pAMPF9L and pAMPF9R contained only one of the two repeats of the *AMA1* region (left and right, respectively).

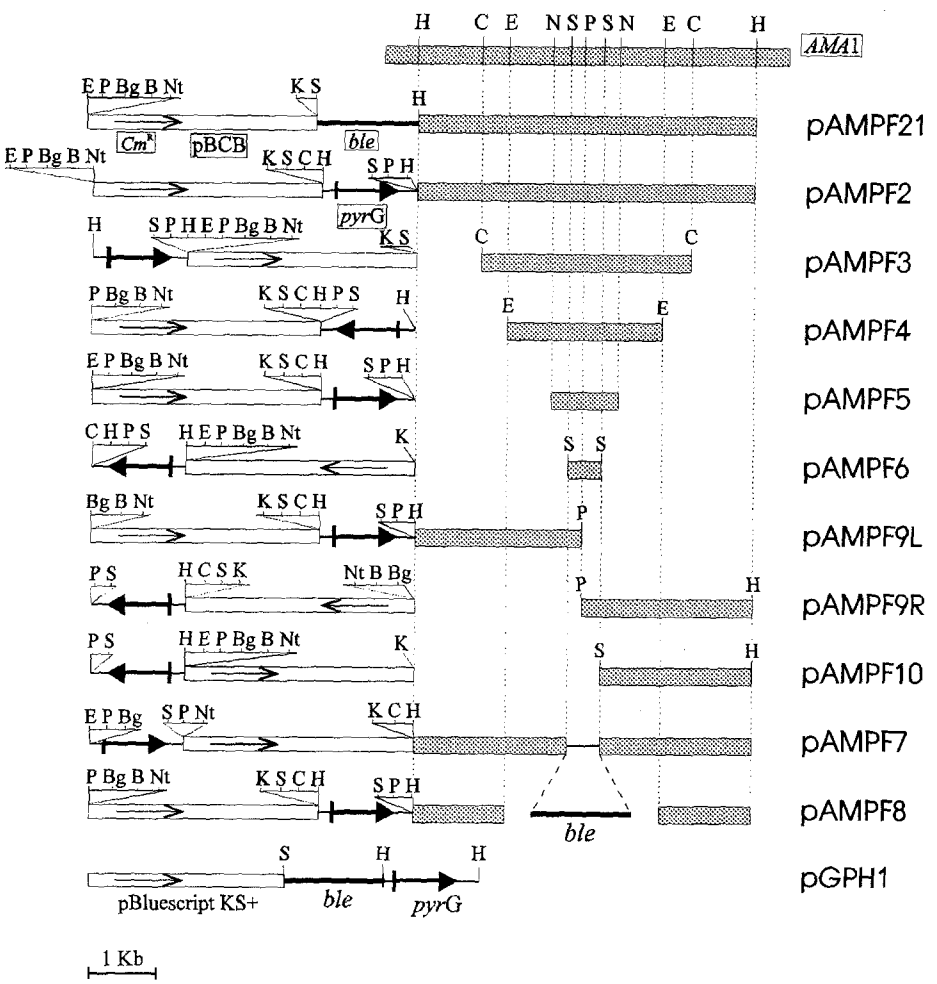
Transformation efficiency with different *AMA1*-derived plasmids

The transformation efficiency of *P. chrysogenum npe6 pyrG* with *AMA1*-derived plasmids was compared under standard conditions (using 1 µg of plasmid DNA and 1×10^7 protoplasts in a final volume of 100 µl with 10 mM aurintricarboxylic acid) with the efficiency obtained with an integrative plasmid (pGPH1) under the same conditions.

About 49% of the protoplasts were able to regenerate and the highest proportion of transformed protoplasts (obtained with pAMPF7) was 5.7%. As shown in Table 1, very high transformation efficiencies per microgram of DNA were obtained with some of the plasmids in three different experiments. As a general rule, the transformation efficiency was higher as the fragment of the *AMA1* region used in the vectors was larger. Only one of the repeats of the

Fig. 1 *Upper.* Plasmids used in this work. pBCB is a modified pBCKS(+) with a *Bgl*II site instead of *Sma*I (all the enzymes of its multiple cloning site are shown). In the rest of the plasmids only the following enzymes are indicated: *Hin*dIII (*H*), *Cla*I (*C*), *Eco*RI (*E*), *Nru*I (*N*), *Sal*I (*S*), *Pst*I (*P*), *Bgl*II (*Bg*), *Bam*HI (*B*), *Not*I (*Nt*) and *Kpn*I (*K*). pULG is a pBluescript KS(+) derivative that contains the *pyrG* gene of *P. chrysogenum* (Cantoral et al. 1988). pHELP was constructed by Gems et al. (1991) and pULJL43 by J. L. Barredo (-Gutiérrez et al. 1991b). The gene *ble* is the phleomycin resistance gene expressed from the promotor of the *pcbC* gene of *P. chrysogenum*; the *cyc* transcriptional terminator from *S. cervisiae* (tcyc1) is indicated by a *solid bar. Lower.* Restriction maps of the series pAMPF2-10, pAMPF21 and pGPH1 in their linear forms. The chloramphenicol resistance marker is indicated by a *thin arrow* inside the open boxes corresponding to the pBCB, and the *pyrG* marker is shown by a *thick arrow* (the thin arrow in pGPH1 corresponds to the Ap^R gene). The sequences corresponding to *AMA1* fragments are indicated by *stippled boxes*


pBCB
3.4 kb
CmR
ColE1 ori
f1(+)ori
lacZ
MCS
BssHII
BssHII
SacI
SacII
BstXI
NotI
XbaI
SpeI
BamHI
BglII
PstI
EcoRI
EcoRV
HindIII
ClaI
SalI
XhoI
ApaI
KpnI
pULG
4.5 kb
ColE1 ori
AmpR
f1(+)ori
pyrG
HindIII
ClaI
BamHI
SalI
EcoRI
HindIII
PstI
SalI
pIC20R
HindIII
HindIII
pHELP
8.0 kb
AMA1
HindIII digestion
pULJL43
4.5 kb
ColE1 ori
AmpR
SalI
pr pcbC
f1(+)ori
PstI
phleoR
tcycl
HindIII
ble
pyrG
HindIII digestion


H C E NSPSN E C H
AMA1
pAMPF21
pAMPF2
pAMPF3
pAMPF4
pAMPF5
pAMPF6
pAMPF9L
pAMPF9R
pAMPF10
pAMPF7
pAMPF8
pGPH1
Cm^R
pBCB
ble
pyrG
ble
pBluescript KS+
ble
pyrG
1 Kb

AMA1 region was sufficient to obtain very high transformation efficiency (more than 100000 transformants per microgram of DNA).

The efficiency of transformation was clearly lower when the *ble* gene was used as a selective marker (pAMPF21) than when *pyrG* was used (pAMPF2), but in all cases it was at least 10-fold higher than with conventional integrative plasmids (Table 1).

Two types of transformants were obtained: (1) clones with normal morphology and good development and sporulation, and (2) small non-sporulating clones that were abortive upon successive replication in minimal medium. Very few, or no, abortive clones were obtained in transformations with plasmids pAMPF2, pAMPF7, pAMPF9L, pAMPF9R and pAMPF10. Most clones of these transformants were well sporulated after 5 days. On the other hand a relatively high number of abortive transformants (from 37 to 82%) were obtained with plasmids pAMPF3, pAMPF4, pAMPF5, pAMPF6 and pAMPF8 and also with the integrative vector pGPH1. Of these, pAMPF3 and pAMPF4 behave as more effective vectors (giving a lower efficiency of abortive transformants and better sporulation efficiency). Interestingly pAMPF6 gave two stable types of morphology: some transformants showed normal morphology whereas other gave irregular and wrinkled colonies that maintained this morphology after repeated transfers in solid medium.

The transformation efficiency of the cephalosporin producer *A. chrysogenum* C10 with plasmids pAMPF21 and the integrative vector pULFR40 (both containing the *ble* marker) gave similar low values, which indicates that *AMA1*-derived plasmids are not particularly efficient vectors for transforming *A. chrysogenum*.

Table 1 Efficiency of transformation of filamentous fungi with different plasmids carrying fragments of the *AMA1* region

Strain	Plasmid	Transformants/ μg DNA[a]
P. chrysogenum npe6 pyrG	pAMPF2	218827
	pAMPF3	24800
	pAMPF4	16640
	pAMPF5	2245
	pAMPF6	3933
	pAMPF7	281349
	pAMPF8	19200
	pAMPF9L	196944
	pAMPF9R	195381
	pAMPF10	187566
	pGPH1[b]	696
	pAMPF21[c]	200
A. chrysogenum C100	pAMPF21[c]	8
	pULFR40[b]	9

[a] Results are the average of three transformation experiments with each of the plasmids. The transformations were carried out using the conditions described in Materials and methods and the number of transformants are given per 10^7 protoplasts
[b] pGPH1 and pULFR40 are integrative vectors
[c] pAMPF21 is similar to pAMPF2 but carries the phleomycin resistance gene as a selective marker

Stability of *P. chrysogenum* transformants with *AMA1*-derived plasmids

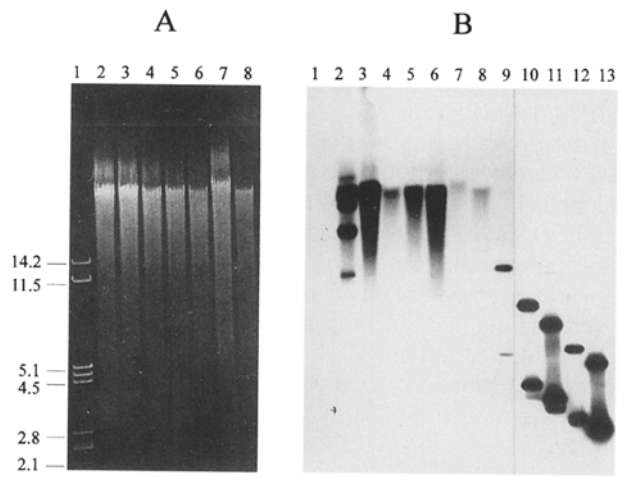
Since loss of the plasmid in *P. chrysogenum npe6 pyrG* transformants results in uridine auxotrophy, mitotic stability after repeated transfers was calculated by comparing the number of clones in MM, or in MM supplemented with uridine, as indicated in Materials and methods. Spores from a single colony of each transformant grown in selective media were collected and seeded again in Power media (which allows poor growth and no sporulation of the auxotrophs). Then, spores from a Petri dish were collected and the plasmid stability determined as described in Materials and methods.

As shown in Table 2, the transformants with the integrative vector pGPH1 were nearly 100% stable (as expected) and 100% stability was also obtained with pAMPF6 and pAMPF8 which suggests that those two vectors may become integrated in the genome. Transformants with all other plasmids showed a different degree of mitotic instability.

Table 2 Mitotic stability after repeated transfers of transformants with the different autonomously replicating and integrative vectors

Plasmids	Number of colonies[a] in Czapeck+Uridine	Number of colonies[a] in Czapeck	Percentage of spores retaining the plasmid (prototrophs)
pAMPF2	128	61	48%
pAMPF3	151	61	40%
pAMPF4	129	114	88%
pAMPF5	140	126	90%
pAMPF6	97	98	100%
pAMPF7	130	21	16%
pAMPF8	134	150	100%
pAMPF9L	124	72	58%
pAMPF9R	120	42	35%
pAMPF10	130	97	75%
pGPH1[b]	110	103	94%
Not transformed	117	0	0%

[a] Results are the average of three experiments with each of the plasmids
[b] pGPH1 is an integrative vector used as a control



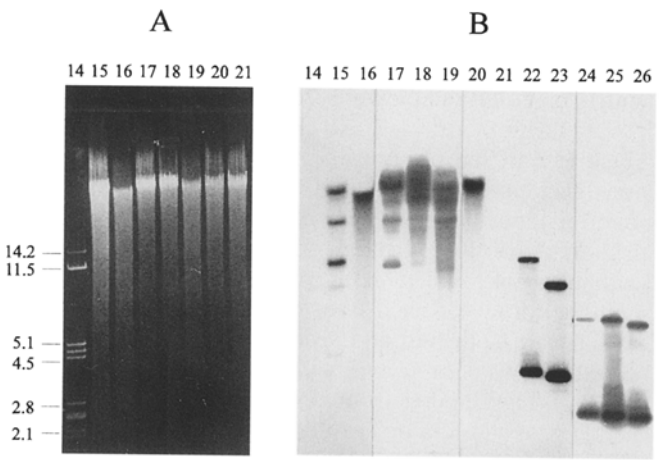


Fig. 2 **A** Total undigested DNA of *P. chrysogenum npe6 pyrG* transformed with *AMA1*-derived plasmids. *Lanes 1 and 14*, size markers; *2*, pAMPF; *3*, pAMPF3; *4*, pAMPF4; *5*, pAMPF5; *6*, pAMPF6 (normal morphology); *7*, pAMPF6 (wrinkled morphology); *8*, pGPH1; *15*, pAMPF7; *16*, pAMPF8; *17*, pAMPF9L, *18*, pAMPF9R, *19*, pAMPF10, *20*, pGPH1; *21*, untransformed control. **B** Hybridization with the *AMA1*-pBCKS(+) probe. *Lanes 1–8* and *14–21* are the same as in **A**. *Lanes 9–13* and *22–26* contain undigested plasmids extracted from *E. coli* (about 6×10^{-3} μg) transformed with the following plasmids; *9*, pAMPF2; *10*, pAMPF3; *11*, pAMPF4; *12*, pAMPF5; *13*, pAMPF6; *22*, pAMPF7; *23*, pAMPF8; *24*, pAMPF9I; *25*, pAMPF9D; *26*, pAMPF10

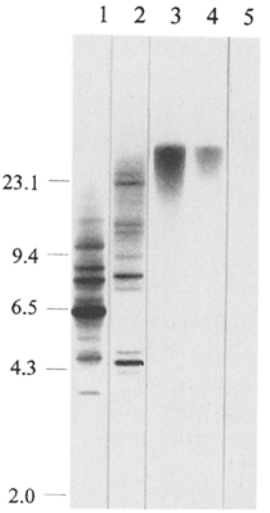


Fig. 3 Hybridization of DNAs of *A. chrysogenum* C10 transformed with pAMPF21 (*lanes 1 and 3*) or pULFR40 (*lanes 2 and 4*), or untransformed (*lane 5*), with the *AMA1*-pBCKS(+) probe. DNA in *lane 1* was digested with *Eco*RI and in *lane 2* with *Bam*HI whereas DNA in *lanes 3, 4 and 5* was undigested. Size markers (Lambda DNA *Hin*dIII fragments) are indicated (in kb) on the left

Fate of plasmid DNA in the transformants

To study if the plasmids replicate autonomously, total DNA was extracted from each transformant grown in liquid MPPY (which does not allow growth of *pyrG* auxotrophs) and hybridized after separation by electrophoresis (without digestion) with a band containing pBCKS(+) *AMA1* as a probe. As shown in Fig. 2, the integrative plasmids (e.g., pGPH1, lanes B8 and B20) hybridize only with chromosomal DNA. This pattern of hybridization was obtained also with total DNA of clones transformed with pAMPF3, 4, 5, 6, and 8, which suggests that they have all integrated in the genome. The different intensity of hybridization in these cases is due to the different number of integrated copies.

Autonomously replicating plasmids were observed in clones transformed with pAMPF2, 7, 9L, 9R and 10. Analysis of the hybridizing signals indicated that in these transformants the hybridization is due to specific bands (different from chromosomal DNA) of high-molecular-weight plasmids. Comparison of the hybridization pattern of *P. chrysogenum* transformants with *E. coli* clones transformed with the same plasmids revealed that autonomously replicating plasmids in the filamentous fungi are always present as multimers, mostly as dimers and trimers.

When total DNAs were digested, the hybridization pattern showed that the autonomously replicating plasmids hybridized with bands with the same mobility as that of the *E. coli* plasmids (data not shown), which indicates that the plasmids have not suffered any rearrangements (see also Fig. 4 below).

AMA1-derived plasmids behave as integrative in *A. chrysogenum* transformants

As shown in Fig. 3 transformants of the cephalosporin producer *A. chrysogenum* C10 with plasmids pULFR40 (integrative) and pAMPF21 (containing the *AMA1* region) hybridized with the pBCKS(+) *AMA1* probe giving a pattern consistent with chromosomal integration (Fig. 4). Both plasmids became integrated in multiple sites within the genome as shown by the bands which hybridize after *Eco*RI digestion.

Estimation of the number of plasmid copies in the transformant

To estimate the number of copies, the DNAs of each transformant shown in Fig. 2A were digested and hybridized with a pBCKS(+) *pyrG* probe that gave hybridization with

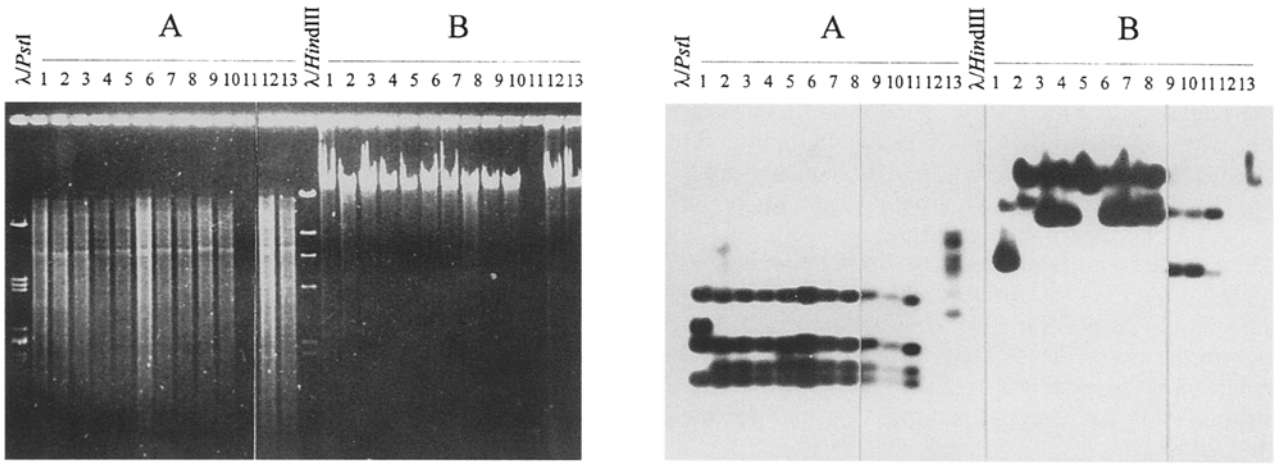


Fig. 4 *Left.* Agarose-gel electrophoresis of total DNAs, digested with *Eco*RI (*A*) or undigested (*B*), of ten different transformants of *P. chrysogenum npe5 pyrG* with pAMPF2 (*lanes 1–10*); *lane 11*, pAMPF2 extracted from *E. coli*; *12*, untransformed control; *13*, integrative transformant with pGPH1. *Right.* Hybridization of panels *A and B* with the *AMA1*-pBCKS(+) probe

(1) the endogenous *pyrG* gene (single copy in *P. chrysogenum* DNA), and (2) the transforming plasmids. This allows a comparative densitometric estimation as indicated in Materials and methods. The endogenous *pyrG* gene digested with *Eco*RI gave two hybridizing bands of 20 and 4.3 kb.

On account of the mitotic stability of the different plasmids (Table 2), we considered that the percentage indicated in Table 2 is indicative of the number of cells within the mycelia carrying the plasmid. Taking these factors into consideration, the number of autonomous plasmid copies per cell was estimated to be about 51 for pAMPF9R, 34–35 for pAMPF2 and pAMPF10, 19 for pAMPF9L, and 10 for pAMPF7. In the case of the integrative plasmids the number of integrated copies ranged from 10–11 copies for pAMPF3 to very few copies (1–2) for pAMPF6.

Lack of significant DNA rearrangements in transformants that maintain the plasmid autonomously

In order to determine whether the autonomously replicating plasmids suffered rearrangements, the DNA of ten transformants of *P. chrysogenum npe5 pyrG* with plasmid pAMPF2 were hybridized (with or without digestion) with pBCKS-*AMA1* as a probe. As shown in Fig. 4, nine out of the ten transformants contained identical plasmids, and the hybridizing bands after DNA digestion coincided with the bands of the same plasmid, pAMPF2, purified from *E. coli*. One of the ten transformants (lane 1) showed a 3.0-kb *Eco*RI band, instead of the original 1.6-kb *Eco*RI band, suggesting that it had suffered a reorganization.

In the undigested total DNA, we could observe two clearly distinct patterns of hybridization: in clones 2 to 8 two different plasmid forms in high copy number were observed, one of which (probably a multimer) migrates close to chromosomal DNA whereas the other moved ahead of it. Clones 9 and 10 showed plasmid forms identical to *E. coli* (monomers). Interestingly in these two clones the number of copies is much lower (probably one or a few copies per cell) than in clones 2 to 8 (Fig. 4).

Clone number 1, which has suffered the rearrangement, also shows low-molecular-weight (*E. coli*-type) monomers. These results suggest that autonomous replication leads frequently to multimers containing multiple copies of the plasmid used for transformation.

Recovery of autonomously replicating plasmids in *E. coli*

If plasmids replicate in an autonomous (non-integrated) manner in *P. chrysogenum*, it should be possible to recover them in *E. coli* by transformation with total DNA isolated from *P. chrysogenum* transformants. Using total DNA from each type of *P. chrysogenum* transformant, *E. coli* clones containing plasmids pAMPF 2, 7, 9L, 9R and 10 were obtained, thus confirming the presence of autonomously replicating plasmids in those *P. chrysogenum* transformants. In contrast, no plasmid could be recovered in *E. coli* from transformants pAMPF 3, 4, 5, 6, and 8 which all contain small fragments of the *AMA1* region. These results agree with the deductions established from Fig. 3 and support the conclusion that pAMPF2, 7, 9L, 9R and 10 replicate autonomously. This method allows one to recover complex constructions in *E. coli* that have been previously introduced in filamentous fungi for expression purposes or for regulatory analysis.

Discussion

The *AMA1* DNA fragment works as an origin for autonomous replication in *A. nidulans*, *Aspergillus niger* (Gems et al. 1991), *Gibberella fujikuroi* (Brückner et al. 1992) and, as shown in this article, in *P. chrysogenum*. The high

efficiency of transformation obtained with autonomously replicating plasmids in *P. chrysogenum*, which is similar to that obtained in *A. nidulans* (Gems et al. 1991), is related to the length of the *AMA1* region used for constructing the different plasmids. One of the two palindromic repeats of *AMA1* (the 2.2-kb *Sal*I-*Hin*dIII fragment present in pAMPF10) is sufficient to confer autonomous replication and a high transformation efficiency.

The mitotic stability of plasmids with a single repeat of the palindrome (35–75% in different plasmids) is similar to the stability of plasmids carrying the entire *AMA1* region with the two repeats. These results suggest that the complete *AMA1* sequence is a bidirectional origin of replication with each repeat serving as a unidirectional origin.

Deletion of the 0.6-kb central fragment located between the repeats does not affect either the ability of the plasmids to replicate autonomously or the efficiency of transformation, but it seems to affect mitotic stability and the plasmid copy number.

In contrast, deletion of any fragment of the 2.2-kb repeat caused the loss of the ability to replicate autonomously and drastically reduced the transformation efficiency. In plasmid pAMPF3 (which lacks the *Hin*dIII-*Cla*I fragment) (Fig. 1), the autonomous replication ability is maintained during several serial transfers in solid medium (Table 2) but is lost in liquid medium, as shown in Fig. 3. This result was confirmed by the inability to recover pAMPF3 in *E. coli* from total DNA of *P. chrysogenum* transformed with this plasmid.

Reorganization of plasmid DNA when maintained autonomously was a rare event and most of the transformants retained the original plasmid configuration. It is interesting that several copies of the plasmid are arranged as multimers. This fact may be beneficial for the amplification of a particular gene. Some other clones maintain single copies of the plasmid (Fig. 4).

Plasmids carrying the *AMA1* region integrate, but apparently do not replicate autonomously, in *A. chrysogenum*, a filamentous fungus which is not closely related to *Aspergillus* and *Penicillium* (Onions and Brady 1987). This result suggests that host factors (which are absent in *A. chrysogenum*) are required for autonomous plasmid replication.

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References

Beggs JD (1978) Transformation of yeast by a replicating hybrid plasmid. Nature 275:104

Bej A, Perlin MH (1991) Acquisition of mitochondrial DNA by a transformation vector for *Ustilago violacea*. Gene 98:135–140

Brückner B, Unkles SE, Weltring K, Kinghorn J (1992) Transformation of *Gibberella fujikuroi*: effect of the *Aspergillus nidulans* AMA1 sequence on frequency and integration. Curr Genet 22:313–316

Bull JH, Smith DJ, Turner G (1988) Transformation of *Penicillium chrysogenum* with a dominant selectable marker. Curr Genet 13:377–383

Cantoral JM, Díez B, Barredo JL, Alvarez E, Martín JF (1987) High-frequency transformation of *Penicillium chrysogenum*. Bio/Technol 5:494–497

Cantoral JM, Barredo JL, Díez B, Martín JF (1988) Nucleotide sequence of the *Penicillium chrysogenum pyrG* (orotidine-5′-phosphate decarboxylase) gene. Nucleic Acids Res 16:8177

Cantoral JM, Gutiérrez S, Fierro F, Gil-Espinosa S, van Liempt H, Martín JF (1993) Biochemical characterization and molecular genetics of nine mutants of *Penicillium chrysogenum* impaired in penicillin biosynthesis. J Biol Chem 268:737–744

Demain AL (1983) Strain exchange between industry and academia. ASM News 49:431

Díez B, Alvarez E, Cantoral JM, Barredo JL, Martín JF (1987) Selection and characterization of *pyrG* mutants of *Penicillium chrysogenum* lacking orotidine-5′-phosphate decarboxylase and complementation by the *pyr4* gene of *Neurospora crassa*. Curr Genet 12:277–282

Fierro F, Gutiérrez S, Díez B, Martín JF (1993) Resolution of four large chromosomes in penicillin-producing filamentous fungi: the penicillin gene cluster is located on chromosome II (9.6 mb) in *Penicillium notatum* and chromosome I (10.4 mb) in *Penicillium chrysogenum*. Mol Gen Genet 241:573–579

Fierro F, Montenegro E, Gutiérrez S, Martín JF (1995) Mutants blocked in penicillin biosynthesis show a deletion of the entire penicillin gene cluster at a specific site within a conserved hexanucleotide sequence. Appl Microbiol Biotechnol (in press)

Gems D, Johnston IL, Clutterbuck AJ (1991) An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. Gene 98:61–67

Gouka RJ, van Hartingsveldt W, Bovenberg RAL, van Zeijl CMJ, van den Hondel CAMJJ, van Gorcom RFM (1993) Development of a new transformant selection system for *Penicillium chrysogenum*: isolation and characterization of the *P. chrysogenum* acetyl-coenzyme A synthetase gene (*facA*) and its use as a homologous selection marker. Appl Microbiol Biotechnol 38:514–519

Gutiérrez S, Díez B, Montenegro E, Martín JF (1991a) Characterization of the *Cephalosporium acremonium pcbAB* gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin-biosynthetic genes and evidence of multiple functional domains. J Bacteriol 173:2354–2365

Gutiérrez S, Díez B, Alvarez E, Barredo JL, Martín JF (1991b) Expression of the *penDE* gene of *Penicillium chrysogenum* encoding isopenicillin N acetyltransferase in *Cephalosporium acremonium:* production of benzylpenicillin by the transformants. Mol Gen Genet 225:56–64

Gutiérrez S, Velasco J, Fernández FJ, Martín JF (1992) The *cefG* gene of *Cephalosporium acremonium* is linked to the *cefEF* gene and encodes a deacetylcephalosporin C acetyltransferase closely related to homoserine O-acetyltransferase. J Bacteriol 174:3056–3064

Hanahan D (1986) Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA Cloning, vol. 1. IRL Press, Oxford, pp 109–135

Hughes K, Case ME, Geever R, Vapnek D, Giles NH (1983) A chimeric plasmid that replicates autonomously in both *Escherichia coli* and *Neurospora crassa*. Proc Natl Acad Sci USA 80: 1053–1057

Kolar M, Punt PJ, van den Hondel CAMJJ, Schwab H (1988) Transformation of *Penicillium chrysogenum* using dominant selection markers and expression of an *Escherichia coli lacZ* fusion gene. Gene 62:127–134

Montenegro E, Fierro F, Fernández FJ, Gutiérrez S, Martín JF (1992) Resolution of chromosomes III and VI of *Aspergillus nidulans*

by pulsed-field gel electrophoresis shows that the penicillin biosynthetic pathway genes *pcbAB, pcbC,* and *penDE* are clustered on chromosome VI (3.0 megabases). J Bacteriol 174:7063–7067

Onions AHS, Brady BL (1987) Taxonomy of *Penicillium* and *Acremonium*. In: Peberdy JF (ed) *Penicillium* and *Acremonium*. Plenum Press, New York, pp 1–36

Peng M, Singh NK, Lemke PA (1992) Recovery of recombinant plasmids from *Pleurotus ostreatus* transformants. Curr Genet 22: 53–59

Peng M, Lemke PA, Singh NK (1993) A nucleotide sequence inolved in replicative transformation of a filamentous fungus. Curr Genet 24:114–121

Perlin MH, Bej AK, Will OH, Jacob RJ (1990) Introdution and maintenance of prokaryotic DNA in *Ustilago violacea*. J Indust Microbiol 5:355–364

Randall T, Rao T, Reddy CA (1989) Use of a shuttle vector for the transformation of the white rot basidiomycete, *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 161:720–725

Randall T, Reddy CA, Boominathan K (1991) A novel extrachromosomally maintained transformation vector for the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J Bacteriol 173:776–782

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Som T, Armstrong KA, Volkert FC, Broach JR (1988) Autoregulation of 2 μm circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell 52:27–37

Struhl K, Stinchcomb DT, Scherer S, Davis RW (1979) High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc Natl Acad Sci USA 76:1035

Tilburn J, Scazzochio C, Taylor GG, Zabicky-Zissman JH, Lockington RA, Davis RW (1983) Transformation by integration in *Aspergillus nidulans*. Gene 26:205–221

Van Heeswicjck R, Roncero MIG (1984) High-frequency transformation of *Mucor* with recombinant plasmid DNA. Carlsberg Res Commun 49:691–702

Van Houten JV, Newton CS (1990) Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol Cell Biol 10:3917–3925

Williamson DH (1985) The yeast ARS element, six years on: a program report. Cell 42:951–958