Subcloning and Nucleotide Sequencing of an ARS Site of *Candida maltosa* Which Also Functions in *Saccharomyces cerevisiae*

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A DNA region exhibiting ARS activity in *Candida maltosa* was subcloned from an isolated 3.8 kb DNA fragment and designated as the TRA region previously [M. Takagi *et al.*, *J. Bacteriol.*, **167**, 551 (1986)], which replicates autonomously not only in *C. maltosa* but also in *Saccharomyces cerevisiae*. It was found that a DNA fragment of about 200 bp (designated as fragment 2) exhibited ARS activity in both yeasts, and nucleotide sequence analysis of this fragment revealed that it contained five 11 bp-sequences which are homologous to the consensus sequence of ARS sites of *S. cerevisiae* [J. R. Broach *et al.*, *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 1167 (1982)] (9 among 11 bp being identical in each).

Plasmids constructed using fragment 2 (pTRA2 and pTRA12) will be useful as cloning vectors, although they showed lower transforming frequencies and lower stabilities in *C. maltosa* than plasmids containing the TRA region (pTRA1 and pTRA11).

Candida maltosa is one of the *n*-alkane-assimilating yeasts. When cells of these yeasts are transferred from glucose- to *n*-alkane-medium, various proteins are induced in the endoplasmic reticulum and peroxisome.^{3,4)} We have been studying the induction mechanisms for these specific proteins in *n*-alkane-grown cells of *C. maltosa*. In a previous paper, it was concluded that the synthesis of at least two major inducible proteins in the endoplasmic reticulum is regulated at the level of transcription.⁵⁾ Detailed investigations on the molecular mechanisms involved in the regulation of gene expression may require the isolation and structural analysis of the genes encoding these proteins. Host-vector systems should be very useful for this purpose. As *C. maltosa* does not contain any plasmids, a DNA fragment exhibiting "ARS" activity ("autonomously replicating sequence") is required to construct vectors.⁶⁾

We have succeeded in isolating an ARS site (a 3.8 kb fragment designated as the TRA region) from the genome of *C. maltosa* which supports the stable replication of a plasmid containing it in *C. maltosa* cells.¹⁾ Using the TRA region and the *LEU2* gene of *S. cerevisiae*, we constructed 11 kb vectors (pTRA1 and pTRA11) for a host, *C. maltosa* J288 (*leu*⁻), a mutant derived from *C. maltosa* IAM12247. During this work, it was found that although neither the replication origin of the 2 μ m plasmid of *S. cerevisiae* nor the ARS1 site isolated from the genome of *S. cerevisiae* can function as a replication origin in *C. maltosa*, the TRA region can also function as an ARS site in *S. cerevisiae*.

In the present investigation, we subcloned the ARS site from the 3.8 kb TRA region (i) to analyze the structure of DNA fragment which functions as an ARS site in both *C. maltosa* and *S. cerevisiae* and (ii) to minimize the size of

plasmids for host-vector systems in *C. maltosa*.

MATERIALS AND METHODS

Strains, plasmids and media. The yeast strains used were *C. maltosa* J288 (*leu*⁻)⁷⁾ and *S. cerevisiae* AH22 (*a leu2-3 leu2-112 his4-519 can1 gal2*).⁸⁾ The synthetic medium for yeasts (SD) contained 0.67% Yeast Nitrogen Base W/O amino acids (Difco), 2% glucose and required supplements. YPD medium (1% yeast extract, 2% Bacto-peptone and 2% glucose) was also used. The bacterial strain used was *E. coli* JA221 (*recA1 leuB6 trpE5 hsdR⁻ lacY*). It was grown in Luria broth. The plasmid vectors used¹⁾ were pTRA1 and pTRA11 (containing pBR322, the *LEU2* gene of *S. cerevisiae* plus the TRA region), pBR-TRA (pBR322 plus the TRA region) and pBR-LEU (pBR322 plus the *LEU2* gene, see Fig. 2).

DNA isolation, transformation and DNA sequencing. *E. coli* JA221 was used as the recipient for the ligated DNA. Restriction enzymes, the Klenow fragment and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. and used under the conditions suggested by the supplier. For quantitative determination of the transformation frequency, each plasmid multiplied in *E. coli* was purified by CsCl-EtBr gradient centrifugation. Transformation of yeast cells was performed by the Li-acetate-method.⁹⁾ The nucleotide sequences were determined by the dideoxy method¹⁰⁾ using M13mp18 and M13mp19 (Takara Shuzo Co., Ltd.).

Subcloning of an ARS site of *C. maltosa* from the TRA region. For the subcloning of the ARS site (see Fig. 1), the TRA region (3.8 kb) was digested with *Hind*III and the resultant 0.6 kb fragment (fragment H) was isolated. The

latter was digested with *Sau*3AI and the resulting two fragments, 0.4 kb (fragment 1) and 0.2 kb (fragment 2), were isolated. These two fragments were separately ligated with pBR-LEU digested with *Bam*HI and *Hind*III to produce pCS27 and pCS28 (see Fig. 2). To produce pTRA2 and pTRA12 containing the *Bam*HI and *Pst*I sites of pBR322 to clone DNA fragments, fragment 2 was inserted into the *Xho*I site of pBR-LEU after the 3'-recessed ends produced on digestion with *Xho*I (for pBR-LEU), and *Hind*III and *Sau*3AI (for fragment 2) had been filled by incubation with deoxynucleoside triphosphates and the Klenow fragment. Only the orientation of the ligation of the two blunt-end fragments differed between pTRA2 and pTRA12 (see Fig. 2).

Plasmid stability. To assay the stability of plasmids in *C. maltosa* grown in a non-selective medium, yeast cells carrying plasmids were grown in the SD medium without leucine, and at the stationary phase they were inoculated into the SD medium containing 2 mM leucine at the inoculation size of 1%. An aliquot was removed at intervals, which was plated onto either the SD agar-medium without leucine or the SD agar-medium containing 2 mM leucine. Plasmid stability was calculated by dividing the number of colonies on the leucine-minus plate by the number of colonies on the leucine-plus plate. The generation of cells after the inoculation was determined by plating an aliquot and then counting the number of colonies on the YPD plate.

RESULTS

Subcloning of the ARS site

In Fig. 1, a restriction map for the TRA region (a *Bam*HI–*Bam*HI fragment) is shown.

FIG. 1. Restriction Maps of the TRA Region (3.8 kb) and Subcloned Fragments of It.

The TRA region, cloned as a *Bam*HI–*Bam*HI fragment, was analyzed using restriction enzymes, *Hind*III and *Xho*I. The 3 fragments obtained on digestion with *Hind*III were separately cloned into YEpl3. In a transformation experiment, it was found that only the *Hind*III–*Hind*III fragment (fragment H) exhibited ARS activity. Fragment H was digested with *Sau*3AI, and the resulting two fragments were separately cloned into pBR-LEU to produce pCS27 and pCS28 (Fig. 2). Only fragment 2, i.e., not fragment 1, exhibited ARS activity, which is about 200 bp in size. The names of the plasmids constructed using each of the fragments are given in parantheses. Abbreviations: B, *Bam*HI; H, *Hind*III; S, *Sau*3AI; X, *Xho*I.

FIG. 2. Plasmids Construction.

To determine which of the two fragments (fragments 1 and 2 in Fig. 1) exhibited ARS activity, each of them was inserted into the *Bam*HI–*Hind*III site of pBR-LEU (7.2 kb) which consists of pBR322 and the *LEU* gene of *S. cerevisiae* to produce pCS27 (7.4 kb) and pCS28 (7.2 kb), respectively. As only pCS28 exhibited ARS activity, fragment 2 was inserted into the *Xho*I site of pBR-LEU to produce cloning vectors, pTRA2 and pTRA12 (both 7.4 kb). Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; X, *Xho*I.

Previously,¹⁾ we reported that the *Xho*I site was located between the left end and the left-side *Hind*III site of the TRA region, but further analysis of the structure revealed that the *Xho*I site was very close to the right end, as shown in Fig. 1.

As fragment H exhibited ARS activity,¹⁾ it was digested with *Sau*3A1, and resulting two fragments (fragments 1 and 2) were ligated with pBR-LEU, which consists of pBR322 and

the *LEU2* gene of *S. cerevisiae*, to produce pCS27 and pCS28, as shown in Fig. 2. *C. maltosa* J288 (*leu*[–]) was transformed with each of these plasmids, and only pCS28 containing fragment 2 gave *Leu*⁺ transformants.

DNA sequence of fragment 2

Fragment 2 was inserted between the *Bam*HI and *Hind*III sites in the multiple cloning region of either M13mp18 or M13mp19, and both strands were completely sequenced. The results are shown in Fig. 3. Computer analysis was performed to find homologous regions to the consensus sequence of ARSs of *S. cerevisiae*.²⁾ Five 11 bp-sequences were found to be identical in 9 bp to the consensus sequence, which are underlined, and these sequences are summarized in the lower part of Fig. 3.

Construction of cloning vectors with fragment 2

To construct cloning vectors smaller than the previously described pTRA1 and pTRA11 (each about 11 kb), fragment 2 was inserted into the *Xho*I site of pBR-LEU in two different orientations to produce cloning vectors pTRA2 and pTRA12 (each about 7.4 kb), as shown in Fig. 2. They contain the *Bam*HI and *Pst*I sites for cloning DNA fragments. The frequency of the transformation of these plasmids into *C. maltosa* was about 1/3 of that in the case of pTRA1 and pTRA11.

The stability of these plasmids in *C. maltosa* grown in a non-selective medium was assayed. As shown in Fig. 4, their stability was lower than that of pTRA1 and pTRA11.

DISCUSSION

To our present knowledge, the size of a DNA fragment long enough to exhibit ARS activity in yeasts is less than 1 kb. The results presented in this paper indicate that a 196 bp-fragment (fragment 2) exhibits ARS activity. As shown in Fig. 3, it has five 11 bp-sequences homologous to the consensus sequence of ARSs of *S. cerevisiae*. At present, it is hard to say which of them are essential or how they

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      10      20      30      40      50      60
GATCAGTTGT ATTTAAGTTG ACAATTTGGT TCACTAAATT TAATTAGTTA GTTGTAAAGTC
CTAGTCAACA TAAATTCAC TGTAAACCA AGTGATTAA ATTAATCAAT CAACATTCAG

      70      80      90      100     110     120
AGTTTTGAAA ATTTAGTCA AATTTTATTA CCAATTTTTT ATGTACACTT AGCAAAAATT
TCAAACTTTT TAAATCAGT TTAAAATAAT GGTTAAAAAA TACATGTGAA TCGTTTTTAA

      130     140     150     160     170     180
GAAAGCACTT TTATACAGTA GTTACAACGA GCATACGTGG TAGTAATGGA TAATCTATGT
CTTTCGTGAA AATATGTCAT CAATGTTGCT CGTATGCACC ATCATTACCT ATTAGATACA

      190     200
GATATATGAT AAGCTT
CTATATACTA TTCGAA

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C. maltosa      5'      3'
1 AGTTGTATTTA (9/11)
2 ATTTAAGTTGA (9/11)
3 AATTAATTTA (9/11)
4 TTTTATGTA (9/11)
5 TTTTATGTACA (9/11)

Consensus
(S. cerevisiae)  A TTTAT G TTT A
                  |   ^   |   |

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FIG. 3. Nucleotide Sequencing of Fragment 2.

Fragment 2 was sequenced and the sequence is shown from the *Sau3A*I site to the *Hind*III site (see Fig. 1). DNA sequences homologous to the consensus sequence of ARSs of *S. cerevisiae* (more than 80% homology) were searched for using a computer, and five sequences were found, which are underlined. Arrowheads indicate the direction of the detected sequence. The 5 homologous sequences are summarized in the lower part, together with the consensus sequence of ARSs of *S. cerevisiae*.

FIG. 4. Stability of pTRA2 and pTRA12 in Comparison to That of pTRA1 and pTRA11 in *C. maltosa* Grown in a Non-selective Medium.

Cells of *C. maltosa* carrying one of the four plasmids, pTRA1 (◆), pTRA11 (◇), pTRA2 (●) or pTRA12 (○), were grown in a non-selective medium. An aliquot was removed at intervals and plated onto plates to determine the total cell number (generations) and the percentage of plasmid-carrying *Leu*⁺ cells. The averages for two independent experiments are presented.

cooperate for the appearance of ARS activity.

It is expected that the smaller the size of a vector, the lower the number of sites for

restriction enzymes is, and thus the greater is the number of restriction enzymes we can use to handle cloned DNA in the vector. The TRA region is 3.8 kb, and pTRA1 and pTRA11 are 11 kb¹⁾ in size. Although these plasmids may not be too large for cloning DNA fragments, it might be desirable to improve them by decreasing their size. So, we have constructed two vectors (pTRA2 and pTRA12) using fragment 2. They can be utilized as cloning vectors in *C. maltosa*, especially for the subcloning and isolation of DNA fragments using various kinds of restriction enzymes for which there are no cutting sites in these plasmids. It is quite easy to find such kinds of suitable restriction enzymes, since the sequences are now known in all the parts of these plasmids (pBR322,¹¹⁾ the *LEU2* gene¹²⁾ and fragment 2).

The low frequency of transformation by these plasmids may not be so disadvantageous for their use in the subcloning and isolation of already cloned DNA fragments. Furthermore,

their low stability in cells grown in a non-selective medium may not be a serious problem in these experiments. These plasmids have the advantage of that they also transform *S. cerevisiae*, although the frequency of transformation is low and the colony sizes of the transformants are small.

It is suggested that during the subcloning the ARS site from the TRA region to fragment 2, some DNA regions were lost, which support efficient transformation and high stability in *C. maltosa* and *S. cerevisiae*. Nevertheless, fragment 2 has an essential DNA region that is functional as an ARS sequence not only in *C. maltosa* but also in *S. cerevisiae*.

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