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Cloning and Characterization of Autonomously Replicating Sequence(ARS) from *Kluyveromyces fragilis*

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An autonomously replicating sequence (Kf-ARS1) of *Kluyveromyces fragilis* was cloned from the genomic library which was constructed using pHN134 as a cloning vector to make a new host-vector system for the production of heterologous protein from *K. fragilis* as a host. The cloning vector pHN134 was composed of Km^r, Ap^r and multiple cloning site in LacZ'. A clone carrying Kf-ARS1 was isolated and the recombinant plasmid was designated as pIKD102. The cloned fragment was 2.3 kb (*EcoRI/EcoRI*) in length. Subcloning experiment showed that the region for ARS activity was 1.5 kb (*SalI/EcoRI*) fragment. It was shown that the Kf-ARS1 was active in *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*.

Yeast is of utility value as a host in the production of heterologous proteins because the modification and the glycosylation of proteins can occur in the organelles such as endoplasmic reticulum and golgi complex, and because the transportation of proteins can occur between organelles as in the case of higher organisms. So there seems to be some limitations in the use of *Escherichia coli* in the production of heterologous proteins. Successful transformation systems have been reported for *S. cerevisiae* (3, 11, 20), *Schizosaccharomyces pombe* (2) and *Kluyveromyces lactis* (7). *K. fragilis* can grow over a wide range of temperature and it is superior to *S. cerevisiae* in the secretion of proteins into medium. At the same time, *K. fragilis* has been regarded as a useful strain in the production of ethanol and sweetener.

The transformation of *S. cerevisiae* without the use of auxotrophic markers, has been achieved with the use of the bacterial β-lactamase gene or the kanamycin resistance (Km^r) gene of Tn903(Tn601) as selection markers (12, 13). *S. cerevisiae* is insensitive to penicillin type antibiotic and only moderately sensitive to Km and chloramphenicol(Cm). G418 sulfate is an aminoglycoside antibiotic which is structurally related to Km and gentamycin, and it shows low antibiotic toxicity in yeast.

It would be possible to transform the wild type yeast strain to have resistance to antibiotic G418 if the organism is sensitive to this antibiotic. Direct selection procedures for transformants resistant to antibiotic G418 were developed for protoplast (21) and intact cells (8). These systems were applied to *K. lactis* and *K. fragilis*, respectively. In vector system, in addition to the selection marker, a suitable replicon is required as a part of the transforming vector.

In 1990, an attempt was carried out to use *K. fragilis* as a host in the production of heterologous protein. But the transformation frequency was very low because an autonomously replicating sequence of *K. lactis* was used as a replicon of vector (18).

For the construction of a new vector for *K. fragilis*, we cloned and determined the sequence of LEU gene of *K. fragilis*. This paper describes the construction of the cloning vector of ARS, the cloning of ARS fragment from *K. fragilis*, and the characterization of its replicon activity in *S. cerevisiae* and *K. fragilis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

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E. coli strains MC1061 (F^- araD139 araAB01 (leu^-) 7679 lacX74 galK rpsL hsdR), C600 (leuB thr thi rk^- mk^- Narr) and JM109 (recA1 supE44 endA1 gyrA96 hsdR17 thi Δ (lac^- proAB), F' [traD36 $proAB^+$ lacIq lacZ M15]) were used as the hosts for all bacterial transformations and plasmid construction. Yeast strains, *S. cerevisiae* DBY746 (trp1-289 his3-1 leu2-3 leu2-12 ura 3-52) and *K. fragilis* Y610 of wild type were used as a host for the expression of ARS activity of cloned fragment. Plasmids pUC19 and YCpG11 were used for the construction of ARS cloning vector of *K. fragilis*. *E. coli* was grown in LB medium (10g polypepton, 5g yeast extract, 5g NaCl and 1g glucose per liter, supplemented with ampicillin 50 μg/ml, X-gal 25 mg/ml in dimethylformamide and IPTG 200 mg/ml, if necessary). Yeast was grown in YEPD medium (10g yeast extract, 20g polypepton and 20g glucose per liter). G418 was added to a final concentration of 200 μg/ml for selection of transformant.

Sensitivity of *K. fragilis* to G418

Antibiotic G418 solution was filtered by Millipore filter to avoid microbial contamination. Various concentrations (0, 50, 100, 150, 200, 250 μg/ml) of G418 were added to the YEPD agar medium, and the cell suspension derived from one colony was inoculated on each YEPD plate with G418 added to a specific concentration.

After incubation for 2-3 days at 30℃, we determined the concentration of G418 in which the colony could not grow.

Plasmid DNA Isolation and Transformation

E. coli plasmid DNA was isolated using alkaline-SDS method (4), and yeast chromosomal DNA was isolated by Cryer's method (6). Transformation of *E. coli* was performed by the conventional $CaCl_2$ method (10). Transformation of yeast was performed with an intact cell (8) with alkali cations and transformants were selected on the YEPD plates containing G418 (200 μg/ml).

Construction of Gene Library

Purified *K. fragilis* chromosomal DNA was digested completely with *Eco*RI, and fractionated with agarose gel electrophoresis. Cloning vector pHN134 digested with *Eco*RI was ligated with fractionated *K. fragilis* chromosome DNA. Recombinant plasmid containing insert DNA was constructed with the transformation of these ligation solutions to *E. coli* JM109.

Cloning of ARS from *K. fragilis*

S. cerevisiae was transformed with gene library and plated on the medium containing G418 as an antibiotic, and the recombinant plasmid was isolated from the colony grown and named pIKD102. Insert DNA was identified by restriction mapping.

Identification of ARS Activity

S. cerevisiae DBY746 transformed with pIKD102 was incubated in YEPD medium for 12 hours, and G418 was added to a concentration of 200 μg/ml in a liquid medium. After about 30 hour incubation, the number of viable cell was estimated and compared with the cell number of transformed cells and untransformed cells (16). pIKD102 isolated from transformed cells in *S. cerevisiae* and *E. coli*, was also compared with agarose gel electrophoresis.

RESULTS

Construction of the Cloning Vector pHN134

For the construction of the cloning vector for an autonomously replicating sequence (ARS), we constructed plasmid pHN134. The strategy for the construction is outlined in Fig. 1. First, both ends of the 1.7 kb *Pvu*II fragment of YCpG11 (17) were filled in with DNA polymerase to make blunt ends, and ligated with pUC19 in *Nar*I site which was blunt ended with DNA polymerase. The resultant plasmid was named pHN134 (4.3

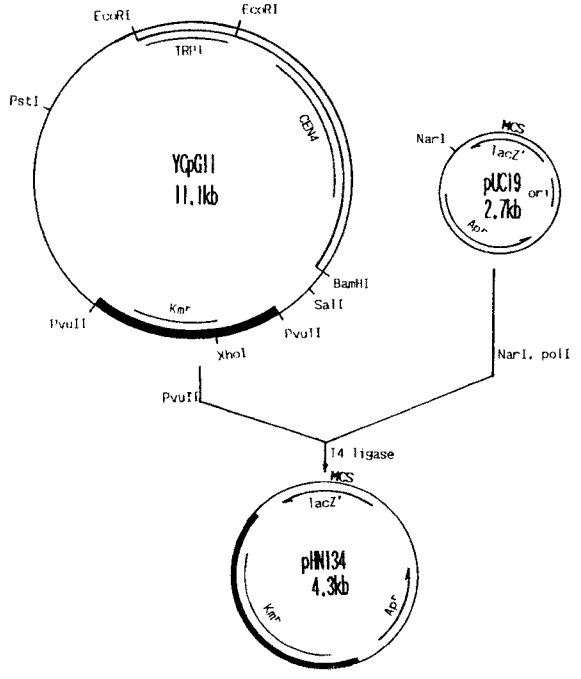

Fig. 1. Construction scheme of Kf-ARS cloning vector pHN134.

Shown is a diagrammatic representation of the pHN134 which is composed of 1.7 kb *Pvu*II fragment derived from Tn903 of YCpG11 and 2.7 kb pUC19 digested by *Nar*I. Km^r gene, Ap^r gene and lacPOZ are designated. This plasmid vector can be used to clone autonomously replicating sequence from G 418 sensitive yeasts. The kanamycin resistance (Km^r) gene encodes a phosphotransferase which can inactivate the aminoglycoside antibiotic G418. But it will be necessary to determine the time of addition of G418 in medium in advance.

Kb) and the restriction map is shown in Fig. 1. Vector pHN134 carries Ap^r and Km^r gene of Tn903, and contains the unique recognition sites for *Sph*I, *Pst*I, *Kpn*I and *Sac*I within MCS.

Sensitivity of *K. fragilis* to G418 Antibiotic

The sensitivity of yeast to G418 depends on the strain. So the colonies derived from a single cell were isolated and their sensitivities to various concentrations of G418 were checked. The lethal concentration of G418 to *K. fragilis* was 200 μg/ml (Fig. 2). The growth of *K. fragilis* was not affected below the concentration of 100 μg/ml, but the growth was inhibited remarkably at the concentration of 150 μg/ml. And at the concentration of 200 μg/ml or more, *K. frgilis* could not grow. So we added G418 to the YEPD plate to the concentration of 200 μg/ml for the selection of transformants with pIKD102.

Cloning of Autonomously Replicating Sequence from *K. fragilis* Gene Library

S. cerevisiae was transformed with the gene library and colonies which could grow in G418 plate were found. Hybrid plasmid containing Kf-ARS1 was isolated from G418 resistant clones and the restriction map was determined by several kinds of restriction endonucleases (Fig. 3). As shown in Fig. 3, *Bam*HI, *Sal*I and *Xba*I sites were identified. These hybrid plasmids were named pIKD 102 (6.6 kb) and pIKD103, the orientation of the inserts are in opposite direction.

Localization of the ARS Activity by Subcloning

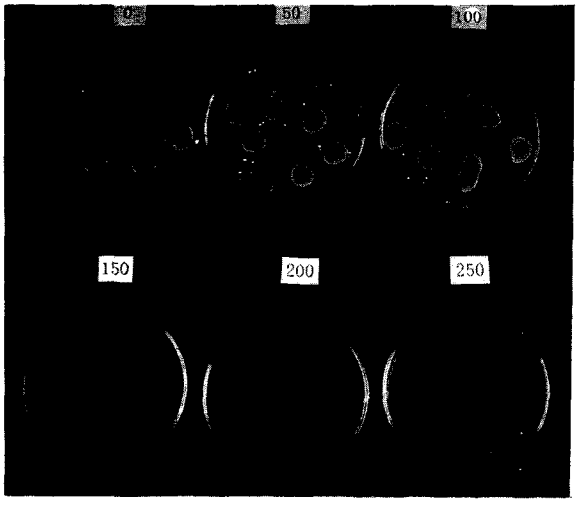

Fig. 2. Sensitivity of *K. fragilis* Y610 to G418 antibiotic.
Various amounts of G418 were added in the plate

0: No G418	150: 150 μg/ml of G418
50: 50 μg/ml of G418	200: 200 μg/ml of G418
100: 100 μg/ml of G418	250: 250 μg/ml of G418

The cloned 2.3 kb fragment was shortened by subcloning to determine the essential region for replication activity. *Sal*I/*Eco*RI fragment of 1.5 kb was identified as the shortest fragment with ARS function (Fig. 4).

Growth Patterns of *S. cerevisiae* Harboring pIKD102

S. cerevisiae has been used extensively as a host for transformation. When the resistance to G418 was examined, the yeast strain was found to be very susceptible to G418 of concentrations of 150 to 500 μg/ml. The addition of G418, however, brought about a significant decrease in the number of viable cells. But, *S. cerevisiae* cells harboring pIKD102 were not affected by the addition of G418. Both the optical density at 660 nm and the number of viable cell remained similar to those of the control cells (without G418) (Fig. 5).

Southern Blot Analysis

K. fragilis chromosomal DNA and plasmid pIKD102 were digested with *Eco*RI, fractionated by a 0.8% agarose gel electrophoresis, and then DNA was transferred to nitrocellulose membrane according to the method of Southern (19). The 2.3 Kb probe fragments were prepa-

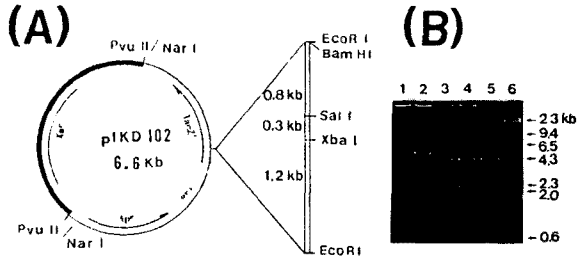

Fig. 3. Restriction map of pIKD102pIKD102 is composed of the cloning vector pHN134 and cloned *K. fragilis* DNA fragment (2.3 kb). Cloned from *K. fragilis*.
(A) Determined restriction map
(B) Pattern of agarose gel electrophoresis
1: *Xba*I, 2: *Sal*I, 3: *Bam*HI, 4: *Eco*RI, 5: pHN134, 6: δDNA digested with *Hin*dIII

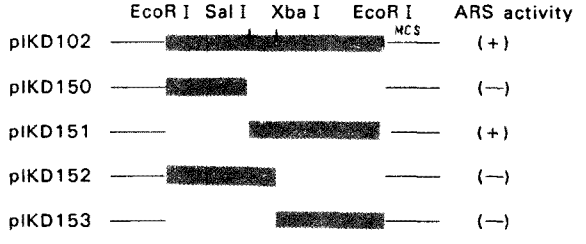

Fig. 4. Identification of ARS activity with cloned fragment.
Subcloned fragments from pIKD102 were ligated to pHN134. The open bars represent the fragments of *K. fragilis*.

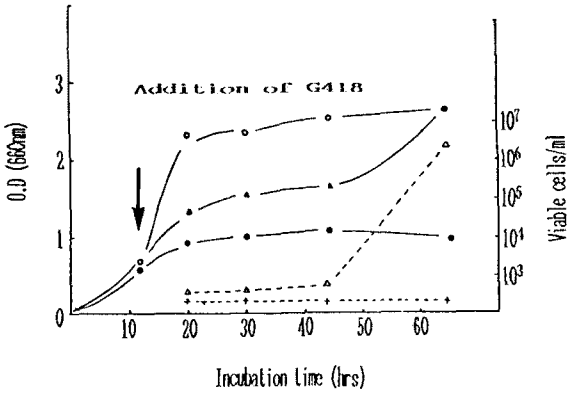

Fig. 5. Effect of G418 on growth and cell viability of *S. cerevisiae* and *S. cerevisiae* harboring pIKD102.
G418 was added (200 μg/ml) to cell culture at 12 hours of incubation.
Absorbance
○−○; *S. cervisiae*
▲−▲; *S. cerevisiae* harboring pIKD102 with addition of G418
●−●; *S. cerevisiae* with addition of G418
Viable cells with addition of G418
△−△; *S. cerevisiae* harboring pIKD102
+−+; *S. cerevisiae*

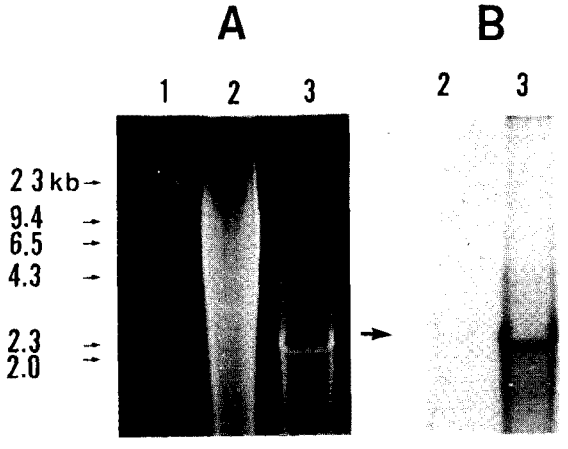

Fig. 6. Southern hybridization of the *K. fragilis* Y610 chromosomal DNA digested with *Eco*RI and pIKD102 containing Kf-ARS.
Photograph of DNA separation by electrophoresis (A) and by auto-radiography (B).
1. λDNA digested with *Hin*dIII
2. *K. fragilis* chromosomal DNA digested with *Eco*RI
3. pIKD102 digested with *Eco*RI

red from pIKD102 by digestion with *Eco*RI, eluted with a Geneclean kit II, and radiolabelled with α-^{35}S-dCTP by the random primer labelling method. As shown in Fig. 6, a distinct hybridization band corresponding to a 2.3 Kb fragment was identified. This result confirmed

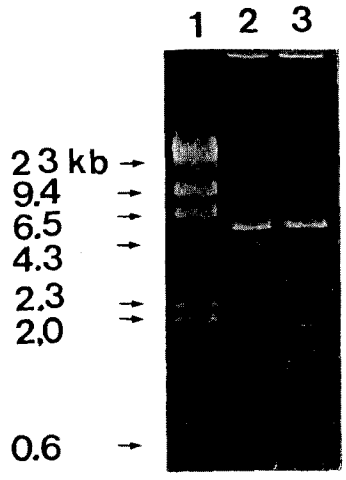

Fig. 7. Expression of Kf-ARS fragment in *S. cerevisiae* and *E. coli*.
Replication capability of Kf-ARS(pIKD151) was confirmed from the transformation and recovery from yeast and *E. coli*.
1. λDNA digested with *Hin*dIII
2. pIKD151 recovered from transformed yeast strain
3. pIKD151 recovered from transformed *E. coli*

Table 1. Numbers of transformants of the four plasmids in *S. cerevisiae* and *K. fragilis*

Transformants/ 10 g DNA/ml	No DNA	YRp7	pHN134	pIKD151
S. cerevisiae	0	0	0	607
K. fragilis	0	0	0	600

that the cloned DNA fragment originated from *K. fragilis* genomic DNA. Thinner band (4.3 Kb) of lane 3 in B panel seems to be a non-specific band.

Estimation of G418 Resistance and ARS Activity with Kf-ARS

Throughout the transformation of *S. cerevisiae* or *K. fragilis* and of *E. coli* with pIKD151, plasmid DNA was isolated from the transformants showing the phenotype of G418 resistance. And then the DNA was checked in *E. coli* and *S. cerevisiae* (Fig. 7). As shown in Fig. 7, pIKD151 could replicate itself in *S. cerevisiae* and *E. coli*.

For the yeast transformation, YRp7 carrying the yeast genomic ARS sequence, pHN134(control) and pIKD151 were used to transform both *S. cerevisiae* and *K. fragilis*. Their transformant numbers at G418 plate per constant DNA concentration are summarized in Table 1. In case of pIKD151, the transformation efficiency of *K. fragilis* was nearly equal to that of *S. cerevisiae* DBY746, but YRp7 and pHN134 could not transform the two species of yeast in the G418 plate.

DISCUSSION

Only a subset of the ARS previously defined for their activity in a plasmid is the real origins in the chromosomes (9) and very few sequences are known to have ARS functions in two independent species of organisms. Although most of ARS fragments were effective in *S. cerevisiae*, ARS fragments originating from other organisms had not been examined for ARS function in their own system. It was found that half of the ARS fragments effective in *S. cerevisiae* and the independently isolated ARS fragments from the chromosomal DNA of *S. pombe*, have ARS function in *S. pombe*. However, the other fragments showing ARS function in *S. pombe* did not function in *S. cerevisiae* and vice versa (2, 14). Similarly, DNA fragments showing ARS function in *S. cerevisiae* but originating from mouse, did not work as ARSs in a mouse cell. *S. cerevisiae* and *Z. rouxii* use similar but different sequences of ARS in pSRI plasmid (1). Four DNA fragments of CfMNPV(Choristoneura fumiferana nuclear polyhedrosis virus) had ARS function in *S. cerevisiae* (15), and a circular plasmid, pKW1, from the *Kluyveromyces waltii* carrying this segment could efficiently transform *K. waltii*, *K. thermotolerans*, but not *K. lactis* (23). The ARS element of *Candida albicans* is an element which is necessary for high frequency transformation and autonomous plasmid replication in both *S. cerevisiae* and *C. albicans* (5). ARS of *K. fragilis* (Kf-ARS1) showed replication functions in *S. cerevisiae* and *K. fragilis* with equal efficiency.

Plasmids carrying the yeast ARS element transformed yeast with high frequency, but the transformants were generally unstable, and the plasmid showed tendency to disappear quite readily. In this study, Kf-ARS1 of *K. fragilis* showed a higher transformation efficiency than the ARS of *K. lactis* with *K. fragilis* under the same conditions (8).

Autonomously Replicating Sequence (ARS) elements are DNA sequences which allow DNA molecules to transform yeast at a high frequency, giving rise to transformants in which the transforming DNA replicates autonomously (22). So, if the optimal transformation condition of *K. fragilis* is elucidate, that will serve as a component of an interesting model system for the study of gene expression and of extracellular secretion of available proteins.

Acknowledgement

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