

## 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 *YIplac211-ANR1* recombinant plasmid, which consists of sequences derived from the yeast integrative vector *YIplac211* and 5.0 kb *ANR1* fragment, showed a  $10^4$ -fold enhancement in transformation efficiency over that found for *YIplac211*, and was easily recovered from the transformed yeast. Genetic analysis of transformants showed that *YIplac211-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 *EcoRI* 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 researching 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* transformation 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  $\mu$ m 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 (YE<sub>p</sub>) shuttle vectors are based on the 2  $\mu$ m 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 YE<sub>p</sub> 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  $\mu$ m in yeast has been found in filamentous fungus, 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 YIplac211 plasmid. The *ANR1* sequence increased the transformation efficiency approximately 10<sup>4</sup>-fold compared to the simple, integrative YIplac211 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<sup>o</sup> ura3 $\Delta$* ) was used in the transformation of yeast to isolate ARS-like sequences of *A. nidulans*. y185 (*MATa ade2-1<sup>o</sup> his4-580<sup>o</sup> leu2-3,112 lys2<sup>o</sup> trp1-1<sup>o</sup> tyr1<sup>o</sup> ura3-52*), 262a (*MATa hom3 thr1*) and 262 $\alpha$ (*MAT $\alpha$  hom3 thr1*) were used for the genetic analysis of y4 transformants.

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

The YIplac211 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 medium 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  $\mu$ g of DNA with 1.0 U of *Sau3AI* 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  $\times$  g. 750  $\mu$ l of each fraction were collected into microfuge tubes. The size of the collected DNA fractions was determined by gel elec-

trophoresis. Fractions of fragments between 2 and 12 kb were used for the construction of the genomic library. These *Sau*3AI fragments were ligated with YIplac211 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  $-70^{\circ}\text{C}$ . For amplification, the primary library cells were grown for 12 h at  $37^{\circ}\text{C}$ . 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  $\text{CaCl}_2$  (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 plasmid DNA from yeast, cells from a selective plate were transferred to microfuge tubes with a wooden toothpick and suspended in 100  $\mu\text{l}$  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  $\mu\text{l}$  of phenol saturated with STE buffer (TE buffer containing 100 mM NaCl) and 15  $\mu\text{l}$  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  $-70^{\circ}\text{C}$ , and centrifuged at  $4^{\circ}\text{C}$  for 5 min. The resulting pellet was washed with 70% ethanol, dried by vacuum, and resuspended in 50  $\mu\text{l}$  of TE. Ten to fifteen  $\mu\text{l}$  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  $37^{\circ}\text{C}$  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  $\mu\text{l}$  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  $\mu\text{l}$  of phenol saturated with STE buffer (TE buffer containing 100 mM NaCl) and 15  $\mu\text{l}$  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 YIplac211 and propagated in JM109. YIplac211-1.7 and YIplac211-2.7 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 YIplac211, respectively. The plasmid YIplac211-4.2 contains the 4.2 kb *Sma*I-*Sca*I fragment of *ANR1* ligated into the *Sma*I site of YIplac211.

For transformation of *A. nidulans*, the pILJ16-4.5 plasmid was constructed from the 4.5 *Eco*RI fragment of YIplac211-*ANR1* ligated into the *Eco*RI site of pILJ16.

### Mitotic stability

Mitotic stability of  $\text{Ura}^+$  transformants were tested as follows. The yeast strain transformed with YIplac211 or YIplac211-*ANR1* from the -Ura plate was inoculated in 5 ml of YPD and incubated for 3 days (more than 20 generations) at  $30^{\circ}\text{C}$ . Two to three hundred cells were inoculated on a YPD plate and incubated for 2 days at  $30^{\circ}\text{C}$ , then the replica was plated on a -Ura plate and YPD plate and the percentage of these colonies retaining uracile prototrophy scored.

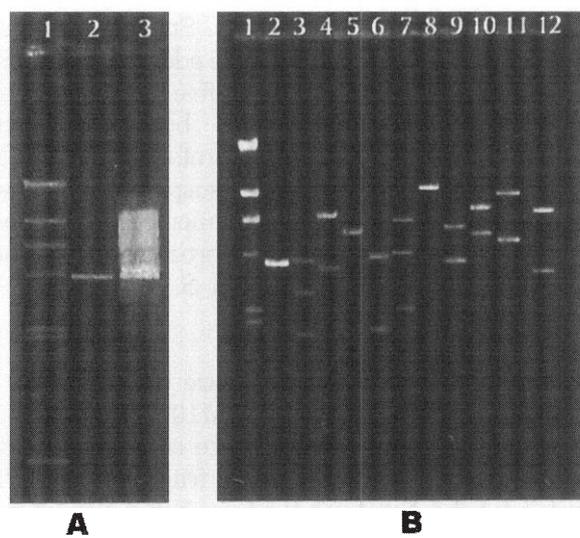
### Meiotic segregation of plasmids

For tetrad analysis, the mated cells were sporulated by incubation on a KAc plate for 4 to 5 days at  $30^{\circ}\text{C}$ . To digest the cell wall of the ascus, 10  $\mu\text{l}$  of Zymolase 60000 (2 mg/ml) were added into 40  $\mu\text{l}$  of water containing approximately  $10^5$  asci and incubated for 10 min at  $25^{\circ}\text{C}$ . After dilution with 500  $\mu\text{l}$  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  $30^{\circ}\text{C}$  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



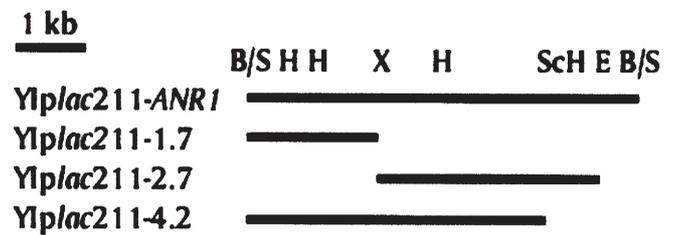
**Fig. 1.** Restriction digestion analysis of genomic library DNAs from *E. coli* transformants. DNAs from *amp*<sup>+</sup> transformants of *E. coli* strain JM109 separated on a 0.6% agarose gel. (A) Lanes: 1,  $\lambda$ /HindIII size marker; 2, YIplac211 DNA from *E. coli* cut with *EcoRI*; 3, genomic library DNAs cut with *EcoRI*. (B) Lanes: 1,  $\lambda$ /HindIII size marker; 2, YIplac211 DNA from *E. coli* cut with *EcoRI*; 3-12, randomly chosen recombinant DNAs from *E. coli* transformants cut with *EcoRI*.

linearized YIplac211, 4,500 ampicillin-resistant colonies were isolated. Plasmid DNA isolated from ten colonies that had been randomly chosen was digested with *EcoRI* 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  $2.6 \times 10^4$  kb for *A. nidulans* (32, 44).

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

One hundred thirty transformants of *S. cerevisiae* y4 (Ura<sup>+</sup>) 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 YIplac211. 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 YIplac211 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 different origin of replication or transformation enhance-



**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, *Hind*III; X, *Xba*I; E, *Eco*RI.

ment DNA fragments. The plasmid designated YIplac211-*ANR1*, carried a 5.0 kb insert DNA fragment in YIplac211.

### Transformation frequency

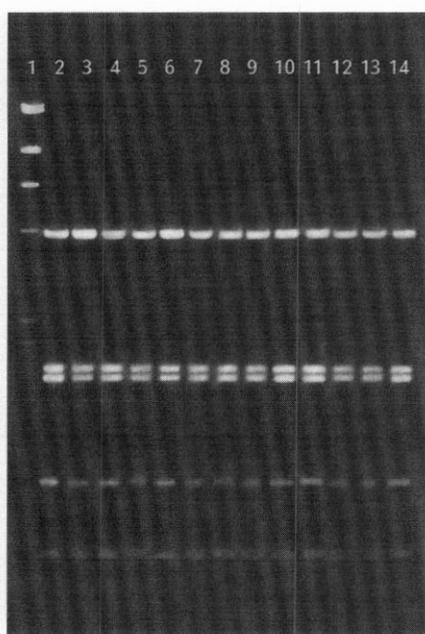
As expected, YIplac211 was able to transform *S. cerevisiae* strain y4 at a very low frequency (less than 5 transformants/ $\mu$ g DNA) because it did not contain the authentic replicating sequence in itself. We could not recover the plasmid DNA from the extract of transformants of *S. cerevisiae*, because this plasmid may integrate into the chromosome. However, the YIplac211-*ANR1* plasmid showed the ability to transform the *S. cerevisiae* y4 strain with a  $10^4$ -fold increase in frequency than the parent YIplac211 plasmid (Table 1). Moreover it was recovered from yeast transformants as unaltered molecules (Fig. 3). This result indicates that the insert might contain the fragment which could exert the

**Table 1.** Comparison of transformation frequencies, doubling time, and stability of the Ura<sup>+</sup> phenotype in *S. cerevisiae* with YIplac211-*ANR1* and its subclones

Plasmids	TF( $\mu$ g <sup>-1</sup> )	DT(h)	Mitotic stability %	
			NS <sup>a</sup>	S <sup>b</sup>
YIplac211- <i>ANR1</i>	$10^4$	2.2	6	13
YIplac211-4.2	$10^4$	2.1	8	13
YIplac211-2.7	$10^4$	2.2	7	16
YIplac211-1.7	$10^4$	2.3	5	13
YIplac211	< 5	1.7	100	100

<sup>a</sup> 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<sup>+</sup> colonies on each plate scored. The tables represent the percentage of these colonies retaining uracil prototrophy.

<sup>b</sup> 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<sup>+</sup> 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 YIplac211-ANR1. Lanes: 1,  $\lambda$ /HindIII size marker; 2, YIplac211-ANR1 DNA from *E. coli* cut with HindIII; 3-14, plasmid DNAs from *S. cerevisiae* transformants cut with HindIII.

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 YIplac211. The subcloned plasmids YIplac211-1.7, YIplac211-2.7 and YIplac211-4.2 with inserts of size 1.7, 2.7 and 4.2 kb respectively (Fig. 2) exerted almost the same transformation frequency as that of YIplac211-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 unselected 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<sup>+</sup>) in transformants obtained with plasmids YIplac211-1.7, YIplac211-2.7, YIplac211-4.2 and YIplac211-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 YIplac211-ANR1 transformants lost the plasmid DNA compared to transformants containing YIplac211 which showed no loss of the plasmid. The mitotic stability of the plasmid YIplac211-1.7, YIplac211-2.7 and YIplac211-4.2 was 5%, 7% and 8%, respectively.

Although cells were grown under selective condition, y4 transformants containing the plasmid YIplac211-1.7, YIplac211-2.7, YIplac211-4.2 and YIplac211-ANR1 showed low stabilities of 13%, 16%, 13%, and 13%, respectively, in comparison to 100% of the YIplac211 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<sup>+</sup> *S. cerevisiae* y4 transformants containing YIplac211-1.7, YIplac211-2.7, YIplac211-4.2 and YIplac211-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 YIplac211. The retarded doubling time of transformants *S. cerevisiae* transformed with the plasmid YIplac211-1.7, YIplac211-2.7, YIplac211-4.2 and YIplac211-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<sup>+</sup> phenotype indicating that the plasmid containing the ANR1 *A. nidulans* DNA fragment may act extrachromosomally in yeast (Table 2). Segregation patterns of Ura<sup>+</sup> progenies from tetrads were not fixed in a 2+:2- pattern. Also, diploids bearing the plasmid YIplac211-1.7, YIplac211-2.7, or YIplac211-4.2 showed the same pattern as diploids bearing the plasmid YIplac211-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 YIplac211-ANR1 and its subclones

Plasmids	Type of segregation					Total number <sup>b</sup> (Ura <sup>+</sup> :Ura <sup>-a</sup> )	
	Ura <sup>+</sup> :Ura <sup>-</sup>						
	4:0	3:1	2:2	1:3	0:4		
YIplac211-ANR1	19	14	18	14	17	168	160
YIplac211-4.2	17	18	20	17	12	179	157
YIplac211-2.7	16	18	10	16	20	154	166
YIplac211-1.7	13	16	20	19	12	159	161
YIplac211	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<sup>+</sup>) on each plate scored.

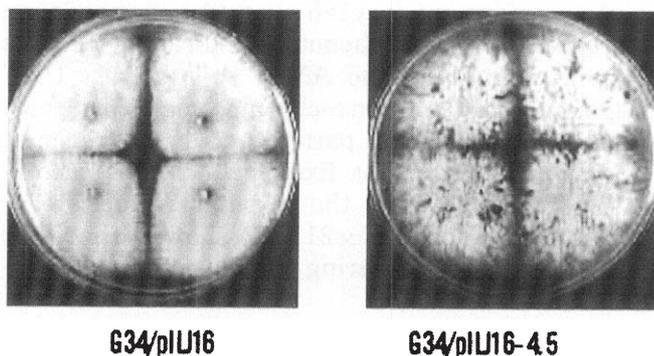
<sup>a</sup> Estimated numbers (Ura<sup>-</sup>) were shown because Ura<sup>-</sup> ascospores could not grow on a -Ura plate.

<sup>b</sup> Because the viability of ascospores was about 80%, the sum of Ura<sup>+</sup> and Ura<sup>-</sup> ascospores were not equal to 400.

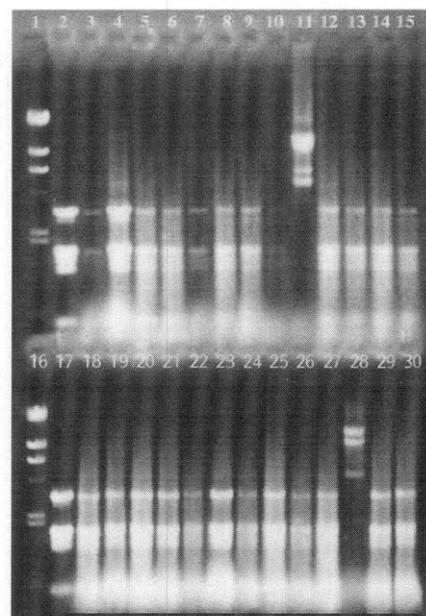
some, Ura<sup>+</sup> progenies from tetrads should segregate 2+:2- predominantly. The occurrence of 4+:0-, 3+:1-, 1+:3-, and 0+:4- tetrads are consistent with the data shown above, indicating that plasmids YIplac211-1.7, YIplac211-2.7, YIplac211-4.2, or YIplac211-ANR1 were not integrated in chromosomes, 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 enhancement activ-



**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**,  $\lambda$ HindIII size marker; **2** and **17**, pILJ16-4.5 DNA from *E. coli* cut with HindIII; **3-15** and **18-30**, plasmid DNAs from *A. nidulans* transformants cut with HindIII.

ity 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  $\mu$ g 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.

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 hyphal 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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