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Co-transformation with autonomous replicating and integrative plasmids in *Penicillium chrysogenum* is highly efficient and leads in some cases to rescue of the intact integrative plasmid

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Abstract

The efficiency of co-transformation in *Penicillium chrysogenum* Wisconsin 54-1255 *pyrG*$^-$ and the fate of the transforming DNA were studied using an integrative (pEF43) and an autonomous replicating plasmid (pAM9L). The results showed a co-transformation frequency of nearly 70% of all transformants tested. The total efficiency of transformation was shown to be dependent on the plasmid marker used as transformant selection (i.e., markers in the integrative or autonomous replicating vector). Analysis of the plasmids re-isolated from several co-transformants showed that different populations of plasmids co-exist in the fungal host. Interestingly, in all co-transformants studied, the integrative plasmid was found to be replicating autonomously without integrating into the host genome. In some cases, co-integrates were formed by recombination between autonomous replicating (pAM9L) and integrative (pEF43) plasmids. However, unexpectedly in some cases, the non-reorganised pEF43 integrative plasmid used in the co-transformation assays was rescued from some co-transformants.
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Keywords: AMA1 sequence; Integrative plasmid recovery; Autonomous replication vector; Co-transformation

1. Introduction

The co-transformation of cells with two different DNA fragments was first observed in *Escherichia coli* (Kretschmer et al., 1975) and then in other organisms such as *Saccharomyces cerevisiae* (Hicks et al., 1978), mammalian cells (Wigler et al., 1979), and filamentous fungi (Díez et al., 1990; Wernars et al., 1987). The effectiveness of co-transformation procedures has led to the development of new techniques based on co-transformation such as gene cloning using "instant gene banks" (Bowyer et al., 1994; Gems et al., 1994), gene replacement or disruption (Kelly et al., 1988; Rudolph et al., 1985; Wernars et al., 1987), and in vivo DNA transfer between vectors (Criswell and Bradshaw, 1998; Ma et al., 1987; Prado and Aguilera, 1994).

Genetic manipulation of fungi using autonomous replicating plasmids has advantages over the integrative system because of the high-frequency of transformation achieved (Fierro et al., 1996; Gems et al., 1991; Aleksenko and Clutterbuck, 1996, 1997) and since it allows recovery of insert-containing plasmid for further genetic analysis. In co-transformation experiments, the use of autonomously replicating plasmids allows transformation even when a selectable gene is not present in the autonomous plasmid (Jiménez and Davies, 1980; Kurtz et al., 1987; Sakai et al., 1984). In some fungal species when an integrative and an autonomously replicating plasmid are used in co-transformation assays, some transformants contain plasmid co-integrates consisting of both autonomously replicating and integrative plasmid sequences (Aleksenko, 1994; Gems and Clutterbuck, 1993; Jiménez and Davies, 1980; Kurtz et al., 1987).

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Penicillium chrysogenum is one of the most economically important microorganisms because it is used for industrial penicillin production (Aharonowitz et al., 1992; Martín et al., 1999). Transformation of this fungus with integrative (Cantoral et al., 1987) or autonomous replicating (Fierro et al., 1996) plasmids has been developed and used for gene cloning, gene disruption (Casqueiro et al., 1999b), and overexpression studies (Bañuelos et al., 2000). In some cases, large non-selectable DNA fragments (e.g., entire gene clusters) (Díez et al., 1990) must be introduced in the host and therefore, co-transformation with a selectable plasmid is a powerful technique for genetic manipulations of this fungus. However, up to now, the fate of DNA following co-transformation of *P. chrysogenum* has not been studied.

Therefore, it was of interest to analyse the genetic stability of co-transformants, the formation of co-integrates, and the possibility of re-isolating genes of interest following co-transformation of *P. chrysogenum* with an integrative and an autonomous replicating plasmid.

2. Materials and methods

2.1. Microorganisms and culture conditions

Escherichia coli DH5α was used for routine DNA manipulation. *E. coli* DH10B was used for plasmid-rescue experiments. *P. chrysogenum* Wisconsin 54-1255 *pyrG*$^{-}$, an uracil auxotroph (Díez et al., 1987) with a frameshift mutation in the *pyrG* gene (Bañuelos et al., 2001), was used as recipient strain in transformation procedures. All fungal strains were grown on plates of Power medium for sporulation (Casqueiro et al., 1999a) and spores were collected from cultures grown for 5 days at 28 °C. Czapek minimal medium was used in phenotype assays. Uridine and phleomycin were added to Czapek medium at a final concentration of 100 and 30 μg/ml, respectively. 5-Fluoro-orotic acid (FOA) was added at a final concentration of 1 mg/ml (Díez et al., 1987) to select *pyrG*$^{-}$ recombinants (strains lacking the OMP-decarboxylase enzyme encoded by *pyrG* are resistant to the toxicity of 5-FOA) (Díez et al., 1987).

2.2. Plasmids and probes

pAM9L (syn. pAMPF9L; Fierro et al., 1993) is an autonomous replicating plasmid carrying the left arm of the *AMA1* sequence of *Aspergillus nidulans* (Gems et al., 1991) and the *P. chrysogenum pyrG* gene as selectable gene (Fig. 1A). pEF43 is an integrative plasmid that contains the bleomycin/phleomycin-resistance gene (*ble*) from *Streptoalloteichus hindustanus* under the control of a fungal promoter (the *cefEF* gene promoter of *Acremonium chrysogenum*) (Fig. 1B). A 2.6-kb *Pst*I fragment of *AMA1* sequence and a 2.3-kb *Xho*I–*Hin*dIII fragment, containing the entire *ble* cassette of pEF43, were used as probes in the hybridisation experiments (Fig. 1).

2.3. Transformation of P. chrysogenum protoplasts

Protoplasts of *P. chrysogenum* Wisconsin 54-1255 *pyrG* were obtained as described previously (Fierro et al., 1993). Transformation was performed according to the procedure of Cantoral et al. (1987) using Czapek plus 1 M sorbitol as protoplast regeneration medium.

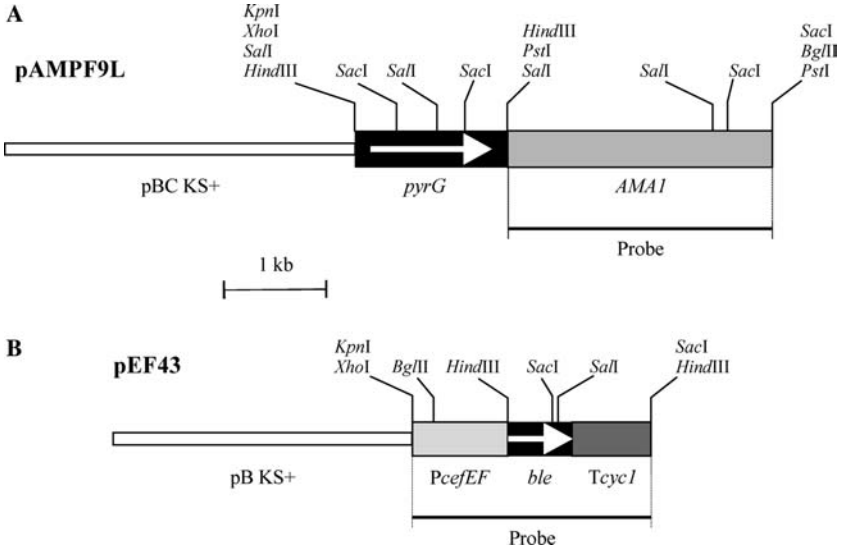

Fig. 1. (A) Physical map of the autonomous-replicating plasmid pAM9L and (B) the integrative plasmid pEF43. The *pyrG* and bleomycin-resistance (*ble*) gene are indicated by white arrows. Note that the *ble* gene is expressed from the *A. chrysogenum cef*EF promoter. *AMA1* corresponds to the autonomous replication region of *A. nidulans*. Tcyc1 is the cyc1 transcriptional terminator of *S. cerevisiae*. Probes used in the Southern blot hybridisations are indicated with solid bars under the corresponding genes.

2.4. Plasmid rescue from P. chrysogenum

Genomic DNA (2–3 μg) from *P. chrysogenum* transformants was used to electroporate competent *E. coli* DH10B cells (Sheng et al., 1995) using a Bio-Rad Gene Pulser. Electroporation conditions were 100 Ω; 2500 V and 25 μF. After electroporation, aliquots of the transformation reaction were spread on LB-ampicillin (50 μg/ml ampicillin) or LB-chloramphenicol (30 μg/ml chloramphenicol).

2.5. Isolation of genomic DNA

Spores from *P. chrysogenum* were inoculated into MPPY medium (Fierro et al., 1993), supplemented with uridine when necessary, and incubated in an orbital shaker at 200 rpm for 48 h at 25 °C. The mycelium was recovered by filtration through Nytal filters and lyophilised. Samples of lyophilised mycelium (500 mg) were suspended in 0.5 ml of 0.18 M Tris–HCl, pH 8.2; 10 mM EDTA; and 1% SDS, treated with 0.5 ml phenol:chloroform:isoamyl alcohol 25:24:1 v/v/v (phenol-CIA), and incubated for 30 min at 50 °C. The phenol-CIA treatment was repeated until the interface was clear. Then, DNA was precipitated with ethanol and re-suspended in TE buffer (Sambrook et al., 1989).

2.6. Southern blot hybridisation and nucleic acid manipulations

Aliquots of genomic DNA (2–4 μg) of *P. chrysogenum* were digested with appropriate restriction endonucleases for Southern blot hybridisations. Genomic or plasmid DNA was separated in 0.8% agarose gels and blotted onto Hybond-N^+ (Amersham-Pharmacia Biotech) membranes using a vacuum blotting system (Pharmacia VacuGene). Digoxigenin labelling, hybridisation, and detection were performed with the Genius Kit (Boehringer–Mannheim) according to the manufacturer's instructions. Hybridisations were done at 65 °C and using as buffer 5× SSC; 0.1% lauryl-sarcosine; 0.02% SDS; and 2% blocking reagent. All other nucleic acid manipulations were carried out by standard methods (Sambrook et al., 1989).

3. Results

3.1. Frequency of transformation of P. chrysogenum Wisconsin 54-1255 pyrG⁻

To study the frequency of co-transformation, *P. chrysogenum* Wisconsin 54-1255 *pyrG*⁻ was transformed, in three independent experiments, with equimolar amounts of pAM9L (autonomous) and pEF43 (integrative) plasmids. Transformation reactions were plated in three different regeneration media: (i) Czapek minimal medium (selection for pAM9L since only transformants complemented with *pyrG* are able to grow in this medium), (ii) Czapek plus phleomycin (selection for the presence of pAM9L and pEF43), and (iii) Czapek supplemented with phleomycin plus uridine (selection for pEF43). Controls were performed by transforming the host strain with each plasmids separately. Plates were incubated for 5 days and the resulting transformants were tested for prototrophy and phleomycin resistance.

Results showed that the frequency of transformation, in co-transformation experiments, was dependent upon the plasmid selected for in the transformation medium. When both plasmids, or only the integrative plasmid (pEF43) were selected for the efficiency of transformation (about 40 transformants per microgram of DNA) was similar to that obtained in the control transformation with the integrative plasmid alone (Table 1). However, selection for the autonomous replicating plasmid marker in the co-transformation experiments led to a high frequency transformation (10^4 transformants/μg of DNA), a result characteristic of a transformation with an autonomous replicating plasmid such as pAM9L (Table 1). The lower efficiency of transformation in co-transformation experiments, as compared to transformation with the autonomous replicating plasmid when the double selection is made, is due to the fact that the phleomycin-resistance conditions are more strict than those for auxotrophy complementation alone, i.e., transformants have to be *pyrG* prototrophs and phleomycin-resistant.

Table 1
Frequency of co-transformation in *P. chrysogenum* using an integrative (pEF43) and an autonomous-replicating plasmid (pAM9L)

Number of transformation experiments	Plasmid	Transformants obtained	Transformants analysed	Co-transformants
3	pEF43	115	70*	0*
3	pAM9L	28,300	52*	0*
3	pEF43 + pAM9L	72	72	70 (97.2%)
3	pEF43 + pAM9L	130	130	91 (70.0%)
3	pEF43 + pAM9L	26,400	1039	719 (69.2%)

Plasmids underlined were those selected for in the regeneration medium.

* A number of separate transformants with either pEF43 or with pAM9L were tested for the presence of the transforming plasmid (no co-transformants).

3.2. Presence of the non-selected plasmid in the co-transformants

To test the presence of the non-selected plasmid in the co-transformation experiments, an analysis of the phenotype of a large number of transformants was performed. Results showed that approximately 70% of them bear both plasmids (Table 1). These results indicated that, in spite of the differences shown in the overall transformation frequency, the efficiency of co-transformation was similar and did not depend on the selectable marker (Table 1). On the other hand, when the presence of both plasmids was selected for, nearly 100% of the colonies from the selection medium were co-transformants (Table 1).

3.3. Phenotypic stability of the transformants

In several fungal species, transformants carrying autonomous replicating plasmids show high mitotic instability and replicating plasmids may be lost after some generations (Fierro et al., 1996; Fournier et al., 1993; Gems et al., 1991; Yang et al., 1994). To study the stability of *P. chrysogenum*, co-transformants carrying both autonomous replicating and integrative plasmids, two co-transformants (named TAR/IN-1 and TAR/IN-2 for autonomously replicating/integrative) and one control transformant (TAR-1) carrying only the autonomous replicating plasmid, were randomly selected. Approximately, 10^6 spores from transformants TAR/IN-1, TAR/IN-2, and TAR-1 were plated in Czapek-FOA medium to study the number of *pyrG*$^-$ derivatives, i.e., transformants that have lost the autonomous replicating plasmid pAM9L. Results showed that a 0.07% of the spores from TAR/IN-1 and TAR/IN-2 transformants and from TAR-1 control transformant were able to grow on FOA-containing medium, indicating that they had lost the autonomous replicating plasmid, resulting in uracil auxotrophy (Table 2). The phenotype of 200 of these FOA-resistant derivatives from TAR/IN-1 and TAR/IN-2 and from TAR-1 was studied, showing that 92.5–99.5% of them were indeed uracil auxotrophs. In the case of the TAR/IN transformants, it was observed that a 95.5–98.5% of the *pyrG*$^-$ derivatives studied had also lost the phleomycin-resistant marker present in the integrative plasmid pEF43 (Table 2).

To test if the phenotypic instability of transformants TAR/IN-1 and TAR/IN-2 occurred also in all the population of co-transformants, a study was done with 30 different TAR/IN co-transformants. After 5 days, it was observed that 28 out of the 30 TAR/IN transformants were able to generate FOA-resistant derivatives at the same frequency described above (0.07–0.1% per generation of spores). The phenotype of 26 FOA^R clones from each of these 28TAR/IN transformants was studied. All clones were uracil (*pyrG*$^-$) auxotrophs, and therefore, it seems that they have lost the autonomous replicating plasmid. Analysis of the phleomycin-resistance phenotype showed that the *pyrG*$^-$ clones obtained from 26 out of 28 TAR/IN transformants (92.8%) had lost also the phleomycin-resistance phenotype present in the integrative plasmid pEF43. These results showed that the instability of the autonomous replicating plasmid is a general phenomenon in the *P. chrysogenum* TAR/IN co-transformants. Interestingly, such instability affects also the integrative plasmid since both of them are lost simultaneously.

3.4. Genetic analysis of TAR/IN co-transformants

In some fungi, co-transformation leads to recombination between the plasmids used. This phenomenon gives rise to co-integrates that, depending on the kind of plasmid used, can integrate in the genome or replicate autonomously in the host. To study what occurs in *P. chrysogenum*, a hybridisation study with eight randomly selected TAR/IN transformants was performed. DNA from the selected transformants (TAR/IN-1–8) and from the plasmids pAM9L and pEF43 as controls was digested with *Bgl*II that cuts once in the pAM9L and pEF43 sequences, and hybridised (i) with a 2.6-kb *Sal*I/*Pst*I probe internal to *AMA1* fragment (Fig. 2A) and

Table 2
Stability of the co-transformants

Strain	Spores tested	FOA resistance[a]	Number of clones tested for phenotype[b]	*pyrG*$^-$/*ble*-resistant[c]	*pyrG*$^-$/*ble*-sensitive[d]
TAR-1	1.16×10^6	1.34×10^3 (0.12 %)	200	—	185 (92.5%)[e,f]
TAR/IN-1	1.42×10^6	1.02×10^3 (0.07 %)	200	1 (0.5%)	197 (98.5%)[2]
TAR/IN-2	1.67×10^6	1.30×10^3 (0.08%)	200	2 (1%)	191 (95.5%)[2]

[a] Derivatives that were able to grow in FOA-containing medium. The percentage of FOA-resistant derivatives over the total number of spores seeded on FOA medium is indicated in parentheses.

[b] 200 FOA-resistant transformants were selected at random from the FOA-containing medium and their phenotypes were determined.

[c] FOA-resistant transformants that were uracil auxotrophs and phleomycin-resistant. Their percentage over the 200 FOA-resistant clones tested is shown in parentheses.

[d] FOA-resistant derivatives that had lost both markers, becoming uracil auxotrophs and phleomycin-sensitive.

[e] Loss of *pyrG*$^+$ phenotype, since TAR-1 strain was transformed only with pAM9L.

[f] The 100% values are completed with false *pyrG*$^-$ phenotype derivatives that showed FOA resistance.

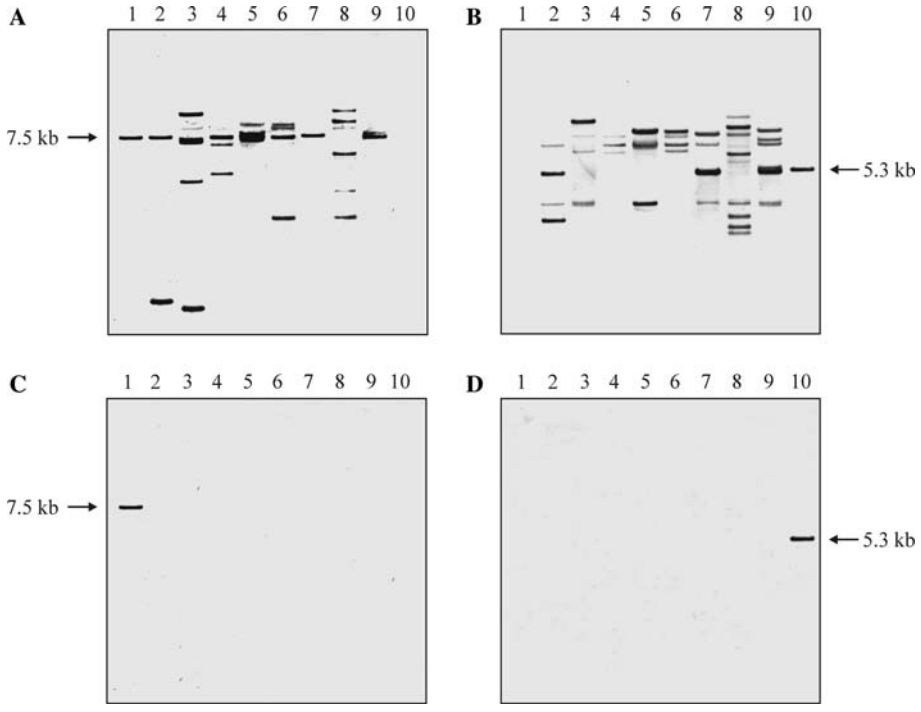

Fig. 2. Southern blot hybridisation of genomic DNA from TAR/IN-1 co-transformants and DAR/IN derivatives. Panels A and B, DNA samples from pAM9L (lane 1), TAR/IN-1–8 co-transformants (lanes 2–9), and pEF43 (lane 10). Plasmids PAM9L and pEF43 were used as positive controls. All DNAs were digested with *Bgl*II and hybridised with probes internal to *AMA1* fragment (panel A) and the *ble* cassette (panel B). Panels C and D, DNA samples from DAR/IN-1 to DAR/IN-8 derivatives (lanes 2–9) and pAM9L (lane 1) and pEF43 (lane 10) plasmids, digested with *Bgl*II and hybridised with the *AMA1* (C) and *ble* (D) probes. The arrows indicate the position of hybridisation bands corresponding to linearised pAM9L (7.5 kb) and pEF43 (5.3 kb).

(ii) with a 2.3-kb *Xho*I–*Pst*I probe corresponding to the *ble* cassette (Fig. 2B). Hybridisation results with the *AMA1* probe showed the presence of positive bands of 7.5 kb in most of the TAR/IN transformants (Fig. 2A) that correspond to a linear form of non-integrated pAM9L plasmid, as it could be deduced by comparison with control pAM9L (Fig. 2A, lane 1). TAR/IN-1, 6, and 8 (Fig. 2B; lanes 2, 7, and 9) showed a genomic hybridisation band with the *ble* probe that corresponds to a linearised and intact non-integrated form of pEF43 (Fig. 2B; lane10). As expected, hybridisation bands with sizes that did not coincide with the pAM9L or pEF43 *Bgl*II restriction pattern were also detected with both *AMA1* (Fig. 2A) and *ble* (Fig. 2B) probes. These complex plasmids are formed possibly by concatenation of pAM9L and pEF43 (Figs. 2A and B) or tandem integration of pF43.

3.5. The integrative plasmid pEF43 does not integrate into the genome of co-transformants

Since most of the *pyrG*$^{-}$ clones obtained from TAR/IN transformants had also lost the phleomycin-resistant marker present in the integrative plasmid pEF43, it seems that pEF43 plasmid is not integrated in most of the TAR/IN transformants, and therefore replicates autonomously. To confirm this hypothesis, a Southern blot hybridisation was performed with genomic DNA from eight *pyrG*$^{-}$/*ble*$^{-}$ derivatives, named DAR/IN-1–8 (obtained from TAR/IN-1–8, respectively). DNA from the eight clones (Figs. 2C and D; lanes 2–9) and from pAM9L (Figs. 2C and D; lane 1) and pEF43 (Figs. 2C and D; lane 10) plasmids as controls was digested with *Bgl*II, blotted, and hybridised with a probe internal to the *AMA1* region (Fig. 2C) and with a probe of the *ble* cassette (Fig. 2D). Results showed that no hybridisation signals in any derivative were obtained with the *ble* and *AMA1* probes (Figs. 2C and D), indicating that pAM9L, and also pEF43, was lost in the DAR/IN clones; therefore, both plasmids could be replicating autonomously in the TAR/IN co-transformants studied.

To study this phenomenon in more detail, a new hybridisation was performed with DNA from TAR/IN-1, TAR/IN-6, and TAR/IN-8 transformants and from pEF43 plasmid as control, using additional restriction enzymes and the *ble* probe. The hybridisation (Fig. 3B) showed a restriction and hybridisation pattern in the TAR/IN transformants similar to that of pEF43 control plasmid (Fig. 3A). These results indicated that transformants TAR/IN-1, TAR/IN-6, and TAR/IN-8 contained free pEF43 plasmid that had neither integrated into chromosomes nor has recombined with pAM9L.

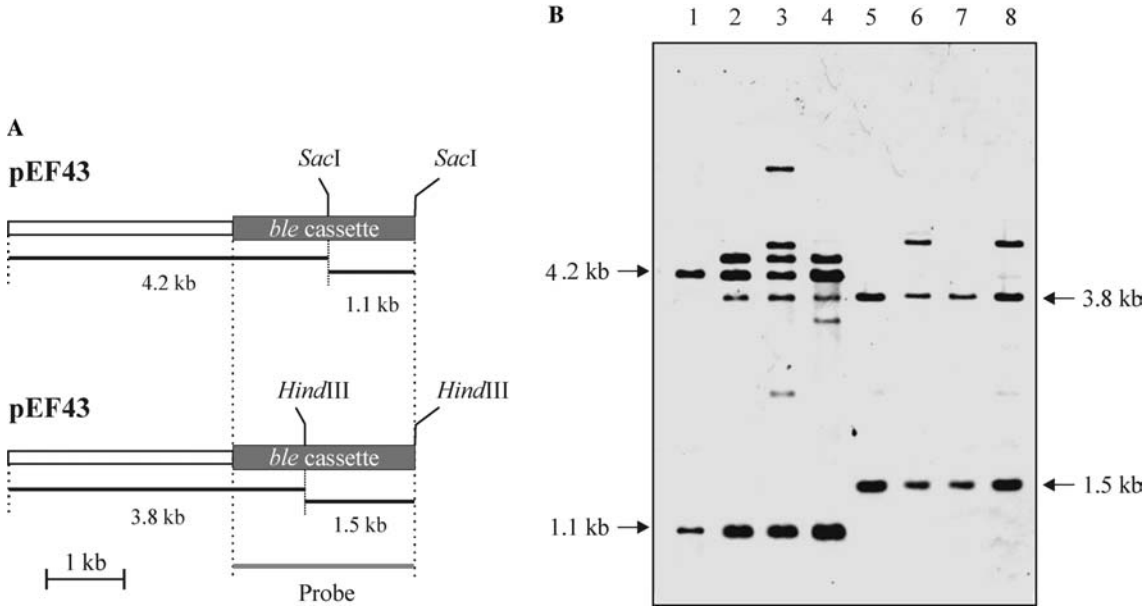

Fig. 3. (A) Restriction map of pEF43. Solid bars indicate the hybridisation bands expected in *Sac*I and *Hin*dIII digestions. (B) Southern blot hybridisation of genomic DNA from Tar/in-1 (lanes 2 and 6), Tar/in-6 (lanes 3 and 7), and Tar/in-8 (lanes 4 and 8) co-transformants and pEF43 plasmid (lanes 1 and 5) with a probe internal to *ble* cassette. DNA samples were digested with *Sac*I (lanes 1–4) and *Hin*dIII (lanes 5–8). Arrows indicate the bands corresponding to the fragments in panel A.

3.6. Re-isolation of AMA1 containing plasmids shows evidence for intermolecular plasmid recombination

Recombination between co-transforming DNA has been reported in other organisms but not in *P. chrysogenum*. We therefore, tested for AMA-1 bearing plasmids carrying both the *ble* and *pyrG* genes which could be generated by recombination between pEF43 and pAM9L.

Autonomous replicating plasmids from the eight Tar/in transformants (Tar/in-1–8) were rescued, using *E. coli* DH10B as bacterial host. Due to the different bacterial-resistance genes (Ap in pEF43 and Cm in pAM9L) present in the original plasmids, it was easy to isolate plasmids derived from pEF43. In several Tar/in transformants, more than one type of plasmid was rescued. As shown in Table 3, plasmids conferring Cm resistance in *E. coli* and with identical restriction and hybridisation pattern to pAM9L were rescued from all transformants (except from Tar/in-7) that did not show the hybridisation band corresponding to linearised pAM9L (Fig. 2A; lane 8). Plasmids conferring Cm or Ap resistance, but with restriction pattern different from those of pAM9L or pEF43 were also re-isolated (Fig. 4; lanes 1–16). Hybridisation analysis with the *AMA1* and *ble* probes (Figs. 4B and C) demonstrated that these plasmids contained both *AMA1* and *ble* sequences, in some cases in the same hybridising fragment (Fig. 4; lanes 1, 4, and 5). These results indicated that these plasmids have been formed by recombination between pAM9L and pEF43.

Table 3
Plasmids re-isolated from Tar/in transformants

Tar/in transformants	Cm resistance		Ap resistance	
	pAM9L	Other[a]	pEF43	Other[a]
Tar/in-1	Yes	1	Yes	1
Tar/in-2	Yes	3	—	1
Tar/in-3	Yes	0	—	0
Tar/in-4	Yes	0	—	1
Tar/in-5	Yes	0	—	0
Tar/in-6	Yes	0	Yes	0
Tar/in-7	—	1	—	1
Tar/in-8	Yes	0	Yes	2

[a] Chloramphenicol- or ampicillin-resistance conferring plasmids that showed restriction patterns different from those of pAM9L or pEF43.

3.7. AMA1-Lacking pEF43 plasmids are rescued from P. chrysogenum co-transformants

As shown above (Table 3), AMA1 containing plasmids conferring resistance to ampicillin and bleomycin (i.e., products of intermolecular recombination between pEF43 and pAM9L plasmids) were rescued in *E. coli* from several Tar/in transformants. However, plasmids conferring Ap resistance, with identical restriction pattern to that of pEF43, were unexpectedly isolated from transformants Tar/in-1, Tar/in-6, and Tar/in-8. Several of these plasmids showed hybridising bands with the *ble* probe corresponding to non-reorganised pEF43 (Fig. 2B, lanes 2, 7, and 9; and Fig. 3B, lanes 2–4 and 6–8).

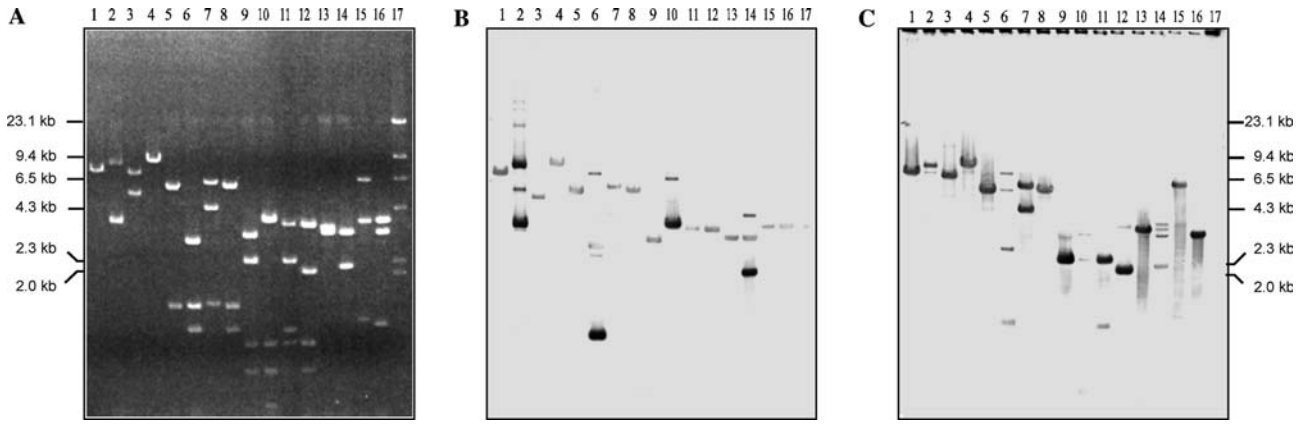

Fig. 4. Southern blot hybridisation of plasmid DNA rescued from TAR/IN transformants, whose restriction pattern was different from that of pEF43 or pAM9L (panel A). Four plasmids were selected, showing chloramphenicol resistance (pC1: lanes 1, 5, 9, and 13; pC2: lanes 2, 6, 10, and 14; and pC3: lanes 3, 7, 11, and 15) or ampicillin resistance (pA1: lanes 4, 8, 12, and 16). DNA samples were digested with *Hin*dIII (lanes 1–4), *Bgl*II (lanes 5–8); *Sal*I (lanes 9–12) and *Sac*I (lanes 13–16), blotted onto nylon membranes and hybridised with a *ble* probe (panel B) and an *AMA1* probe (panel C).

A Southern blot analysis was performed to further characterise the rescued pEF43-like plasmids and to test if they contained *AMA1* sequences that could explain their autonomous replication ability. Rescued plasmids, named pEF-I, pEF-II, and pEF-III (from *P. chrysogenum* TAR/IN-1, TAR/IN-6, and TAR/IN-8, respectively), and pEF43 control plasmid were digested with several restriction enzymes and hybridised with *AMA1* and *ble* probes (Fig. 5). Results with the *ble* probe showed a hybridisation pattern identical to that of the pEF43 control plasmid (Fig. 5B). On the other hand, hybridisation with the *AMA1* probe did not show positive signals in the control pEF43 plasmid, but *AMA1*-hybridising bands could be observed that did not correspond to any of the bands of the pEF43-like plasmids observed in the ethidium bromide-stained agarose gel (Fig. 5C). These results suggested the presence of *AMA1* containing sequences rescued from TAR/IN transformants, simultaneously with pEF43, but at a much lower concentration, since they could not be detected in the agarose gel (Fig. 5A).

To identify these *AMA1*-like containing sequences, DNA from pEF-I, II, and III plasmids (isolated from TAR/IN-1, TAR/IN-6, and TAR/IN-8 transformants, respectively) was used to transform *E. coli* DH5α. Transformations were plated separately in ampicillin and chloramphenicol-containing media, obtaining colonies in both conditions. Plasmid DNA was isolated, and hybridisation analyses were performed; again using probes internal to the *ble* and *AMA1* sequences (Figs. 6A–C). Plasmid pEF43 was isolated from ampicillin-resistant colonies and, in this case, it did not show hybridisation with *AMA1* probe. On the other hand, chloramphenicol-resistant colonies were shown to contain a plasmid with a restriction and hybridisation pattern similar to that of pAM9L. These results proved that both plasmids, pEF43 and pAM9L, were simultaneously isolated from *P. chrysogenum* transformants,

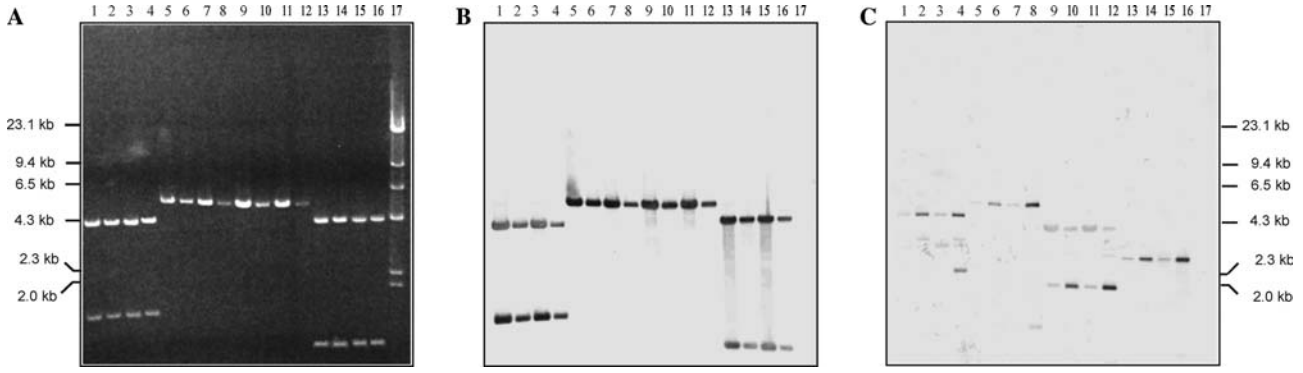

Fig. 5. Analysis of three pEF43-like plasmids (A) rescued from TAR/IN-1 (pEF-I; lanes 2, 6, 10, and 14), TAR/IN-6 (pEF-II; lanes 3, 7, 11, and 15) and TAR/IN-8 (pEF-III; lanes 4, 8, 12, and 16), and the control plasmid pEF43 (lanes 1, 5, 9, and 13). Plasmids were digested with *Hin*dIII (lanes 1–4), *Bgl*II (lanes 5–8); *Sal*I (lanes 9–12), and *Sac*I (lanes 13–16) and hybridised with a *ble* probe (panel B) and an *AMA1* probe (C). Lane 17, molecular size markers.

Fig. 6. Restriction (A) and hybridisation analysis of ampicillin-resistant (lanes 1–10) and chloramphenicol-resistant (lanes 11–17) plasmids isolated from pEF43-like plasmid preparations by re-transformation in *E. coli*. Ampicillin-resistant plasmids obtained from pEF-I (plasmids in lanes 1–3), pEF-II (plasmids in lanes 4–6), and pEF-III (lanes 7–9), and the control plasmid pEF43 (lane 10), were digested with *Bgl*II. In the same way, Cm-resistant plasmids purified from pEF-I (lanes 11 and 12), pEF-II (lanes 13 and 14), and pEF-III (lanes 15 and 16), and the control plasmid pAM9L (lane 17) were also digested with *Bgl*II. Then, DNA samples were hybridised with probes internal to *ble* gene (B) and *AMA1* fragment (C).

where they could be associated replicating autonomously.

4. Discussion

Co-transformation has been used to introduce non-selectable DNA in yeasts and filamentous fungi such as *S. cerevisiae* (Siliciano and Tatchell, 1984), *Candida albicans* (Kurtz et al., 1987), and *A. nidulans* (Wernars et al., 1987).

In *A. nidulans*, a high-frequency of co-transformation has been reported when an integrative and an autonomous replication plasmid containing the *AMA1* sequence (so-called helper plasmid) are used in the transformation assay. Recombination between co-transforming plasmids has been shown to occur in this fungus, giving rise to autonomously replicating co-integrates (Aleksenko, 1994; Gems and Clutterbuck, 1993).

In this work, we show that co-transformation is an effective procedure to introduce non-selectable DNA in *P. chrysogenum*. A high-frequency of co-transformation (nearly 70%) was achieved independently of the plasmid selected when transforming with pAM9L and pEF43 plasmids. This co-transformation frequency is similar to those observed in other filamentous fungi such as *A. nidulans* (Wernars et al., 1987) and in the yeasts *S. cerevisiae* (Hicks et al., 1978) and *Schizossaccharomyces pombe* (Sakai et al., 1984).

Although there were no differences in the co-transformation efficiency, the total transformation frequency was shown to be dependent on the plasmid selected for in the regeneration medium. When the autonomous replicating plasmid marker was selected for, a high frequency of transformation was obtained, a known characteristic of transformations with an autonomous replication plasmid (Fierro et al., 1996). However, when the integrative plasmid marker was selected for the transformation frequency decreased to similar levels to those obtained in an integrative transformation. In other microorganisms such as *S. cerevisiae* (Jiménez and Davies, 1980), *A. nidulans* (Gems and Clutterbuck, 1993), *S. pombe* (Sakai et al., 1984), and *C. albicans* (Kurtz et al., 1987), a high-frequency of transformation was observed, even when the integrative plasmid bears the selectable marker.

In this work, we provide evidence showing that co-integrate formation from co-transforming plasmids occurs in *P. chrysogenum*, giving rise to complex autonomous replicating plasmids. Results of Southern blot hybridisation and plasmid rescue assays showed that in each *P. chrysogenum* TAR/IN co-transformant co-exists in different populations of plasmids, as occurs in other fungi (Aleksenko, 1994; Gems and Clutterbuck, 1993; Jiménez and Davies, 1980; Sakai et al., 1984) and higher organisms (Katz and Ratner, 1988; Riggs and Bates, 1986; Stinchcomb et al., 1985).

A very interesting finding is the observation that autonomous replicating plasmid pAM9L is present in co-transformants as a non-integrated element, as shown by hybridisation and re-isolation experiments. Additionally, the pEF43 integrative plasmid has been shown to be present as non-integrated element into the genome nor recombined with pAM9L in some of the studied co-transformants (3 out of 8). Intact pEF43 was re-isolated from those *P. chrysogenum* transformants in which a hybridisation band corresponding to a non-reorganised pEF43 was observed. Although we used an *E. coli* strain which is a *recA* mutant, the possibility that pEF43 re-isolation from TAR/IN transformants was due to resolution in *E. coli* of recombinant plasmids could not be excluded. However, the presence of the band (Fig. 2) corresponding to no integrated nor recombined pEF43

suggested the possibility that the pEF43 could be somehow kept autonomously.

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References

Aharonowitz, Y., Cohen, G., Martín, J.F., 1992. Penicillin and cephalosporin biosynthetic genes: structure, organisation, regulation, and evolution. Annu. Rev. Microbiol. 46, 461–495.

Aleksenko, A.Y., 1994. Cointegration of transforming DNAs in *Aspergillus nidulans*: a model using autonomously-replicating plasmids. Curr. Genet. 26, 352–358.

Aleksenko, A.Y., Clutterbuck, A.J., 1996. The plasmid replicator AMA1 in *Aspergillus nidulans* is an inverted duplication of a low-copy-number dispersed genomic repeat. Mol. Microbiol. 19, 565–574.

Aleksenko, A.Y., Clutterbuck, A.J., 1997. Autonomous plasmid replication in *Aspergillus nidulans*: AMA1 and MATE elements. Fungal Gen. Biol. 21, 373–387.

Bañuelos, O., Casqueiro, J., Gutiérrez, S., Martín, J.F., 2000. Overexpression of the *lys1* gene in *Penicillium chrysogenum*: homocitrate synthase levels, α-aminoadipic acid pool and penicillin production. Appl. Microbiol. Biotechnol. 54, 69–77.

Bañuelos, O., Casqueiro, J., Gutiérrez, S., Martín, J.F., 2001. Intrachromosomal recombination after targeted monocopy integration in *Penicillium chrysogenum*: stabilization of the direct repeats to prevent loss of the inserted gene. Curr. Genet. 39, 231–236.

Bowyer, P., Osbourn, A.E., Daniels, M.J., 1994. An "instant gene bank" method for heterologous gene cloning: complementation of two *Aspergillus nidulans* mutants with *Gaeumannomyces graminis* DNA. Mol. Gen. Genet. 242, 448–454.

Cantoral, J.M., Díez, B., Barredo, J.L., Alvarez, E., Martín, J.F., 1987. High-frequency transformation of *Penicillium chrysogenum*. Bio/Technology 5, 494–497.

Casqueiro, J., Bañuelos, O., Gutiérrez, S., Hijarrubia, M.J., Martín, J.F., 1999a. Intrachromosomal recombination in *Penicillium chrysogenum*: gene conversion and deletion events. Mol. Gen. Genet. 261, 994–1000.

Casqueiro, J., Gutiérrez, S., Bañuelos, O., Hijarrubia, M.J., Martín, J.F., 1999b. Gene targeting in *Penicillium chrysogenum*: disruption of the *lys2* gene leads to penicillin overproduction. J. Bacteriol. 181, 1181–1188.

Criswell, T.L., Bradshaw, S., 1998. Transfer of P1 inserts into a yeast–bacteria shuttle vector by co-transformation mediated homologous recombination. Nucleic Acids Res. 26, 3611–3613.

Díez, B., Alvarez, E., Cantoral, J.M., Barredo, J.L., Martín, J.F., 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.

Díez, B., Gutiérrez, S., Barredo, J.L., van Solingen, P., van der Voort, L.H.M., Martín, J.F., 1990. The cluster of penicillin biosynthetic genes. J. Biol. Chem. 265, 16358–16365.

Fierro, F., Gutiérrez, S., Díez, B., Martín, J.F., 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, 573–578.

Fierro, F., Kosalková, K., Gutiérrez, S., Martín, J.F., 1996. Autonomously replicating plasmids carrying the AMA1 region in *Penicillium chrysogenum*. Curr. Genet. 29, 482–489.

Fournier, P., Abbas, A., Chasles, M., Kudla, B., Ogrydziak, D.M., Yaver, D., Xuan, J.W., Peito, A., Ribet, A.M., Feynerol, C., Gaillardin, C., 1993. Colocalization of centromeric and replicative functions on autonomously replicating sequences isolated from the yeast *Yarrowia lipolytica*. Proc. Natl. Acad. Sci. USA 90, 4912–4916.

Gems, D.H., Clutterbuck, A.J., 1993. Co-transformation with autonomously-replicating helper plasmids facilitates gene cloning from an *Aspergillus nidulans* gene library. Curr. Genet. 24, 520–524.

Gems, D.H., Johnstone, I.L., Clutterbuck, A.J., 1991. An autonomously replicating plasmids transform *Aspergillus nidulans* at high frequency. Gene 98, 61–67.

Gems, D., Aleksenko, A., Belenky, L., Robertson, S., Ramsden, M., Vinetski, Y., Clutterbuck, A.J., 1994. An 'instant gene bank' method for gene cloning by mutant complementation. Mol. Gen. Genet. 242, 467–471.

Hicks, J.B., Hinnen, A., Fink, G.R., 1978. Properties of yeast transformation. Cold Spring Harbor Symp. Quant. Biol. 43, 1305–1313.

Jiménez, A., Davies, J., 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. Nature 287, 869–871.

Katz, K.S., Ratner, D.I., 1988. Homologous recombination and the repair of double-strand breaks during co-transformation of *Dictyostelium discoideum*. Mol. Cell. Biol. 8, 2779–2786.

Kelly, R., Miller, S.M., Kurtz, M.B., 1988. One-step gene disruption by co-transformation to isolate double auxotrophs in *Candida albicans*. Mol. Gen. Genet. 214, 24–31.

Kretschmer, F.J., Chang, A.C., Cohen, S.N., 1975. Indirect selection of bacterial plasmids lacking identifiable phenotypic properties. J. Bacteriol. 124, 225–231.

Kurtz, M.B., Cortelyou, M.W., Miller, S.M., Lai, M., Kirsch, D.R., 1987. Development of autonomously replicating plasmids for *Candida albicans*. Mol. Cell. Biol. 7, 209–217.

Ma, H., Kunes, S., Schatz, P.J., Botstein, D., 1987. Plasmid construction by homologous recombination in yeast. Gene 58, 201–216.

Martín, J.F., Casqueiro, J., Kosalková, K., Marcos, A.T., Gutiérrez, S., 1999. Penicillin and cephalosporin biosynthesis: mechanism of carbon catabolite regulation of penicillin production. Antonie van Leeuwenhoek 65, 227–243.

Prado, F., Aguilera, A., 1994. New in vivo cloning methods by homologous recombination in yeast. Curr. Genet. 25, 180–183.

Riggs, C.D., Bates, G.W., 1986. Stable transformation of tobacco by electroporation: evidence for plasmid concatenation. Proc. Natl. Acad. Sci. USA 83, 5602–5606.

Rudolph, H., Koenig-Rauseo, I., Hinnen, A., 1985. One-step gene replacement in yeast by co-transformation. Gene 36, 87–95.

Sakai, K., Sakaguchi, J., Yamamoto, M., 1984. High-frequency co-transformation by copolymerization of plasmids in the fission yeast *Schizosaccharomyces pombe*. Mol. Cell. Biol. 4, 651–656.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sheng, Y., Mancino, V., Birren, B., 1995. Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucleic Acids Res. 23, 1990–1996.

Siliciano, P.G., Tatchell, K., 1984. Transcription and regulatory signals at the mating type locus in yeast. Cell 37, 969–978.

Stinchcomb, D.T., Shaw, J.E., Carr, S.H., Hirsh, D., 1985. Extrachromosomal DNA transformation of *Caenorhabditis elegans*. Mol. Cell. Biol. 5, 3484–3496.

Wigler, M., Sweet, R., Sim, G.K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., Axel, R., 1979. Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell 16, 777–785.

Wernars, K., Goosen, T., Wennekes, B.M., Swart, K., van den Hondel, C.A., van den Broek, H.W., 1987. Co-transformation of *Aspergillus nidulans*: a tool for replacing fungal genes. Mol. Gen. Genet. 209, 71–77.

Yang, V.W., Marks, J.A., Davis, B.P., Jeffries, T.W., 1994. High-efficiency transformation of *Pichia stipitis* based on its URA3 gene and a homologous autonomous replication sequence, ARS2. Appl. Environ. Microbiol. 60, 4245–4254.