Curr Genet (1993) 24: 520–524

Current Genetics

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Co-transformation with autonomously-replicating helper plasmids facilitates gene cloning from an *Aspergillus nidulans* gene library

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Received: 24 June 1993

Abstract. Autonomously-replicating, marker-less "helper" plasmids were added to transformations of *Aspergillus nidulans* with plasmids which normally transform by chromosomal integration. This resulted in as much as a 200-fold increase in transformation efficiency. Recovery of autonomously-replicating plasmid co-integrates indicated that co-transformation involves recombination between integrating and helper plasmids, which occurs at a high frequency. Increasing DNA sequence-homology between pairs of plasmids used in simultaneous transformations enhanced co-transformation efficiency. Using helper plasmids and an *A. nidulans* gene library in a normally-integrating vector, the genes *adC* and *adD* were cloned as part of such a co-integrate. In effect, the addition of helper plasmid converts an integrating into an autonomously-replicating gene library in vivo.

Key words: Co-transformation – Gene cloning – DNA recombination – Plasmid replication – *Aspergillus nidulans*

Introduction

High-frequency co-transformation of selected and unselected DNA fragments is a well-established phenomenon. Initially seen in *Escherichia coli* (Kretschmer et al. 1975), it was interpreted as reflecting the presence of a tiny subpopulation of highly-competent cells. This model was invoked to explain co-transformation in budding yeast (Hicks et al. 1978), mammalian cells (Wigler et al. 1979), and many other organisms. Since transformation with replicating plasmids is much more efficient than with integrating ones (Beggs 1978; Gems et al. 1991), it is possible that the limiting step in integrative transformation is recombination with the chromosome. In this case, if recombination between plasmids is relatively frequent, co-transformation can be explained by the integration of co-integrates previously formed by plasmid-plasmid recombination.

We here demonstrate that if one of the co-transforming plasmids is capable of autonomous replication, co-transformation is very efficient and leads to the formation of replicating recombination products of the two plasmids employed. We have made use of this observation by constructing helper plasmids for addition to an *A. nidulans* plasmid gene library, and have employed this combination to clone the closely-linked *adD and adC* genes.

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Materials and methods

Aspergillus strains and culture. All strains were derived from the Glasgow stocks, and standard growth media and culture techniques were used (Pontecorvo et al. 1953; Clutterbuck 1974). The strains employed were DHG019 (*yA2 pabaA1*; *argB2 methH2*; *trpC801 brlA42*), DHG135 (*biA1*; *methH2 argB2*; *trpC801*), G135 (*luA1 yA2*; *adD3*), G225 (*yA2*; *adC1*; *pyroA4*), G34 (*yA2*; *methH2 argB2*) and G196 (*trpB403 papaA1 yA2*).

E. coli strains and transformation. E. coli strains DH1 and DH5 (F^-, *recA1*, *endA1*, *gyrA96*, *thiA1*, *hsd17*, *supE44*) were used for re-isolation of plasmids from *A. nidulans* and plasmid propagation. Competent cells for transformation were either prepared using a modification of the $CaCl_2$/RbCl method (Sambrook et al. 1989) as described in Johnstone et al. (1985) [yield (DH1): $5 \times 10^6 - 1 \times 10^7$ transformants/μg pUC8] or purchased from Gibco-BRL [yield (DH5): 1×10^8 transformants/μg pBR322]. For plasmid rescue from *Aspergillus*, 100–500 ng of total DNA from an *Aspergillus* transformants was added to 100 μl of competent *E. coli* cells. Up to 25 plasmid-containing DH1 or DH5 colonies were obtained when plating on L-agar contaning 50 μg/ml of ampicillin.

Aspergillus transformations. Transformation was as previously described (Johnstone et al. 1985). In double transformations 200–300 ng of each transforming DNA species was added per 10^6 protoplasts used. This represents near-saturating levels of DNA (Gems et al. 1991).

Plasmids. pACYC184, derived from the P15-A cryptic miniplasmid, is described by Chang and Cohen (1978). pUC8 and pIC-20R, both derived from pBR322, are described by Vieira and Messing (1982) and Marsh et al. (1984) respectively. The plasmid pTA11 was kindly provided by Dr. T. H. Adams of the University of Athens, Georgia, USA. pILJ16 is described by Johnstone et al. (1985). pMS1 was constructed by Moira Stark in this laboratory.

Testing mitotic stability of transformant phenotypes. In the case of ARG$^+$ transformants this was as described in Gems et al. (1991). Conidia from TRP$^+$ transformants were plated on *Aspergillus* complete medium (CM) supplemented with tryptophan (4 mM) to give a density of approximately 50 colonies per plate. These were stab-inoculated in an ordered array onto tryptophan-supplemented CM, and then tested by replica plating onto *Aspergillus* minimal medium with or without tryptophan.

Isolation of DNA. Plasmid DNA was isolated from bulk cultures by standard means (Birnboim and Doly 1979) and purified by density-gradient ultracentrifugation (Sambrook et al. 1989). *Aspergillus* DNA was prepared using the method of Raeder and Broda (1985). DNA prepared in this way was used for Southern blots, but in the case of DNA to be used for plasmid rescue a further phenol extraction, followed by two chloroform extractions, and ethanol-precipitation were carried out.

Results

Co-transformation of *A. nidulans* with autonomous and integrating plasmids

Aspergillus strains transformed with the autonomously-replicating plasmid ARp1 have been shown to be mitotically unstable, 65% of conidial (asexual) progeny having lost the plasmid (Gems et al. 1991). An *argB2 trpC801* strain of *A. nidulans* (DHG019) was simultaneously transformed with ARp1 (Fig. 1), which carries the gene *argB*$^+$, and pTA11, which contains *trpC*$^+$ but lacks a fungal origin of replication. Selection for TRP$^+$, in five transformations using pTA11 plus ARp1 gave on average 4000 transformants per 10^6 protoplasts, compared to 50 transformants per 10^6 protoplasts with pTA11 alone, an 80-fold increase in transformation efficiency. Equimolar quantities of plasmids were used. Eighty-five percent of such TRP$^+$ co-transformants proved also to be ARG$^+$, and both phenotypes were found to be highly unstable in most transformants, usually being lost together rather than separately (Table 1). Both these characteristics point to genetic linkage of pTA11 to the ARp1 replicon.

In order to utilise this enhancement of transformation by the addition of a replicating plasmid, three subclones of ARp1 were constructed (Fig. 1). These 'helper' plasmids contain a fungal origin of DNA replication but no selectable *Aspergillus* gene. pHELP1 contains the *AMA1* inverted repeat in the pBR322-based vector pIC-20R; pHELP2, comprising *AMA1* inserted into the non-pBR322-based pACYC184, was constructed to test the importance of DNA sequence homology for co-transformation (pILJ16 and pTA11 both contain pBR322-based vector sequences); pHELP3 contains only one arm of *AMA1*, and was made to test whether both arms are required for autonomous replication.

These plasmids were used in conjunction with the origin-less *argB*$^+$ plasmid pILJ16 (Johnstone et al. 1985)

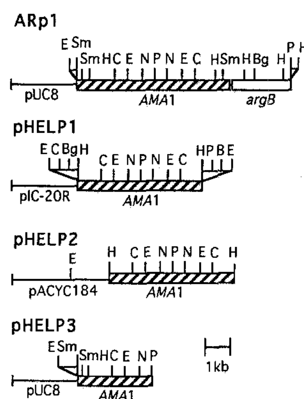

Fig. 1. Linear restriction maps of ARp1 and helper plasmid derivatives pHELP1, pHELP2 and pHELP3. pACYC184 (Chang and Cohen 1978) has almost no sequence homology to the pBR322-based pUC8 and pIC-20R (Gems et al. 1991). pHELP1 and pHELP2 were formed by ligating the 5.2-kb *Hin*dIII fragment of *AMA1* into pIC-20R and pACYC184 respectively. pHELP3 was formed by *Pst*I digestion of ARp1, followed by ligation to itself. *Single lines*, bacterial sequences; *boxes*, *A. nidulans* sequences. Abbreviations: *B*, *Bam*HI; *Bg*, *Bgl*II; *C*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *N*, *Nru*I, *P*, *Pst*I; *Sm*, *Sma*I

Table 1. Stability of the ARG$^+$ and TRP$^+$ phenotypes in pTA11-ARp1 co-transformants of *A. nidulans* strain DHG019 (*argB2*) *trpC801*). Twenty-four progeny each of eight ARG$^+$TRP$^+$ transformants were examined (all retained the parental markers *methH2* yA2 and *brlA42*)

Transformant	Number of progeny			
	ARG$^+$TRP$^+$	ARG$^+$TRP$^-$	ARG$^-$TRP$^+$	ARG$^-$TRP$^-$
1	24	0	0	0
2	8	0	0	16
3	1	2	0	21
4	4	0	0	20
5	24	0	0	0
6	4	1	3	16
7	8	0	1	15
8	21	1	0	2

in co-transformations of an *A. nidulans argB2* strain (G34). Addition of pHELP1, pHELP2 and pHELP3 was found to result in 200-fold, 13-fold and 100-fold increases respectively in efficiency over transformation with pILJ16 alone (approximately 60 ARG$^+$ transformants per 10^6 protoplasts at near-saturating levels of transforming DNA) (Table 2). This increase is comparable to that obtained with ARp1, which transforms with a 250-fold higher efficiency than pILJ16 (Gems et al. 1991). All transformants tested showed vegetative instability, indicative of the presence of autonomously-replicating plasmids; among conidial progeny of transformants with pILJ16 and pHELP1, pHELP2 and pHELP3, 79.2%, 63.3% and 84.5% respectively had lost the ARG$^+$ phenotype (total sample size: 1594). Southern analysis of DNA prepared from these transformants grown under selective conditions (probe: pUC8) revealed the presence of large, extrachromosomal plasmids (data not shown), and a plasmid co-integrate of pILJ16 and pHELP2, des-

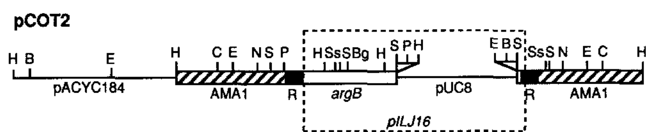

Fig. 2. Linear restriction map of pCOT2. This is the product of non-homologous recombination between pHELP2 and pILJ16. The pILJ16 component of this plasmid cointegrate is indicated by a *dashed line box*. *Single lines*, bacterial sequences; *open boxes*, *argB* sequences; *shaded boxes*, *AMA1* sequences; *black boxes*, duplicated sequence. Abbreviations for restriction sites as in Fig. 1, and *Ss*, *Sst*I

Table 2. Frequency of transformation of strain G34 to ARG$^+$ using pILJ16 and helper plasmids pHELP1, pHELP2 and pHELP3

Transformation	Number of transformants per 10^6 protoplasts with plasmid added to pILJ16			
	None	pHELP1	pHELP2	pHELP3
1	66	–	–	6 500
2	30	3 000	100	5 000
3	100	20 000	300	–
4	40	13 000	2 000	–

ignated pCOT2, was re-isolated into *E. coli* strain DH1 from an unstable pILJ16/pHELP2 transformant. The restriction map (Fig. 2) indicates that it is the product of a single non-homologous recombination event between pILJ16 and the *AMA*1 region of pHELP2. It also suggests that an 0.8-kb duplication has occurred at the point of recombination. These results indicate that the effect of autonomously-replicating plasmids on transformation with normally-integrating plasmids is the result of recombination between the two.

The greater enhancement of transformation by pHELP1 and pHELP3 compared to pHELP2 may reflect the greater sequence homology between the former two plasmids and pILJ16, all three of which contain pBR322-based plasmids. The similarity of pACYC184 to the pUC plasmids was checked by comparing their sequences using the MacMolly Compare program, and the G.C.G. program Pileup. No significant DNA sequence similarity was detected, apart from one 12-base pair sequence (position 85–96 in pACYC184). A higher frequency of recombination resulting from increased DNA sequence homology would parallel the behaviour of many integrating plasmids which, in *A. nidulans*, generally integrate by homologous recombination (Yelton et al. 1984; Upshall 1986), but at a lower frequency may also recombine with non-homologous chromosomal regions.

Helper plasmids do not enhance transformation efficiency where the transforming gene sequence is incomplete

Transformations were carried out using pMS1, a 4.3-kb subclone of pILJ16, which contains only part of the *argB* gene. pMS1 may only transform *Aspergillus* to ARG$^+$ by recombination with the *argB2* mutant chromosomal allele (Birse 1989). Transformations with pMS1 alone gave 0.7 ARG$^+$ transformants/10^6 protoplasts. Addition of pHELP1 or pHELP3 did not increase the transformation frequency, confirming that the effect of the helper plasmids is independent of chromosomal integration, which they are unable to enhance.

These results suggest that co-transformation results from recombination between transforming plasmids, occurring at a rate greater than that of plasmid integration into the chromosome, and resulting in accumulation of plasmid heterodimers or oligomers. That the low frequency of recombination between chromosome and transforming plasmid may be a rate-limiting step in transformation is suggested by the high frequency of transformation of autonomously-replicating plasmids, generally 2–4 orders of magnitude higher than that of integrating plasmids (Beggs 1978; Gems et al. 1991).

Re-transformation with an integrating plasmid of an Aspergillus transformant already containing an autonomously-replicating plasmid

Transforming plasmids appear to recombine with each other much more readily than with the chromosome. To test whether this recombinogenicity was due to a specific property of transforming DNA, protoplasts were prepared from *A. nidulans* strain DHG135 (*argB2 trpC801*) which had previously been transformed to ARG$^+$ with the autonomously-replicating ARp1-derivative pDHG25 (Gems et al. 1991). These were then re-transformed with pTA11 (*trpC*$^+$), selecting for ARG$^+$TRP$^+$. The frequency of transformation was found to be approximately the same as control co-transformations of strain DHG135 with pDHG25 plus pTA11. Thus it appears that recombination to give co-integrate is as effective when the replicating component is resident in the host cell as when it is used as co-transforming DNA. This suggests that inter-plasmid recombination is not determined by conditions specific to transformation, e.g., the reaction of the cell to exogenous or naked DNA, but is a feature of plasmids *per se* e.g., circularity and the potential for supercoiling.

Cloning of adC and adD by co-transformation of a gene bank in pIJ16 with pHELP3

For this experiment we used pHELP3 because it is the smallest helper plasmid (5.8 kb), which we believed would facilitate recovery of cointegrates into *E. coli*. An *adD3* strain of *A. nidulans* (G135) was simultaneously transformed with pHELP3 and an *A. nidulans* gene bank in the integrating vector pILJ16 (construction described in Johnstone et al. 1985), selecting for AD$^+$. Two AD$^+$ colonies were obtained, TAD1 and TAD2, and in both cases the AD$^+$ phenotype was mitotically highly unstable (98.5% and 93.2% respectively of progeny were AD$^-$, total sample size: 384). Southern analysis of uncut chromosomal DNA showed the presence of a free-plasmid component of approximately 20 kb (data not shown). DNA was prepared from TAD1 after subculturing through three rounds of asexual reproduction under se-

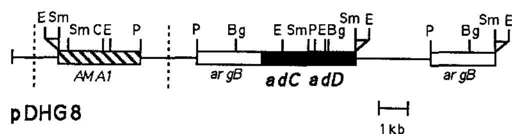

Fig. 3. Linear restriction map of pDHG8. *Single lines*, bacterial sequences; *boxes*, *A. nidulans* sequences; *dashed lines*, deduced sites of non-homologous recombination. Abbreviation for restriction enzymes as Fig. 1

lective conditions. When this DNA was used uncut for plasmid rescue into *E. coli* strain DH5, 13 amp^R colonies were obtained and single-colony gel analysis showed that all contained similar-sized plasmids. Plasmid DNA prepared from one such colony was designated pDHG8. This transformed *A. nidulans* strain G135 (*adD3*) to AD$^+$ at a frequency of up to 18000 AD$^+$ colonies per 10^6 protoplasts transformed. An *adC1* strain (G225) was also transformed to AD$^+$, suggesting that pDHG8 contains both *adC* and *adD*, which map 0.1 map units apart (Foley et al. 1965). We isolated a larger DNA fragment (17.8 kb) hypbridising to pDHG8 from an *A. nidulans* genomic DNA lambda library. This clone was able to complement both *adC*$^-$ and *adD*$^-$ mutants, and also, at low frequency, mutants for the adjacent spore colour gene, *ygA* (yellow-green). This confirms that pDHG8 contains DNA from the *adC-adD-ygA* region rather than a suppressor. The *ygA* locus maps 0.1 map units distal to *adD*. Restriction mapping showed pDHG8 to be an 18.4-kb heterotrimer formed from one molecule of pILJ16, one of pHELP3, and one of pILJ16 containing a 3.6-kb library insert including *adC* and *adD* (Fig. 3). This heterotrimer appears to be the product of homologous recombination between pILJ16 and pILJ16 plus a library insert, and non-homologous recombination between the vector component of one of these and that of pHELP3.

Discussion

In this study we have shown that co-transformation can occur as the result of recombination between transforming plasmids. Recombination between transforming DNA species is seen in a wide variety of eukaryotic organisms. In *Schizosaccharomyces pombe* the addition of an autonomous plasmid carrying the *URA1* gene to transformations of a *leu1 ura1* strain with the *Saccharomyces cerevisiae LEU2* gene on an integrative plasmid resulted in up to a 280-fold increase in the efficiency of transformation to *LEU*$^+$ (Sakai et al. 1984). All *LEU*$^+$ transformants were also found to be *URA*$^+$, and proved to contain autonomously-replicating oligomers comprising both plasmids. Similar properties have been observed in numerous other fungi e.g., *S. cerevisiae* (Jimenez and Davies 1980), *Candida albicans* (Kurtz et al. 1987), and *Yarrowia lipolytica* (Gaillardin et al. 1985).

Integration of co-transforming DNAs at the same chromosomal site has been demonstrated in *A. nidulans* (Wernars et al. 1987) and other fungi. This implies either a tendency for the second plasmid to be targeted to a chromosomal site where one plasmid is already integrated, or else co-integrate formation between transforming plasmids before recombination with the chromosome. Support for a limited (two-fold) increase in transformation rates and targeting of a previously-integrated plasmid has been found in *Leptosphaeria maculans* (Farman and Oliver 1992), but our evidence suggests that inter-plasmid recombination is a much more important factor in co-transformation.

Multimerisation of transforming plasmids in transformations of most protozoa, plants and animals results in the formation of large integrated tandem repeats of transforming plasmids in most cases, e.g., in *Dictyostelium discoideum* (Nellen and Firtel 1985), *Nicotinia tabacum* (Riggs and Bates 1986), *Xenopus laevis* (Rusconi and Schaffner 1981), and mammalian cells (Wigler et al. 1979). It is well established that, in transformations of mammalian cells, independently-introduced DNA species which integrate into the chromosome are usually found linked to each other (Anderson et al. 1984; Perucho et al. 1980), and that this linkage results predominantly from homologous recombination (Botchan et al. 1976; Folger et al. 1982; De Saint Vincent and Wahl 1983; Pomerantz et al. 1983; Small and Scangos 1983). It was shown by Folger et al. (1985) that every transforming DNA molecule participates in at least one recombination event.

Recombination between transforming DNAs may be utilised in order to facilitate gene cloning by transformation with a gene bank in an integrating vector, which normally transforms at a lower frequency, by effectively converting it in vivo to autonomous replication. There are three obvious advantages to this method: (1) transformation efficiency is increased by two orders of magnitude; (2) transforming sequences are not linked to the chromosome and are present at a high copy number (Gems et al. 1991), and therefore easier to reisolate into *E. coli*; (3) given that complementation occurs by a gene unlinked to the chromosome, the entire gene, including essential regulatory regions, must be present. It should be noted that in our experience, not all gene bank co-transformants could be recovered in *E. coli*, therefore it was essential to keep a number of the original fungal transformants. We assume that non-recovery was due to disruption of vector sequences essential for propagation in bacteria.

Acknowledgements. We thank Iain Johnstone for helpful discussion, Bernie Cohen for criticism of the manuscript, and Moira Stark and Mary Burke for invaluable technical advice. This work was funded by a SERC studentship grant to D.H.G.

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Communicated by J.D. Beggs