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Isolation of an Autonomously Replicating DNA Sequence from *Aspergillus nidulans*

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Using yeast, *Saccharomyces cerevisiae*, and the integrate vector system, we have isolated and characterized an autonomously replicating sequence (ARS) from *Aspergillus nidulans*. The DNA fragment, designated *ANR1*, is 5.0 kb in size and maintained free from the chromosome in *S. cerevisiae*. The YIp*lac*211-*ANR1* recombinant plasmid, which consists of sequences derived from the yeast integrative vector YIp*lac*211 and 5.0 kb *ANR1* fragment, showed a 10^4-fold enhancement in transformation efficiency over that found for YIp*lac*211, and was easily recovered from the transformed yeast. Genetic analysis of transformants showed that YIp*lac*211-*ANR1* could be over 96% cured when cultured over 20 generations in complete medium and thus suggests that this sequence is mitotically unstable. In *A. nidulans*, recombinant plasmid pILJ16-4.5 which carries the 4.5 kb *Eco*RI fragment of *ANR1* showed a 170-fold enhancement in transformation efficiency compared to that of the integrative vector pILJ16.

Key words: ***Aspergillus nidulans*, ARS, *ANR1***

The ascomycetous fungi are widely used as tools for basic research and in the production of many valuable materials to humans. They are also used in the production of foods, beverages, antibiotics, organic acids, and industrial enzymes (27). In nature, they are important as agents of deterioration and decay. Some of those are opportunistic pathogens in immunocompromised individuals, whereas others are animal and plant pathogens. *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Neurospora crassa* and *Aspergillus nidulans* are well-known experimental species used in studies of basic biological processes. Their small sizes, haploid genomes, simple nutritional requirements, and rapid life cycles are reasons for their wide use. Unicellular yeast such as *S. cerevisiae* are valuable in researdching many biological processes, but they are less useful for the study of differentiation in multicellular organisms. By contrast, the lower eukaryote, *A. nidulans*, is a favorable organism for the research of differentiation and the life cycle in which sexual, asexual, and parasexual modes of reproduction are included. The features of its life cycle and genetic system make the organism particularly useful both for classical genetic research and for the application of new technological advances in molecular genetics (17, 30).

Transformation caused by foreign DNA in *S. cerevisiae* has been achieved by Hinnen *et al.* (23) and Beggs (9), and *N. crassa* has been transformed by Case *et al.* (14). *A. nidulans* trasnformation system was established in the early 1980s (6, 7, 8, 26, 27, 39, 42, 43, 44). Through the transformation experiments, knowledge about transformed DNAs and selection of transformants were accumulated (1, 5, 22, 28, 30, 41). Based on this knowledge methods were developed by which the genome of fungi was easily manipulated. Recombinant plasmids for fungal transformation were developed, and they consist of genes which can be used to differentiate transformants and non-transformants, and bacterial sequences that can act as an origin of replication and as a selectable marker in *E. coli*.

In transformants, transformed DNAs can exist either in the chromosome by homologous or heterologous recombination or free from chromosome. The yeast integrative plasmid YIp cannot replicate autonomously and must be integrated into chromosomal DNA to subsist in the yeast cell. By contrast, replicator-containing plasmids (YRp, YCp, YLp, and others in yeast) replicate by themselves and can be maintained independent of chromosomal DNA (24, 34, 35, 37). In addition to replicator sequences found in chromosomal and mitochon-

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drial DNA, some strains of *S. cerevisiae* naturally carry 2 μm plasmid DNA in the nucleus. It has a typical chromatin structure and its replication is generally under the same genetic control as chromosomal DNA replication (16). Autonomously replicating episomal plasmid (YEp) shuttle vectors are based on the 2 μm plasmid (9). Various cloning and expression vectors in *S. cerevisiae* such as YRp containing chromosomal, autonomously replicating sequence (ARS), YCp containing ARS and centromere sequence (CEN) and YLp containing ARS, CEN and telomere sequence (TEL)(38), have been developed and widely used. Only ARS containing plasmids such as YEp and YRp show mitotic instability in a few generations, whereas YCp vectors, an ARS-plasmid containing a CEN sequence, are maintained for more than twenty generations as one or two copies per cell (16, 34). YACs (yeast artificial chromosome) are useful in cloning large sizes of the DNA fragment in yeast (12).

Unfortunately, no native nuclear plasmid such as 2 μm in yeast has been found in filamentous fungis, and yeast centromeres have no function in filamentous fungi (11, 40). Many workers have tried to isolate ARS-like sequences in *A. nidulans* (7, 18) and other organisms (36). Ballance and Turner (7) isolated an *A. nidulans* sequence, *ans1*, which can enhance the transformation frequency considerably relative to simple plasmids such as pDJB1, but are unable to autonomously replicate in *A. nidulans*. Also an ARS-like sequence was not found in the mitochondrial genomic DNA of *A. nidulans* (7, 10). Gems *et al.* (18) isolated a sequence (*AMA1*) of chromosomal origin which confers the ability to replicate on plasmids which carry it. The *AMA1* sequence give a 250~2000 fold increase in transformation frequency. The average copy number is 10 to 30 copies per haploid genome, and replicator *AMA1* is an inverted duplication of a low-copy-number dispersed genomic repeat known as mobile *Aspergillus* transformation enhancers (MATEs)(4, 18). The inverted duplication, *AMA1*, is apparently specific to the Glasgow strain (3) and a recombinant sequence constructed during the process of transformation (2, 3).

In this paper we report the isolation of a *Aspergillus nidulans* replicator (*ANR1*) sequence which can act as a plasmid replicator. The *ANR1* was isolated from *S. cerevisiae* transformants which had been transformed with *A. nidulans* genomic library constructed in a yeast integrative YIp*lac*211 plasmid. The *ANR1* sequence increased the transformation efficiency approximately 10^4-fold compared to the simple, integrative YIp*lac*211 plasmid in *S. cerevisiae*.

Materials and Methods

Strains and plasmids

The *A. nidulans* strain FGSC4 (wild type) was used for the preparation of intact, genomic DNA molecules. G34 (*yA2*; *argB2 methH2*) was used for transformation of *A. nidulans*. *S. cerevisiae* y4 (*MATa ade1 his2 leu2-3,112 trp1-1^a ura3*Δ) was used in the transformation of yeast to isolate *ARS-like sequences* of *A. nidulans*. y185 (*MAT*a *ade2-1^o his4-580^a leu2-3,112 lys2^o trp1-1^a tyr1^o ura3-52*), 262a (*MATa hom3 thr1*) and 262α(*MAT*α *hom3 thr1*) were used for the genetic analysis of y4 transformants.

E. coli JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ *(lac-proAB)* F'[*traD36 proAB$^+$ lacI^q lacZ*Δ*M15*]) was used for the subcloning experiments.

The YIp*lac*211 plasmid, an integrating vector of *S. cerevisiae* (19), was used to construct a genomic library of *A. nidulans*. In addition, the pILJ16, plasmid an integrating vector of *A. nidulans* (kindly provided by Dr. Clutterbuck), was used for the construction of the recombinant plasmid in the transformation of *A. nidulans*.

Media and growth conditions

E. coli and yeast were grown as described (21, 31, 33). -X (SC, synthetic complete medium lacking one supplement; -Ura, SC mediun lacking uracil) was used to confirm the genotypes of *S. cerevisiae* strains. Standard complete (CM) and minimal (MM) media (20) for growth of *A. nidulans* were supplemented with 70 mg/L of methionine and/or 100 mg/L of arginine, respectively (1).

Preparation of plasmid and *Aspergillus* DNA

Bacterial plasmid DNA was prepared by the alkali lysis method (32). Total *Aspergillus* DNA was isolated from FGSC4 as described previously (29).

Construction of genomic library

A. nidulans genomic DNA fragments were generated by partial digestion of 50 μg of DNA with 1.0 U of *Sau*3AI for 10 min at 37°C. After phenol extraction and ethanol precipitation, the pooled DNA was subjected to sucrose density gradient ultracentrifugation (Beckman, table-top ultracentrifuge) as follows. The digested DNA was heated to 65°C for 5 min to dissociate any DNA aggregates before centrifugation. The DNA solution was carefully layered on top of the sucrose gradient and centrifuged at 20°C for 24 h at 85,000 × g. 750 μl of each fraction were collected into microfuge tubes. The size of the collected DNA fractions was determined by gel elec-

tophoresis. Fractions of fragments between 2 and 12 kb were used for the construction of the genomic library. These *Sau*3AI fragments were ligated with YIp*lac*211 plasmid DNA that had been treated with *Bam*HI and calf intestinal alkaline phosphatase (Poscochem, Korea) and were used to transform *E. coli* JM109 to obtain ampicillin resistance.

The primary library harvested from the transformation plates was transferred into LB containing 20% glycerol and stored at −70°C. For amplification, the primary library cells were grown for 12 h at 37°C. The plasmid library DNA was isolated using the alkaline lysis procedure followed by cesium chloride density gradient ultracentrifugation (32).

Transformation of *E. coli*, *S. cerevisiae*, and *A. nidulans*

E. coli and yeast transformation was performed by $CaCl_2$ (32) and a modified lithium acetate method of Itoh *et al.*, respectively (25). *A. nidulans* transformation was performed by the method of Yelton *et al.* (43).

Plasmid recovery from *S. cerevisiae* and *A. nidulans*

For recovery of plsmid DNA from yeast, cells from a selective plate were transferred to microfuge tubes with a wooden toothpick and suspended in 100 µl of extraction buffer (0.1 M Tris–HCl pH 9.0; 0.1 M NaCl; 0.5% SDS; 1 mM EDTA). Sterile glass beads were added (0.40~0.50 mm in diameter) to the meniscus of the cell suspension. Fifty of phenol saturated with STE buffer (TE buffer containing 100 mM NaCl) and 15 µl of chloroform/isoamyl alcohol (25/1) were added to the cell suspension. This mixture was then vortexed vigorously and microfuged at room temperature for 2~3 min. The upper phase was collected and extracted repeatedly until the interphase became clear. Two volumes of ethanol were added to the upper phase, precipitated for 5 min at −70°C, and centrifuged at 4°C for 5 min. The resulting pellet was washed with 70% ethanol, dried by vacuum, and resuspended in 50 µl of TE. Ten to fifteen µl of DNA solution were used for *E. coli* transformation.

For recovery of plasmid DNA from *A. nidulans*, conidia were inoculated (10^6 conidia/ml) to selective liquid medium and incubated at 37°C for 18 h with vigorous shaking. The mycelia were harvested by filtration onto socks mesh (BYC, Korea), rinsed completely with deionized water, and dried by blotting absorbent paper towels. The dried mycelia were ground to fine powder using a mortar and pestle cooled in liquid nitrogen. About 100 mg of powder of mycelia was transferred to a microfuge tube and suspended in 300 µl of extraction buffer (50 mM Tris–HCl, pH 8.0, 0.1 M EDTA). Sterile glass beads (0.40~0.50 mm in diameter) were added to the meniscus of the cell suspension. Forty-five µl of phenol saturated with STE buffer (TE buffer containing 100 mM NaCl) and 15 µl of chloroform/isoamyl alcohol (25:1) were added to the cell suspension. This was then vortexed vigorously and microfuged at room temperature for 2~3 min. The processes after these steps were the same as used in *S. cerevisiae*.

Construction of plasmids

Plasmids for subcloning were constructed in YIp*lac*211 and propagated in JM109. YIp*lac*211-1.7 and YIp*lac*211-2.7 plasmids were constructed from the 1.7 kb and 2.7 *Eco*RI-*Xba*I fragment of *ANR1* ligated into the *Eco*RI-*Xba*I site of YIp*lac*211, respectively. The plasmid YIp*lac*211-4.2 contains the 4.2 kb *Sma*I-*Sca*I fragment of *ANR1* ligated into the *Sma*I site of YIp*lac*211.

For transformation of *A. nidulans*, the pILJ16-4.5 plasmid was constructed from the 4.5 *Eco*RI fragment of YIp*lac*211-*ANR1* ligated into the *Eco*RI site of pILJ16.

Mitotic stability

Mitotic stability of Ura^+ transformants were tested as follows. The yeast strain transformed with YIp*lac*211 or YIp*lac*211-*ANR1* from the -Ura plate was inoculated in 5 ml of YPD and incubated for 3 days (more than 20 generations) at 30°C. Two to three hundred cells were inoculated on a YPD plate and incubated for 2 days at 30°C, then the replica was plated on a −Ura plate and YPD plate and the percentage of these colonies retaining uracile prototrophy scored.

Meiotic segregation of plasmids

For tetrad analysis, the mated cells were sporulated by incubation on a KAc plate for 4 to 5 days at 30°C. To digest the cell wall of the ascus, 10 µl of Zymolase 60000 (2 mg/ml) were added into 40 µl of water containing approximately 10^5 asci and incubated for 10 min at 25°C. After dilution with 500 µl water, one loop of the spore suspension was streaked on to a YPD plate. Four-spored asci were separated to each spore using a micromanipulator and incubated at 30°C for 2 days. The segregation pattern of the genetic marker on the plasmid was analysed.

Results and Discussion

Genomic library of *A. nidulans*

Through the bacterial transformation with the ligation mixture of *A. nidulans* genomic DNA and

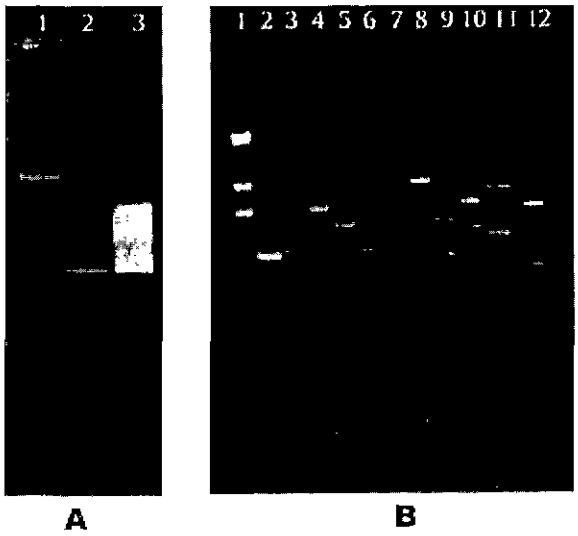

Fig. 1. Restriction digestion analysis of genomic library DNAs from *E. coli* transformants. DNAs from amp^+ transformants of *E. coli* strain JM109 separated on a 0.6% agarose gel. (**A**) Lanes: **1**, λ /*Hin*dIII size marker; **2**, YIp*lac*211 DNA from *E. coli* cut with *Eco*RI; **3**, genomic library DNAs cut with *Eco*RI. (**B**) Lanes: **1**, λ /*Hin*dIII size marker; **2**, YIp*lac*211 DNA from *E. coli* cut with *Eco*RI; **3-12**, randomly chosen recombinant DNAs from *E. coli* transformants cut with *Eco*RI.

linearized YIp*lac*211, 4,500 ampicillin-resistant colonies were isolated. Plasmid DNA isolated from ten colonies that had been randomly chosen was digested with *Eco*RI and subjected to agarose gel electrophoresis. Results showed that all of the plasmid DNA contained an insert 2 to 12 kb in size(Fig. 1). The average size of inserted DNA fragments was about 7 kb, therefore, the library may contain more than 70% of genomic equivalents, assuming a genome size of 2.6×10^4 kb for *A. nidulans* (32, 44).

Isolation of ARS-like fragment from *A. nidulans*

One hundred thirty transformants of *S. cerevisiae* y4 (Ura^+) with the genomic library of *A. nidulans* were obtained. At first, ten which were randomly chosen from these transformants were used in the recovery of the recombinant plasmid containing the *Aspergillus* DNA fragment in YIp*lac*211. Using a method utilized in preparation of plasmid from yeast, one putative origin of DNA replication from the *A. nidulans* genome, *ANR1*, was isolated on the basis of their ability to confer on YIp*lac*211 replicating in *S. cerevisiae*.

A restriction map of *ANR1* is shown in Fig. 2. The restriction sites present in *ANR1* fragment were different from those of *ans1* (7) and *AMA1* (18) indicating that this fragment contained differdent origin of replication or transformation enhancement DNA fragments. The plasmid designated YIp*lac*211-*ANR1*, carried a 5.0 kb insert DNA fragment in YIp*lac*211.

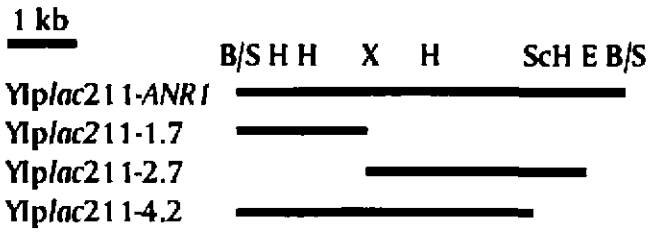

Fig. 2. Restriction map of isolated DNA fragment *ANR1* from *A. nidulans*. Only insert fragments are shown here. B/S, *Bam*HI and *Sau*3AI junction; H, *Hin*dIII; X, *Xba*I; E, *Eco*RI.

Transformation frequency

As expected, YIp*lac*211 was able to transform *S. cerevisiae* strain y4 at a very low frequency (less than 5 transformants/μg DNA) because it did not contain the authentic replicating sequence in itself. We could not recover the plasmid DNA from the extract of transformants of *S. cerevisiae*, because this plasmid may integrate into the chromosome. However, the YIp*lac*211-*ANR1* plasmid showed the ability to transform the *S. cerevisiae* y4 strain with a 10^4-fold increase in frequency than the parent YIp*lac*211 plasmid (Table 1). Moreover it was recovered from yeast transformants as unaltered molecules (Fig. 3). This result indirates that the insert might contain the fragment which could exert the

Table 1. Comparison of transformation frequencies, doubling time, and stability of the Ura^+ phenotype in *S. cerevisiae* with YIp*lac*211-*ANR1* and its subclones

Plasmids	TF(μg^{-1})	DT(h)	Mitotic stability %	
			NS[a]	S[b]
YIp*lac*211-*ANR1*	10^4	2.2	6	13
YIp*lac*211-4.2	10^4	2.1	8	13
YIp*lac*211-2.7	10^4	2.2	7	16
YIp*lac*211-1.7	10^4	2.3	5	13
YIp*lac*211	< 5	1.7	100	100

[a] One y4 transformant strain from the -Ura plate was inoculated in 5 ml of YPD and incubated for 2 days (it is equivalent to more than 20 generations) at 30°C. Two to three hundreds cells were inoculated on each YPD agar plate and incubated for 2 days at 30°C. The cells were replica plated on -Ura plate and YPD plate and the number of Ura^+ colonies on each plate scored. The tables represent the percentage of these colonies retaining uracil prototrophy.

[b] Transformant strains were inoculated in 5 ml of -Ura (selective medium) broth and incubated for 2 days (more than 20 generations) at 30°C. Two to three hundreds cells per plate were inoculated on YPD and -Ura plate and incubated for 2 days at 30°C. The number of Ura^+ colonies on each plate was scored. The tables represent the percentage of these colonies retaining uracil prototrophy (10). TF, transformation frequency; DT, doubling time; NS, nonselective; S, selective.

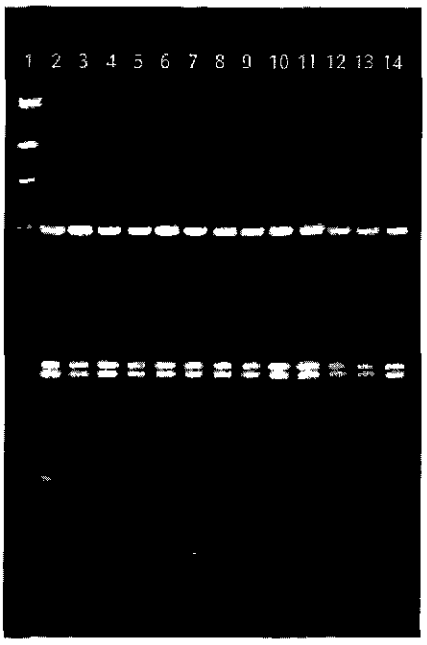

Fig. 3. Restriction digestion analysis of recovered plasmid DNAs from *S. cerevisiae* transformed with the plasmid YIp*lac*211-*ANR1*. Lanes: **1**, λ/*Hin*dIII size marker; **2**, YIp*lac*211-*ANR1* DNA from *E. coli* cut with *Hin*dIII; **3-14**, plasmid DNAs from *S. cerevisiae* transformants cut with *Hin*dIII.

activity of transformation enhancement as well as self-replication in the host.

To identify the element that confers the transformation enhancement, we have subcloned the *ANR1* fragment into YIp*lac*211. The subcloned plasmids YIp*lac*211-1.7, YIp*lac*211-2.7 and YIp*lac*211-4.2 with inserts of size 1.7, 2.7 and 4.2 kb respectively (Fig. 2) exerted almost the same transformation frequency as that of YIp*lac*211-*ANR1*. These subclones were also easily isolated from the transformants suggesting that they existed as an extrachromosomal form (data not shown).

Plasmid stability in transformants

Plasmid DNA can be maintained in cells when it contains the centromere sequence (CEN) or it is integrated into the host chromosome. Otherwise, it will be lost at the unselective condition during the division of the host cells (8, 10, 18). Therefore to obtain indirect evidence that plasmid DNA is extrachromosomally located in the cell, the plasmid stability during cell growth at the unselected condition was determined.

Table 1 summarizes the data on the mitotic stability of the plasmid that was scored by uracil prototrophy (Ura^+) in transformants obtained with plasmids YIp*lac*211-1.7, YIp*lac*211-2.7, YIp*lac*211-4.2 and YIp*lac*211-*ANR1*. Transformants containing the *ANR1* fragment or its subclones were extremely unstable during mitotic growth in complete media. Following growth for 72 h in the presence of uracil, 94% of YIp*lac*211-*ANR1* transformants lost the plasmid DNA compared to transformants containing YIp*lac*211 which showed no loss of the plasmid. The mitotic stability of the plasmid YIp*lac*211-1.7, YIp*lac*211-2.7 and YIp*lac*211-4.2 was 5%, 7% and 8%, respectively.

Although cells were grown under selective condition, y4 transformants containing the plasmid YIp*lac*211-1.7, YIp*lac*211-2.7, YIp*lac*211-4.2 and YIp*lac*211-*ANR1* showed low stabilities of 13%, 16%, 13%, and 13%, respectively, in comparison to 100% of the YIp*lac*211 vector in the host. The low stability of recombinant plasmids in *S. cerevisiae* would be due to the low efficiency of plasmid transfer from the mother cell to the daughter cell during mitosis. Vectors bearing the replication origin or transformation enhancement element without the CEN or TEL sequence show very low mitotic stability (10, 18), therefore, the DNA fragment in the recombinant plasmid isolated in this study does not seem to have the activity of the CEN or TEL sequence.

Growth rate analysis revealed that Ura^+ *S. cerevisiae* y4 transformants containing YIp*lac*211-1.7, YIp*lac*211-2.7, YIp*lac*211-4.2 and YIp*lac*211-*ANR1* had a doubling time of 2.3, 2.2, 2.1, and 2.2 h, respectively, compared to 1.7 h for transformants containing YIp*lac*211. The retarded doubling time of transformants *S. cerevisiae* transformed with the plasmid YIp*lac*211-1.7, YIp*lac*211-2.7, YIp*lac*211-4.2 and YIp*lac*211-*ANR1* would also be due to the low efficiency of plasmid transfer.

Meiotic segregation of plasmids

If the plasmid exists as more than two copies in the host cell and replicates as a chromosome-independent mode, the meiotic segregation pattern of the plasmid will not show mendelian inheritance. Tetrad analysis of diploids constructed by mating of a y4 transformant to y185 showed non-mendelian segregation of Ura^+ phenotype indicating that the plasmid containing the *ANR1 A. nidulans* DNA fragment may act extrachromosomally in yeast (Table 2). Segregation patterns of Ura^+ progenies from tetrads were not fixed in a 2+:2- pattern. Also, diploids bearing the plasmid YIp*lac*211-1.7, YIp*lac*211-2.7, or YIp*lac*211-4.2 showed the same pattern as diploids bearing the plasmid YIp*lac*211-*ANR1*.

Previous studies have shown that a sequence included in the chromosome segregates with the chromosome 2+:2- through meiosis (16). If the *URA* sequence had integrated into the chromo-

Table 2. Meiotic segregation of yeast plasmids YIp*lac*211-*ANR1* and its subclones

Plasmids	Type of segregation Ura$^+$:Ura$^-$					Total number[b] (Ura$^+$:Ura$^-$[a])	
	4:0	3:1	2:2	1:3	0:4		
YIp*lac*211-*ANR1*	19	14	18	14	17	168	160
YIp*lac*211-4.2	17	18	20	17	12	179	157
YIp*lac*211-2.7	16	18	10	16	20	154	166
YIp*lac*211-1.7	13	16	20	19	12	159	161
YIp*lac*211	0	0	78	0	0	156	156

Strains y4 transformed with each plasmid were crossed with the y185 strain. One hundred tetrads per diploid were dissected on an -Ura plate, incubated for 4 days at 30°C, and the number of colonies (Ura$^+$) on each plate scored.

[a] Estimated numbers (Ura$^-$) were shown because Ura$^-$ ascospores could not grow on a -Ura plate.

[b] Because the viability of ascospores was about 80%, the sum of Ura$^+$ and Ura$^-$ ascospores were not equal to 400.

some, Ura$^+$ progenies from tetrads should segregate 2+:2- predominantly. The occurrence of 4+:0-, 3+:1-, 1+:3-, and 0+:4- tetrads are consistent with the data shown above, indicating that plasmids YIp*lac*211-1.7, YIp*lac*211-2.7, YIp*lac*211-4.2, or YIp*lac*211-*ANR*1 were not integrated in chromosomes, but remained as free forms.

Recovery of plasmid DNA from the cellular extract of *A. nidulans* transformants

There is no report about episomal plasmid in *A. nidulans*. To recover the integrated plasmid DNA from *A. nidulans* transformants, we have to isolate whole genomic DNA, cut with a restriction enzyme which does not cut the transformed plasmid, ligate it into any replicating plasmid and then select the right clone. This forced us to construct an episomal plasmid in *A. nidulans*, and such a plasmid is recovered from transformants easily. To test whether the *ANR1* fragment exerted the transformation enhan- cement activity in *A. nidulans*, the plasmid pILJ16-4.5 *ANR1* fragment was inserted into the integrating pILJ16 plasmid. The resulting pILJ16-4.5 plasmid was used to transform *A. nidulans* G34. The transformation frequency of pILJ16-4.5 in *A. nidulans* was an average of 13,500 transformants per 10^6 protoplasts per μg of DNA. The frequency was 170-fold higher than pILJ16 meaning that the insert DNA fragment in pILJ16-4.5 may possess transformation enhancement activity in *A. nidulans* as well.

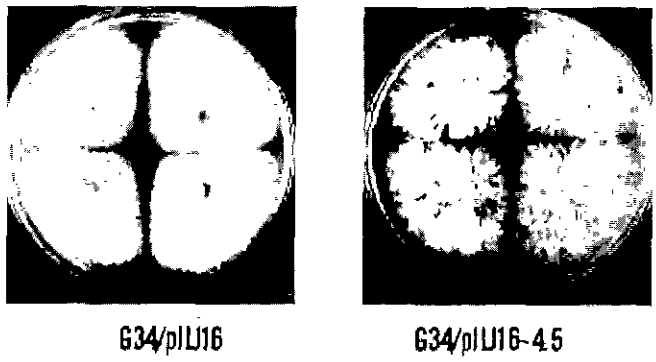

Fig 4. Comparison of growth of *A. nidulans* G34 transformants transformed with pILJ16 and pILJ16-4.5 on selective media.

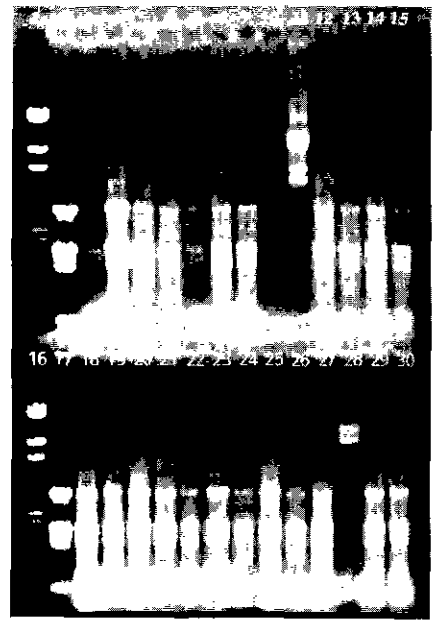

Fig. 5. Restriction digestion analysis of recovered plasmid DNAs from *A. nidulans* transformants. Lanes: **1** and **16**, λ/*Hin*dIII size marker; **2** and **17**, pILJ16-4.5 DNA from *E. coli* cut with *Hin*dIII; **3-15** and **18-30**, plasmid DNAs from *A. nidulans* transformants cut with *Hin*dIII.

The plasmid DNAs were recovered from twenty-six *E. coli* transformants which had been transformed with the cellular extract of *A. nidulans* G34 bearing the pILJ16-4.5 plasmid. Two out of twenty-six recovered plasmid DNAs showed a different restriction pattern from that of the initial plasmid (Fig. 5). This implies that about eight percent of transforming DNAs could be rearranged during the transformation of *A. nidulans*.

The morphological characteristic of the *A. nidulans* transformant transformed with the pILJ16-4.5 plasmid was distinguished from that of the G34 transformant transformed with the pILJ16 plasmid (Fig. 4). The ragged phenotype of colony margin of G34 bearing pILJ16-4.5 would be due to the irregularity of plasmid transfer during hypal growth.

The *ANR1* sequence isolated in this study would be potentially used as a cloning vector for *A. nidulans*. However, the activity of the *ANR1* sequence

in *A. nidulans* remains to be investigated.

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References

1. **Aleksenko, A.Y.** 1994. Cointegration of transforming DNAs in *Aspergillus nidulans:* a model using autonomously-replicating plasmids. *Curr. Genet.* **26**, 352-358
2. **Aleksenko, A.Y. and A.J. Clutterbuck.** 1995. Recombinational stability of replicating plasmids in *Aspergillus nidulans* during transformation, vegetative growth and sexual reproduction. *Curr. Genet.* **28**, 87-93.
3. **Aleksenko, A.Y. and A.J. Clutterbuck.** 1996. The Plasmid Replicator AMA1 in *Aspergillus nidulans* is an inverted duplication of a low-copy-number dispersed geno-mic repeat. *Mol. Microbiol.* **19**, 565-574.
4. **Aleksenko, A.Y. and A.J. Clutterbuck.** 1997. Autonomous Plasmid Replication in *Aspergillus nidulans:* AMA1 and MATE Elements. *Fungal Gene. and Biol.* **21**, 373-387.
5. **Almasan A. and N.C. Mishra.** 1990 Characterization of a novel plasmid-like element in *Neurospora crassa* derived mostly from the mitochondrial DNA. *Nucleic Acids Res.* **18**. 5871-5877.
6. **Ballance, D.J., F.P. Buxton and G. Turner.** 1983 Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. *Biochem. Biophys. Res. Commun* **112**, 284-289.
7. **Ballance, D.J., and G. Turner.** 1985. Development of a high frequency transforming vector of *Aspergillus nidulans*. *Gene* **36**, 321-331.
8. **Barnes, D.E and D.W. MacDonald.** 1986. Behaviour of recombinant plasmids in *Aspergillus nidulans:* structure and stability. *Curr. Genet.* **10**. 767-775.
9. **Beggs, J.D.** 1978. Transformation of yeast by a replicating hydrid plasmid. *Nature* **275**, 104-109.
10. **Beri, R.K., E.L. Lewis and G. Turner.** 1988. Behaviour of a replicating mitochondrial DNA sequence from *Aspergillus amstelodami* in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. *Curr. Genet.* **13**, 479-486.
11. **Boylan, M.T., M.J. Holland and W.E. Timberlake.** 1986. *Saccharomyces cerevisiae* centromere CEN11 dose not induce chromosome instability when integrated into the *Aspergillus nidulans* genome. *Mol. Cell. Biol.* **6**, 3621-3625.
12. **Bruke, D.T., G.F. Carle and M.V. Olson.** 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236**, 806-812.
13. **Buxton, F.P. and A. Radford.** 1984. The transformation of mycelial spheroplasts of *Neurospora crassa* and the attempted isolation of an autonomous replicator. *Mol. Gen. Genet.* **196**, 339-344.
14. **Case, M.E., M. Schweizer, S.R. Kushner and N. H. Giles.** 1980. Efficient transformation of N. crassa by utilizing hybrid plasmid DNA. *Proc. Natl. Acad. Sci. USA* **76**, 5259-5263.
15. **Chan, C.S. M., and B.K. Tye.** 1980. Autonomous replication sequences in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **74**, 6329-6333.
16. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. *Nature* **287**, 504-509.
17. **Clutterbuck, A.J.** 1974. *Aspergillus nidulans* In "Handbook of Genetics" (R. C. King, ed.) **1**, 447-510. Plenum, New York.
18. **Gems, D., J.L. Johnstone and A.J. Clutterbuck.** 1991. An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. *Gene* **98**, 61-67.
19. **Gietz, R.D. and Sugino, A.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527-534.
20. **Harsani, I., I. A. Granek and D. W. Mackenzie.** 1976. Genetic damage induced by ethyl alcohol in *Aspergillus nidulans Mutation res.* **48**, 51-74.
21. **Hartwell. L.H.** 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* **93**, 1662-1670.
22. **Hicks, J.B., A. Hinnen and G.R. Fink.** 1978. Properties of yeast transformation. *Cold Spring Harbor Symp. Quant. Biol.* **XLIII**, 1305-1313.
23. **Hinnen, A., J.B. Hicks and G.R. Fink.** 1978. Transformation of yeast. *Proc. Natl. Acad. Sci. USA* **75**, 1929-1933.
24. **Hsiao, C-H., and J. Carbon.** 1979. High-frequency transformation of yeast by plasmids containing the cloned yeast *ARG4* gene. *Proc. Natl. Acad. Sci. USA* **76**, 3829-3833.
25. **Ito, H., Y. Fukuda, K. Murata and A. Kimura.** 1983. Transformation of intact yeast cell treated with alkali cations. *J Bacteriol.* **153**, 163-168.
26. **Johnstone, I.L.** 1985. Transformation of *Aspergillus nidulans*. *Microbiol. Sci.* **2**, 307-311.
27. **Johnstone, I.L., S. G. Hughes and A.J. Clutterbuck.** 1985. Cloning of an *Aspergillus nidulans* developmental gene by transformation. *EMBO J.* **4**, 1307-1311.
28. **Mishra, N.C., and E.L. Tatum.** 1973. Non-Mendelian inheritance of DNA-induced inositol independence in *N. crassa*. *Proc. Natl. Acad. Sci. USA* **70**, 3875-3879.
29. **Osmani, S.A., G.S. May and N.R. Morris.** 1987. Regulation of the mRNA levels of *nimA*, a gene for the G_2-M transition in *Aspergillus nidulans*. *J. Cell Biol.* **104**, 1495-1504.
30. **Pontecorvo, G., J.A. Roper, L.M.Hemmons, K.D. Macdonal and A.W.J. Bufton.** 1953. The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**, 141-238.
31. **Rambosek, J., and J. Leach.** 1987. Recombinant DNA in filamentous fungi; Progress and prospects. *CRC Crit. Rev. Biotechnol.* **6**, 357-393.
32. **Sambrook, J., E.F. Fritch and T. Maniatis.** 1989. Molecular cloning : a laboratory manual, 2nd edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
33. **Sherman, F., G.R. Fink and J.B. Hicks.** 1982. Methods in Yeast Genetics (Cold Spring Harbor), New York: *Cold Spring Harbor Laboratory Press*.
34. **Smith, J. and J. Pateman (eds).** 1977. Genetics and Physiology of *Aspergillus*, pp361-539, *Academic Press*.
35. **Stinchomb, D.T., C, Mann and R. W. Davis.** 1982. Centromeric DNA from *Saccharomyces cerevisiae*. *J. Mol. Biol.*

158, 157-179.

36. **Stinchomb, D.T., K. Struhl and R.W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. *Nature* **282**, 39-43.
37. **Stinchomb, D.T., M. Thomas, J. Kelly, E. Selker and R.W. Davis.** 1980. Eukaryotic DNA segments capable of autonomous replication in yeast. *Proc. Natl. Acad. Sci. USA* **77**, 4559-4563.
38. **Struhl, K., D.T. Stinchomb, S. Scherer and R.W. Davis.** 1979. High-frequency transformation of yeast: Autonomous replication of hybrid DNA molecules. *Proc. Natl. Acad. Sci. USA* **76**, 1035-1039.
39. **Szostak, J.W. and E.H. Blackburn.** 1982. Cloning yeast telomeres on linear plasmid vectors. *Cell* **29**, 245-255.
40. **Tilburn, J., C. Scazzocchio, G.G. Taylor, J H. Zabicky-Zissman, R.A. Lockingston and R.W. Davies.** 1983. Transformation by integration in *Aspergillus nidulans*. *Gene* **26**, 205-221.
41. **Timberlake, W.E. and M.A. Marshall.** 1989. Genetic engineering in filamentous fungi. *Science* **244**, 1313-1317.
42. **Turner, G. *et al*** *1985. Development of cloning vectors and* a marker for gene replacement techniques in *Aspergillus nidulans*. In "Molecular Genetics of Filamentous Fungi" (W. E. Timberlake ed.) 15-28. New York.
43. **Yelton, M.M., J.E. Hamer and W.E. Timberlake.** 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc. Natl. Acad. Sci. USA* **81**, 1470-1474.
44. **Yelton, M.M., W.E. Timberlake and C.A. M.J.J. van den Hondel.** 1985. A cosmid for selecting genes by complementation in *Aspergillus nidulans*; selection of the developmentally regulated *yA* locus. *Proc. Natl. Acad. Sci USA 82, 834-838.*