High Expression Vectors for a *Zygosaccharomyces rouxii* Host

Toshiaki IMURA, Ikuyo UTATSU and Akio TOH-E*

Department of Fermentation Technology, Hiroshima University,
Shitami, Saijo, Higashihiroshima 724, Japan

Received October 24, 1988

We evaluated the expression of three yeast genes in *Zygosaccharomyces rouxii* using *lacZ* as a reporter gene; they were the glyceraldehyde-3-phosphate dehydrogenase gene of *Z. rouxii* (*GAP-Zr*), that of *S. cerevisiae* (*GAP-Sc*) and the *PHO5* gene of *S. cerevisiae*. The *GAP-Sc* promoter showed high expression in the *Z. rouxii* host as well as in the *S. cerevisiae* host. The *GAP-Zr* promoter showed a comparable level of expression as the *GAP-Sc* both in the *S. cerevisiae* host and the *Z. rouxii* host. The transcription initiation site of the *GAP-Zr* promoter in the heterologous host seems to be the same as that in the homologous host. The *PHO5* promoter showed constitutive expression, as efficient as in the case of the *GAP-Zr* promoter, in the *Z. rouxii* host. All the promoters mentioned above can be used as sources of a high expression vector in the *Z. rouxii* host.

Zygosaccharomyces rouxii is one of the yeasts which are used in the field of fermentation industry. Two plasmids, pSR1 and pSB3, have been found in *Z. rouxii*^{1,2)} and used for constructing various composite plasmids. A transformation method involving a *leu*⁻ strain of *Z. rouxii*, ME3, has been developed.^{2a)} By using these plasmid vectors and a host strain, a heterologous gene can be introduced into *Z. rouxii*. To express a foreign gene in *Z. rouxii* efficiently, however, it is necessary to place the gene under the control of an appropriate promoter which functions in *Z. rouxii*. Development of expression vectors and *Z. rouxii* hosts will be useful for improving *Z. rouxii* strains which are in practical use.

In the previous study, we cloned and sequenced one of the structural genes for glyceraldehyde-3-phosphate dehydrogenase (*GAP*) from *Z. rouxii*.³⁾ Since the *GAP* gene is known to be as one of the highly expressed genes in *S. cerevisiae*⁴⁾ and since the codon usage of deduced amino acid sequence of the *GAP* gene of *Z. rouxii* (*GAP-Zr*) is strongly biased, this gene is supposed to be a highly expressed gene. In this communication, we present evidence showing that the promoter of

the *GAP-Zr* gene shows as efficient expression as *GAP-Sc* in *Z. rouxii*, as well as in *S. cerevisiae*. The *GAP-Sc* and *PHO5* promoters of *S. cerevisiae* also show high expression in *Z. rouxii*.

Materials and Methods

Organisms. The yeasts, bacterial strains and plasmids used in this study are listed in Table I. *Z. rouxii* ME3 harbors plasmid pSR1. ME3 [pSB3] contains plasmid pSB3 in addition to pSR1, and both plasmids are maintained stably in it.⁵⁾ The *leu*⁻ phenotype of ME3 can be complemented with the *LEU2* gene of *S. cerevisiae*.

Media. For yeasts: YEPD contained 2% Polypepton (Daigo Eiyo), 1% yeast extract (Oriental Yeast) and 2% glucose. SD contained 0.67% yeast nitrogen base without amino acids (DIFCO) and 2% glucose. For cultivation of *Z. rouxii*, 5% NaCl was added to YEPD and SD. Leucine (20 µg/ml) was added as necessary. For *E. coli*: nutrient broth and L-broth were prepared as described.⁶⁾ McConkey agar (Daigo Eiyo) was used for selecting Lac⁺ *E. coli*. Ampicillin (50 µg/ml) or tetracycline (10 µg/ml) was added as appropriate.

Techniques for nucleic acids. Plasmid isolation, gel electrophoresis, construction of plasmids *in vitro* and extraction of RNA from yeast were carried out as described.⁷⁾ DNA sequencing was performed by the Maxam-Gilbert

* To whom reprint requests should be addressed.

Table I. LIST OF STRAINS AND PLASMIDS

Organism	Genotype or character	Reference or source
<i>Zygosaccharomyces rouxii</i>		
ME3	Leu ⁻	5)
ME3 (pSB3)	Leu ⁻	5)
<i>Saccharomyces cerevisiae</i>		
K12-2A (cir ⁰)	<i>MAT_a leu2 ura3</i>	Our stock
<i>Escherichia coli</i>		
JA221	F ⁻ <i>leuB6 trpΔE5 recA hsdR lacY</i>	16)
MC1066	F ⁻ <i>leuB trpC9830Δ(lac)X74 strA pyrF74::Tn5 (Km^r)</i>	17)
Plasmids		
YIp32	<i>LEU2 Ap^r</i>	18)
YIp33	<i>LEU2 Ap^r</i>	18)
pMC1871	<i>lac'Z Ap^r</i>	17)
pMC1415	<i>lac'Z TRP1 ARS1 Ap^r</i>	Arias-Martinez and Casadaban
pAT87	<i>PHO5 Tc^r</i>	Our stock
pGC1	<i>GAP-Sc-lacZ Ap^r</i>	T. Oshima
pGAP1-Zr	<i>GAP-Zr Ap^r</i>	3)
pSR1	Cryptic	1)
pSB3	Cryptic	2)

method.⁸⁾ The primer extension reaction was carried out as described by Hahn *et al.*⁹⁾

Determination of β -galactosidase activity. The assay for β -galactosidase activity was that as described by Miller⁶⁾ with partial modification. A transformant to be tested was grown at 28°C in the selective medium with shaking. An appropriate volume of the culture was taken at the mid-log phase, a part of which was used for measuring OD_{660 nm} and a part for computing the fraction of cells retaining the plasmid. Cells were harvested from a given amount of the culture, washed with 1 ml of water and then resuspended in 1 ml of Z-buffer (1 mM MgSO₄, 0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.05 M β -mercaptoethanol, pH 7.0). After adding 50 μ l of chloroform and 20 μ l of 0.1% SDS, the mixture was vortexed and then incubated at 28°C 5 min. The reaction was started by adding 0.2 ml *o*-nitrophenyl- β -D-galactoside (4 mg/ml) to the above mixture. The incubation was continued until a pale yellow color developed. The reaction was stopped by adding 0.5 ml of 1 M Na₂CO₃. After removing the chloroform, the cells were spun down and the absorbance at 420 nm of the resulting supernatant was determined. The enzyme unit was defined as follows:

$$\text{units} = 1000 \times \text{OD}_{420 \text{ nm}} / (\text{OD}_{660 \text{ nm}} \times t \times v)$$

where *t* is the incubation time (minutes) and *v* is the culture volume (ml) for the enzyme assay.

Stability of the plasmids. A portion of a culture, taken

as described above, was diluted appropriately and then spread on YEPD or YEPD containing 5% NaCl, depending on the host. After 3~4 days incubation, colonies were replica-plated onto plates containing a synthetic medium without leucine to determine the number of Leu⁺ colonies.

Results

Construction of plasmids containing the *lacZ* fusion gene

To determine the extent of expression of the *GAP-Zr* promoter, a *GAP-Zr-lacZ* fusion gene was constructed. The 700bp *Pst*I-*Hpa*I fragment containing the *GAP-Zr* promoter (from pGAP1-Zr), the 3.2 kb *Sma*I-*Sal*I fragment carrying the *lacZ* gene (from pMC1871) and the 5.2 kb *Sal*I-*Pst*I fragment carrying the *LEU2* gene (from YIp33) were ligated to obtain pTI46 (not shown). pTI46 was used for the construction of pTI48, pTI49, pTI51 and pTI52 (Fig. 1). The *PHO5-lacZ* fusion gene was constructed as shown in Fig. 2. First, pAT190 was constructed by ligating the 2 kb *Pst*I-*Bam*HI fragment carrying the *PHO5* gene (from pAT87), the 6 kb *Pst*I-*Sal*I fragment carrying the *lacZ* gene (from pMC1415)

Fig. 1. Structures of Plasmids Containing a *lacZ*-fusion Gene.

Filled boxes indicate the yeast promoters and open boxes the *lac* gene. The hatched boxes indicate the *LEU2* gene. The length of *lac* DNA used varied; in pTI48, pTI49, pTI51 and pTI52, the *lac* DNA contains only *lacZ*. In the rest of the plasmids, the *lac* DNA contains *lacY* and *lacA* in addition to *lacZ*. Both *GAP-Zr* and *GAP-Sc* contain a *HpaI* site, however, the position of the *HpaI* site in *GAP-Zr* is different from that in *GAP-Sc*. Therefore, the length of the N-terminal amino acids fused to *lacZ* is different in *GAP-Zr-lac* and *GAP-Sc-lac*. An arrow shown on the *lacZ* gene indicates the direction of the transcription. Arrows on the plasmid segment (thick line) indicate the position of IR. Thin lines indicate the pBR322 sequences. An arrow, Ap, indicates the orientation of the Ap gene. E, *EcoRI*; K, *KpnI*; Hp/Sm, *HpaI/SmaI*; S/X, *SalI/XhoI*; P, *PstI*.

and the 4kb *SalI*–*BamHI* fragment from pBR322. In this plasmid, the *PHO5* and *lacZ* genes orientated in the same direction. pAT190 was linearized with *KpnI*, which cuts inside the coding region of the *PHO5* gene, and digested partially with *Bal-31* nuclease and then with *SmaI*. The resulting DNA mixture was treated with T4-DNA ligase and then used as donor DNA for the transformation of a *lac*[−] *E. coli* host (MC1066) from *lac*[−] to *lac*⁺. The transformation mixture was spread on a plate of McConkey agar containing ampicillin. Red colonies were isolated as transformants

containing the *PHO5*–*lacZ* fusion gene. The nucleotide sequence at the junction between the *PHO5* and *lacZ* genes was determined by the Maxam–Gilbert method⁸⁾ to confirm the in-frame joining of the *PHO5* and *lacZ* genes (Fig. 3). One of the isolates, pAT335, was subjected to further study. The *HindIII*–*SalI* fragment carrying the fusion gene was replaced with the 600 bp *HindIII*–*SalI* fragment of YIp33 to construct pTI47 (not shown). This plasmid was used for constructing pTI50 and pTI60 (Fig. 1). A *GAP-Sc*–*lacZ* fusion was also constructed and employed as a reference.

Fig. 2. Construction of the *PHO5-lacZ* Fusion Gene.

Filled boxes indicate the DNA containing the *PHO5* gene. Open boxes indicate *lacZ*. Arrows inside the circles indicate the direction of orientation of the gene. The junction between *PHO5* and *lacZ* is shown by a wavy line.

Fig. 3. Nucleotide Sequence Surrounding the *PHO5-lacZ* Junction.

The 580 bp *Bam*HI fragment was excised from pAT335 DNA and its 5'-termini were labelled with 32 P as described.⁸⁾ The 32 P-labelled *Bam*HI fragment was then digested with *Bst*EII and the resulting 200 bp fragment containing the *PHO5-lacZ* junction, which is shown as a vertical line, was sequenced by the Maxam-Gilbert method. The 17mer, which was used for the primer extension experiments, is the stretch of nucleotides underlined. The vertical arrowheads indicate the transcription start sites determined in the *S. cerevisiae* host. The big one indicates the major initiation site. The initiator ATG of the *PHO5* protein is boxed.

The 7.6 kb *Hind*III-*Sal*I fragment carrying the *GAP-Sc-lacZ* fusion gene was excised from pGC1 and replaced with the 600 bp *Hind*III-*Sal*I fragment of YIp33 to construct pTI55 (not shown). This plasmid was used for constructing pTI61 and pTI62 (Fig. 1). Plasmids containing the *lacZ* fusion gene thus constructed had a further insertion at their *Sal*I site of either pSB3 or pSR1 linearized by *Xho*I digestion (Fig. 1). These plasmids can all propagate in *E. coli*, *S. cerevisiae* and *Z. rouxii*.

Production of β -galactosidase by transformants containing the fusion gene

Each test plasmid was introduced into the indicated host strains of *S. cerevisiae* or *Z. rouxii* by selecting *Leu*⁺ transformants. Two independent transformants were subjected to the quantitative enzyme assay (Table II). The transformants to be tested were grown in the selective medium and the cells were harvested at the log phase, and the β -galactosidase activity was measured. The fraction of cells

Table II. EXPRESSION OF THE *GAP-lacZ* AND *PHO5-lacZ* FUSION GENES

Plasmid	Fusion gene	Vector	Host ^a	β -Galactosidase units (A)	Fraction of cells retaining plasmid (B)	A/B
pTI48	<i>GAP-Zr-lacZ</i>	pSB3	ME3 [pSB3]	643	0.652	986
				560	0.385	1450
pTI49	<i>GAP-Zr-lacZ</i>	pSB3	ME3 [pSB3]	863	0.495	1740
				1350	0.661	2060
pTI51	<i>GAP-Zr-lacZ</i>	pSR1	ME3	966	0.551	1750
				940	0.596	1580
pTI52	<i>GAP-Zr-lacZ</i>	pSR1	ME3	932	0.553	1690
				1320	0.605	2180
pTI61	<i>GAP-Sc-lacZ</i>	pSB3	ME3 [pSB3]	502	0.538	933
				478	0.422	1130
pTI62	<i>GAP-Sc-lacZ</i>	pSR1	ME3	455	0.589	770
				505	0.614	822
pTI50	<i>PHO5-lacZ</i>	pSB3	ME3 [pSB3]	517	0.457	1130
				444	0.371	1200
pTI60	<i>PHO5-lacZ</i>	pSR1	ME3	463	0.660	702
				547	0.624	877
pTI48	<i>GAP-Zr-lacZ</i>	pSB3	K12-2A [cir ⁰]	71	0.068	1040
				65	0.033	1970
pTI49	<i>GAP-Zr-lacZ</i>	pSB3	K12-2A [cir ⁰]	133	0.095	1400
				79.1	0.070	1130
pTI61	<i>GAP-Sc-lacZ</i>	pSB3	K12-2A [cir ⁰]	76.2	0.103	740
				82.5	0.079	1040

^a ME3 [pSB3] was used as a host for pSB3-based plasmids. On the other hand, ME3 was used as a host for pSR1-based plasmids, since ME3 contains pSR1 as a resident plasmid. *Z. rouxii* transformants were grown in SD containing 5% NaCl. *S. cerevisiae* transformants were cultivated in a synthetic medium without leucine.

carrying the plasmid was also determined as described under **Materials and Methods**. pSB3-based plasmids carrying the *GAP-Zr-lacZ* or *GAP-Sc-lacZ* genes (pTI48, pTI49 or pTI61) could replicate in a *S. cerevisiae* host, K12-2A[cir⁰], but were unstably maintained. This instability is due to the absence of the partitioning mechanism for pSB3 in the heterologous host rather than to their inefficient replication in *S. cerevisiae*.⁵⁾ Three plasmids showed low but similar stability in the *S. cerevisiae* host. Transformants carrying pTI48 or pTI49, both of which carry the *GAP-Zr-lacZ* fusion gene, produced higher levels of β -galactosidase than transformants carrying pTI61, which carries the *GAP-Sc-lacZ* fusion gene. K12-2A[cir⁰] carrying the 2 μ m DNA-based plasmid with the *GAP-Sc-lacZ* fusion gene showed an A/B value of 1130, which is similar to that shown by pTI61 in the same host. This result indicates that the promoter of

the *GAP-Zr* gene shows high expression in the *S. cerevisiae* host, as the promoter of the *GAP-Sc* gene does. Moreover, expression driven by the *GAP-Zr* promoter is comparable to or higher than that driven by the *GAP-Sc* promoter in *Z. rouxii*. Since similar levels of β -galactosidase were produced by the *GAP-Sc-lacZ* gene in the two hosts, we concluded that the *GAP-Zr* promoter supports the high expression of the *lacZ* gene in *Z. rouxii* as well as in *S. cerevisiae*. *Z. rouxii* transformants carrying pTI50 or pTI60 produced comparable amounts of β -galactosidase to those produced by strains carrying the *GAP-lacZ* gene. Constitutive expression of the *PHO5-lacZ* gene in *Z. rouxii* was unexpected because a positive factor(s) is needed for derepression of the *PHO5* promoter in *S. cerevisiae*.¹⁰⁾ The *PHO5-lacZ* fusion gene, when introduced into the *S. cerevisiae* host, only produced β -galactosidase in low-Pi medium, at a similar level to

that in the present study (data not shown).

Mapping of the transcriptional start site of the *GAP-Zr* gene

In the previous section, it was shown that the *GAP-Zr* promoter works satisfactorily well in both a homologous and a heterologous host. We tested whether or not the same transcriptional start site of *GAP-Zr* gene is selected in *S. cerevisiae* and *Z. rouxii*. pTI52, which is a pSR1-based plasmid carrying the *GAP-Zr-lacZ* fusion gene, was introduced into the *S. cerevisiae* host and the *Z. rouxii* host. Total RNA was extracted from K12-2A[pTI52] and ME3[pTI52] cells. RNA (60 μ g) thus obtained was annealed with 2.7 ng of 5'-³²P-labelled 17mer (d(GTTTTCCCAGTCACGAC)), whose 5' end is located 39 bases downstream from the *GAP-Zr-lacZ* junction, and then the primer extension reaction was carried out. Fig. 4 shows that the transcrip-

tion of *GAP-Zr* starts at -58 and -59 in *Z. rouxii* and at -58 in *S. cerevisiae*.

Discussion

An increasing number of yeast genes which can be expressed in a heterologous yeast host have been reported in recent years.¹¹⁻¹³ Each promoter of such genes is a possible donor of a promoter for heterologous expression, however, the promoter function in a heterologous host has not been studied in detail in most cases. In the case of two distantly related yeasts, *Schizosaccharomyces pombe* and *S. cerevisiae*, the extent of heterologous expression is lower than that of homologous expression and the transcriptional start site of the gene is changed in the heterologous host.^{14,15} On the other hand, the *GAP-Zr* promoter showed high expression in the *S. cerevisiae* host and, moreover, it initiated transcription at the same site(s) in homologous and heterologous hosts.

The constitutive expression of the *PHO5* promoter in *Z. rouxii* can be explained in several ways. One obvious explanation is that the *PHO5* promoter sequence contains, by chance, a promoter which can be expressed constitutively in *Z. rouxii*. However, it is still possible to assume the presence of a positive factor which can recognize the genuine UAS of the *PHO5* gene in *Z. rouxii*. In this context, it should be emphasized that the genomic DNA of *Z. rouxii* contains a sequence which is hybridizable, although weakly, with the *PHO4* gene of *S. cerevisiae* (unpublished result). Further work is necessary to elucidate the mechanism underlying expression of the *PHO5* promoter in *Z. rouxii*.

At present, three strong promoters functioning in *Z. rouxii* are available. However, it is still worthwhile obtaining controllable promoters in addition to constitutive ones such as those described in this paper.

Acknowledgments. Part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Fig. 4. Determination of the Transcription Initiation Site of the *GAP-Zr* by Primer Extension.

Total RNA was obtained from K12-2A [cir⁰, pTI52] (lane 1), ME3 [pTI52] (lane 2) and ME3 (lane 3). The primer extension reaction was carried out after annealing the RNA with the ³²P-labelled 17mer primer as described under **Materials and Methods**. A known sequence ladder was run in the same gel. The numbers at the left-hand side indicate the lengths and the number at the right-hand side the transcription initiation site used in both *S. cerevisiae* and *Z. rouxii*, which corresponds to -58 bp upstream of the translation initiation codon of the *GAP-Zr* gene.

References

- 1) A. Toh-e, S. Tada and Y. Oshima, *J. Bacteriol.*, **151**, 1380 (1982).
 - 2) A. Toh-e, H. Araki, I. Utatsu and Y. Oshima, *J. Gen. Microbiol.*, **130**, 2527 (1984). 2a) K. Ushio, H. Tatsumi, H. Araki, A. Toh-e and Y. Oshima, *J. Ferment. Technol.*, **66**, 481 (1988).
 - 3) T. Imura, I. Utatsu and A. Toh-e, *Agric. Biol. Chem.*, **51**, 1641 (1987).
 - 4) J. P. Holland and M. J. Holland, *J. Biol. Chem.*, **254**, 9839 (1979).
 - 5) A. Toh-e and I. Utatsu, *Nucl. Acids Res.*, **13**, 4267 (1985).
 - 6) J. Miller, "Experiments in Molecular Genetics," Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.
 - 7) A. Toh-e and T. Shimauchi, *Yeast*, **2**, 129 (1986).
 - 8) A. Maxam and W. Gilbert, *Methods in Enzymol.*, **65**, 499 (1980).
 - 9) S. Hahn, E. T. Hoar and L. Guarente, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 8562 (1985).
 - 10) A. Toh-e, Y. Ueda, S. Kakimoto and Y. Oshima, *J. Bacteriol.*, **113**, 727 (1973).
 - 11) C. Kohchi and A. Toh-e, *Mol. Gen. Genet.*, **203**, 89 (1986).
 - 12) R. Roggenkamp, H. Hansen, M. Eckart, Z. Janowicz and C. P. Hollenberg, *Mol. Gen. Genet.*, **202**, 302 (1986).
 - 13) S. Das, E. Kellermann and C. P. Hollenberg, *J. Bacteriol.*, **158**, 1165 (1984).
 - 14) P. R. Russell, *Nature*, **301**, 167 (1983).
 - 15) P. R. Russell, *Gene*, **40**, 125 (1985).
 - 16) L. Clarke and J. Carbon, *J. Mol. Biol.*, **120**, 517 (1978).
 - 17) M. M. Casadaban, A. Martinez-Arias, S. K. Shapira and J. Chou, *Methods in Enzymol.*, **100**, 293 (1983).
 - 18) D. Botstein, C. Falco, S. Stewart, M. Brennan, S. Scherer, D. Stinchcomb, K. Struhl and R. W. Davis, *Gene*, **8**, 17 (1979).
-