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Cloning and Expression of *Kluyveromyces fragilis* β-Galactosidase Gene in *Saccharomyces cerevisiae*

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A gene coding for the β-galactosidase (lactase) of *Kluyveromyces fragilis* UCD 55-55 was isolated by complementation in *Escherichia coli* YMC9. From the plasmid library made from *Sau*3A-digested chromosomal DNA, one positive clone was selected. The cloned gene for β-galactosidase was on 7.3 kilobase pair DNA fragment, and a slightly low level of β-galactosidase enzyme activity was detected in *E. coli*. It was also confirmed that the cloned gene comes from *K. fragilis* by DNA-DNA hybridization and immunochemical blotting experiments. In order to construct a new yeast strain having the metabolic ability for lactose, the cloned gene for *K. fragilis* β-galactosidase was inserted in yeast vector YEp24 and YRp17, and transformed into *Saccharomyces cerevisiae* YNN27 and M1-2B. The yeast transformants showed the nearly the same β-galactosidase productivity as level of *K. fragilis* when uninduced, but these could not utilize lactose as a sole carbon source, presumably due to the lack of lactose transport system. Nevertheless, a slightly higher ethanol productivity was achieved by these transformants than *S. cerevisiae* or *K. fragilis*, in the medium containing glucose and lactose.

For the construction of a hybrid yeast strain which is capable of fermenting lactose and tolerant to high concentrations of ethanol, the fusant yeast strain have been made by protoplast fusion between the yeast *Kluyveromyces* species containing a gene coding the enzyme β-galactosidase which can ferment lactose, and *Saccharomyces cerevisiae* which is more tolerant to high concentrations of ethanol than *Kluyveromyces* species. The resulting fusant could ferment lactose to high concentrations of ethanol because it carried the genetic characteristics of both parental yeast strains (8, 11, 19, 25).

Recently, using the recombinant DNA techniques, several genes from *Kluyveromyces* species have been cloned by complementation of auxotrophic markers in strains of *Escherichia coli* (3-5, 16). Specifically, the β-galactosidase (lactase; E.C. 3.2.1.23) gene from *Kluyveromyces lactis*, a heterothallic yeast, was initially cloned in 1978 (5). Furthermore, up to seven unlinked genes have been reported to be involved in the utilization of lactose in *K. lactis*. One of these genes is the structural gene for enzyme β-galactosidase (*Lac4*), another codes for the permease, and the remaining genes play important roles in the regulation of enzyme synthesis (21). Subsequent studies revealed that the regulation of *Lac4* gene expression is somewhat complex and probably involves the galactose/lactose induction system (18, 20) as well as the catabolite repression (7, 10).

In the case of *K. fragilis*, an inducible enzyme β-galactosidase has been shown to contain two major subunits with molecular weights of 90 kilodaltons (kDa) and 120 kDa (13), whereas the β-galactosidase from *K. lactis* is composed of two identical subunits of 135 kDa (6) as likely as the procaryotic β-galactosidase of *E. coli* which consists of four identical subunits each with 116 kDa. Based on electron microscopic study, it has been also reported that β-galactosidase from *K. fragilis* is composed of 9 to 10 subunits (13). Moreover, comparisons of amino acid compositions indicated that the physicochemical properties of β-galactosidase from *K. fragilis* might be quite different from those of enzymes from bacteria and other yeasts (27). The enzyme from *K. fragilis* contains about 30% more methionine than the bacterial enzymes.

We report here on the cloning and characterization of a gene coding for β-galactosidase from *K. fragilis*. We

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also describe our attempts to construct the recombinant yeasts by introducing this gene into yeast - *E. coli* shuttle vectors and expressing the gene in *S. cerevisiae*.

MATERIALS AND METHODS

Microorganisms and Media

The microbial strains used throughout this work are summarized in Table 1. Yeast cells were maintained on YPD medium composed of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. *E. coli* strains were cultivated on Luria-Bertani (LB) broth medium, and used as hosts in transformation experiments. If neccessary, ampicillin was added into culture broth at concentration of 50 μg/ml, tetracycline at 15 μg/ml, isopropyl-β-D-thiogalactopyranoside (IPTG) at 100 μM, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 0.004%. For the assay of β-galactosidase activity, *E. coli* cells were cultured on M9 minimal medium containing 0.5% glucose supplemented with 100 μM of IPTG or lactose, and yeast strains were grown on Yeast Nitrogen Base (YNB; Difco Laboratories, MI, U.S.A.) medium with either 0.2% carbon sources. In ethanol fermentation of the recombinant yeasts, 10% of seed culture on YPD medium was inoculated into Erlenmeyer flasks with the fermentation media (pH 5.0), and cultivated statically at 30℃ for 4 days.

Preparation of Chromosomal DNAs

The chromosomal DNA from *K. fragilis* UCD55-55 was prepared by the procedure of Sherman et al. (22). The yeast cells were lysed with Zymolyase 60,000 (60,000 unit/g, Seikagaku Kogyo, Ltd, Tokyo, Japan), followed by treatment with pancreatic ribonuclease and pronase E. The chromosomal DNA of *E. coli* YMC9 was prepared according to the procedure of Rodriguez and Tait (17). The ethanol-precipitated DNAs were dried, dissolved, and stored in TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) over 10 μl of chloroform until used.

Construction of Genomic Library and Gene Cloning in *E. coli*

The chromosomal DNA of *K. fragilis* was partially digested with *Sau*3A enzyme, and fractionated by ultracentrifugation on 10~40% sucrose gradient (14). The fractions containing 4~10 kilobase pairs (kb) fragments were pooled and dialyzed against TE buffer. The recombinant plasmid library was constructed by ligating *K. fragilis* genomic DNA fragments into *Bam*HI-cut and bacterial alkaline phosphatase (BAP)-treated pBR322 plasmid. The resulting plasmids were transformed into *E. coli* YMC9 or JM83 competent cells made by the calcium chloride method described previously (9), and the transformed bacterial cells were spread on LB plates containing ampicillin, IPTG, and X-gal. The preparation of chimeric plasmid DNA from a selected clone (pFR-1) was carried out as described by Maniatis *et al.* (14).

Gene Cloning in *S. cerevisiae*

In order to introduce the cloned β-galactosidase gene into *S. cerevisiae*, first of all, the plasmid pFR-1 was partially cleaved by *Cla*I and *Sal*I, and 8.25 kb fragment was electroeluted and purified. After this fragment was ligated with *Cla*I/*Sal*I-cut and BAP-treated YRp17 (24) fragment of 5.4 kb, the resulting plasmid YRpFR-1 was transformed into *E. coli* YMC9 or JM83 competent cells. On the other hand, *Pvu*I-cut pFR-1 plasmid was filled up to make blunt ends and recut with another restriction enzyme *Sal*I. After electroeluting 6.9 kb fragment from agarose gels, the purified fragment was inserted into the large fragment of *Pvu*II/*Sal*I-cut and BAP-treated YEp24 plasmid (2) having 6.35 kb to construct YEpFR-1 plasmid. The amplified plasmids in *E. coli* cells were isolated and transformed again into *S. cerevisiae* YNN27 or M1-2B according to the method of Sherman *et al.* (22). The positive clones were selected on YPD medium containing 0.004% X-Gal.

Southern Analysis

The *Xba*I single-cut plasmid which carries the *K. fragilis*

Table 1. The microbial strains used in this work

Strains	Genotype	Source
Kluyveromyces fragilis UCD 55-55	Wild type for β-galactosidase	Ref. (13)
Saccharomyces cerevisiae YNN27	α *trp1-289 ura3-52 gal2*	Genetic Engineering Research Institute (KCTC1547)
Saccharomyces cerevisiae M1-2B	*a trp1-289 ura3-52 his*	Genetic Engineering Research Institute (KCTC7037)
Escherichia coli YMC9	*F endA1 thi1 hsdR17 supE44* △(*argF lac*)*205*(*U169*) λ	Ref. (1)
Escherichia coli JM83	*ara* △(*pro-lac*) *rpsL thi* φ*80 dlacZ* △*M15* λ	Ref. (26)

β-galactosidase gene in pBR322 (pFR-1) was labelled with 7-biotin-deoxyadenosine triphosphate using a nick translation system (BRL Life Technologies, Inc., MD, U.S.A.) for preparation of the probe in Southern hybridization. The chromosomal DNAs (5 μg) of *K. fragilis* or *E. coli* were digested with several different restriction endonucleases, and the DNA fragments were separated on 0.7% agarose gel. The DNA fragments were transferred to nitrocellulose BAB5 (Schleicher & Schuell, Inc., NH, U.S.A.) and were hybridized with the prepared probe at 60℃ for 4 hours according to the procedure of Maniatis *et al.* (14). The post-hybridization wash was performed under high stringent conditions for 1 hour at 60℃ twice in 0.2 × SSC buffer (30 mM sodium chloride, 3.4 mM sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS). The hybridized bands on the nitrocellulose filter were visualized using BlueGENE nonradioactive nucleic acid detection system (BRL Life Technologies, Inc.), which consists of streptavidine-alkaline phosphatase conjugate capable of binding with the biotin moiety of the nick translated probe, and the alkaline phosphatase substrates, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indolyl phosphate (BCIP).

Immunological Analysis

For the study of protein identity of yeast and bacterial β-galactosidases by immunoblotting, the protein solutions were prepared by the 10 minute-sonication of yeast cells cultivated on YPD medium or of *E. coli* cells on LB medium, and applied onto nitrocellulose paper BSB5. The bound proteins were first treated with mouse anti-*E. coli* β-galactosidase monoclonal antibody (BRL Life Technologies, Inc.) as the primary antibody and then with goat anti-mouse IgG (H+L) conjugated with alkaline phosphatase as the secondary antibody (BRL Life Technologies, Inc.). Using ProtoBlot Western Blot AP System (Promega Corp., WI, U.S.A.), the bound antibody was detected by colorizing with the substrates of alkaline phosphatase, NBT and BCIP.

Analysis of Fermentation Broths

The β-galactosidase activities in the microbial cells were measured by the procedure of Miller (15). The units of enzyme were expressed in nmole of o-nitrophenol generated per minute per 1 optical density (OD) of cells or 1 mg of protein when using o-nitro-phenylgalactoside as the substrate. The protein contents were measured by the Folin-Lowry method (12) from the supernatant obtained after lysing the cells with the ultrasonic dismembrator (Fisher Scientific Co., NJ, U.S.A.) for 10 minutes. The concentrations of the produced ethanol were determined by the gas chromatographic method using Gas Chromatograph (Model 5890) of Hewlett Packard S.A. (Switzerland) (11). The biomass of the yeast cells was measured as turbidance at 600nm.

RESULTS

Molecular Cloning of the β-Galactosidase Gene from *Kluyveromyces fragilis*

The plasmid library constructed by ligating *Sau*3A-cut *K. fragilis* DNA into pBR322 was transformed into *E. coli* YMC9 which has a deletion in *lacZ* coding for the enzyme β-galactosidase. Positive recombinant colonies were screened on the basis of complementation of the Lac phenotype of host *E. coli*; the production of blue pigment of a colony by degradation of X-gal in the medium would indicate expression of a β-galactosidase gene in the host bacteria. Among 13,000 colonies screened, one blue colony was found, probably containing the β-galactosidase gene from *K. fragilis*. To further verify this, the plasmid of the positive clone, named pFR-1, was isolated and transformed into another Lac host, *E. coli* JM83. All the transformants gave blue colonies on LB plate containing X-gal, too.

In order to characterize this clone, the plasmid pFR-1 was recovered and digested with several restriction endonucleases to construct the restriction map of the insert. Restriction mapping revealed that the size of the insert is 7.3 kb, and that one of the *Bam*HI sites is missing at the *Bam*HI/*Sau*3A cloning junction (Fig. 1). The restriction analysis showed that the insert has one recognition site for *Cla*I, *Pvu*II, *Sal*I, *Xba*I and *Xho*I, two sites recognized by *Bam*HI, *Bgl*II and *Pst*I, and four *Eco*RI sites.

The combined molecular weight of β-galactosidase subunits of *K. fragilis* is around 210 kDa (13) and the estimated size of the coding region would be about 5.9 kb. Therefore, the complete β-galactosidase gene of *K. fragilis* could be contained in this insert of 7.3 kb. This explanation could be supported by the fact that the β-galactosidase activity was fully lost when the DNA sequences in the front of *Cla*I site (0.9 kb) was deleted (data not shown).

Southern Analysis

In DNA-DNA hybridization analysis using *Xba*I-cut pFR-1 plasmid as a probe, the chromosomal DNA of

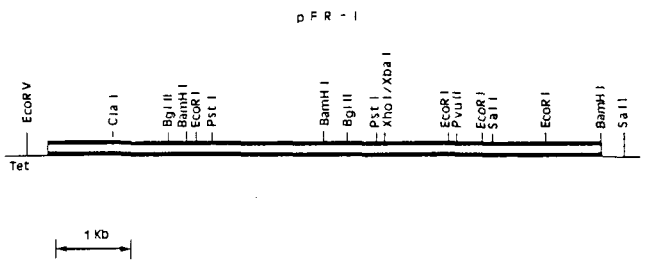

Fig. 1. Restriction map of the *Kluyveromyces fragilis* DNA insert in plasmid pFR-1.
The restriction sites for *Xho*I and *Xba*I were too close to determine their order.

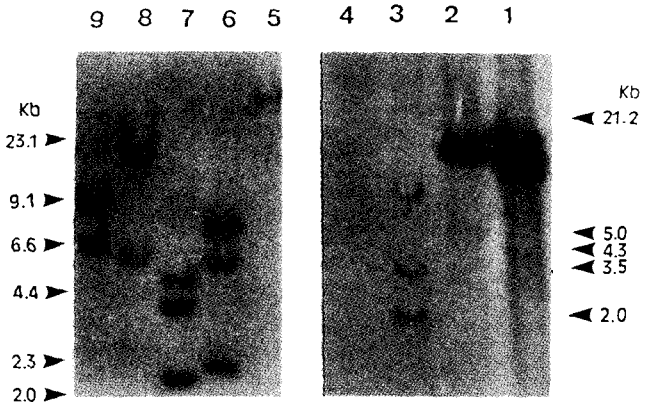

Fig. 2. Southern hybridization of pFR-1 with different chromosomal DNAs.

After electrophoresis of *K. fragilis* or *E. coli* chromosomal DNAs (5 μg) digested with different restriction enzymes on 0.7% agarose gel along with *Xba*I-cut pFR-1 and *Sal*I-cut pKR-1B, the separated DNA fragments were transferred to nitrocellulose paper and hybridized with biotin-labelled probe pFR-1. Lane 1; *Xba*I-cut pFR-1 plasmid, lane 2; *Sal*I-cut pKR-1B plasmid, lane 3; *Bam*HI-cut *K. fragilis* UCD55-55 chromosomal DNA, lane 4; *Bam*HI-cut *E. coli* YMC9 chromosomal DNA, lane 5; uncut *K. fragilis* UCD55-55 chromosomal DNA, lane 6; *Bgl*II-cut *K. fragilis* UCD55-55 chromosomal DNA, lane 7; *Pst*I-cut *K. fragilis* UCD55-55 chromosomal DNA, lane 8; *Sal*I-cut *K. fragilis* UCD55-55 chromosomal DNA, lane 9; *Xho*I-cut *K. fragilis* UCD55-55 chromosomal DNA.

K. fragilis digested with restriction enzyme *Bam*HI gave 3 bands - 8.0 kb, 3.7 kb, and 1.9 kb (Fig. 2, lane 3). These results are consistent with the results of restriction mapping. Two small bands represent the DNA segments within the insert of pFR-1 (Fig. 1), and the largest band corresponds to the chromosomal fragment containing an end fragment of the insert in our clone. In contrast, genomic DNA from *E. coli* YMC9 did not show any strong complementarity with the cloned plasmid when cut by *Bam*HI (Fig. 2, lane 4). This confirms that the β-galactosidase gene which has been cloned comes from *K. fragilis*.

In order to further confirm the DNA sequence of pFR-1, the genomic DNA of *K. fragilis* was digested with several different restriction enzymes and then hybridized with the probe prepared from pFR-1. As seen in Fig. 2, the results were consistent with the results of restriction analysis. In case of cutting with *Bgl*II (Fig. 2, lane 6), chromosomal DNA fragment gave 3 DNA bands, one of 2.4 kb that corresponds to the internal fragment in the insert and two larger bands containing genomic DNA segments with each end fragment of the inserted gene. A similar result was also obtained after digesting with *Pst*I (Fig. 2, lane 7); one small band for internal segment of 2.2 kb and two larger bands containing the termini of the gene. The *Sal*I-cut and *Xho*I-cut chromosomal DNA fragments (Fig. 2, lanes 8 and 9) appeared to have two large bands because these two restriction enzymes have unique recognition sites in the insert. Thus, it can

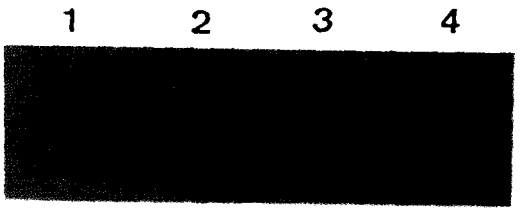

Fig. 3. Immunochemical blotting of β-galactosidases with anti-*E. coli* β-galactosidase antibody.

Total proteins (6 μl) from the cell lysates by sonication were loaded on nitrocellulose paper, and treated with mouse anti-*E. coli* β-galactosidase antibody and then with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L). 1; *E. coli* JM83 harboring pUC18 plasmid, 2; *E. coli* JM83 harboring pFR-1 plasmid, 3; *E. coli* YMC9 harboring pFR-1 plasmid, 4; *K. fragilis* UCD55-55.

be concluded that the β-galactosidase gene of our clone has not been rearranged compared to *K. fragilis* genomic DNA, and finally that the gene for β-galactosidase in our clone came from the chromosome of *K. fragilis*.

The β-galactosidase gene (*Lac4*) of K. lactis, which has been already cloned by Dickson's group (5) was also probed with our clone. The *Sal*I-cut pKR-1B, the plasmid carrying the gene from *K. lactis*, very strongly hybridized with pFR-1 (Fig. 2, lane 2), which implies that these two cognate genes have a strong DNA sequence similarity.

Immunological Analysis

The protein identity of the β-galactosidase enzymes from *E. coli* clone and *K. fragilis* was immunochemically tested using commercially available anti-*E. coli* β-galactosidase monoclonal antibody. As seen in Fig. 3, this antibody neither bound well with β-galactosidase from *K. fragilis* nor the enzymes from *E. coli* YMC9 and JM83 carrying pFR-1 plasmid, although there was a strong interaction with the protein of *E. coli* JM83 harboring pUC18 plasmid capable of producing *E. coli* own β-galactosidase. This result does not directly indicate that the β-galactosidase enzyme of our clone is the same as that of *K. fragilis*, but strongly suggest that both enzymes are quite different from the *E. coli* β-galactosidase.

Cloning of *K. fragilis* β-Galactosidase Gene into *S. cerevisiae*

In order to construct the recombinant *S. cerevisiae* strains capable of utilizing lactose, yeast - *E. coli* shuttle vector YEp24 and YRp17 were employed as cloning vehicles. The 8.25 kb fragment isolated from the pFR-1 plasmid which contains the entire sequence of the cloned β-galactosidase from *K. fragilis* was introduced into *Sal*I/*Cla*I-digested YRp17 fragment having 5.4 kb. As described in Materials and Methods section, the 6.9 kb fragment of pFR-1 plasmid carrying the full sequences of β-galactosidase was inserted into 6.35 kb fragment of *Pvu*II/ *Sal*I-cut YEp24 plasmid. After the transformation of the resulting plasmids in *E. coli* YMC9 or JM83, the cells grown on the LB medium containing ampicillin were

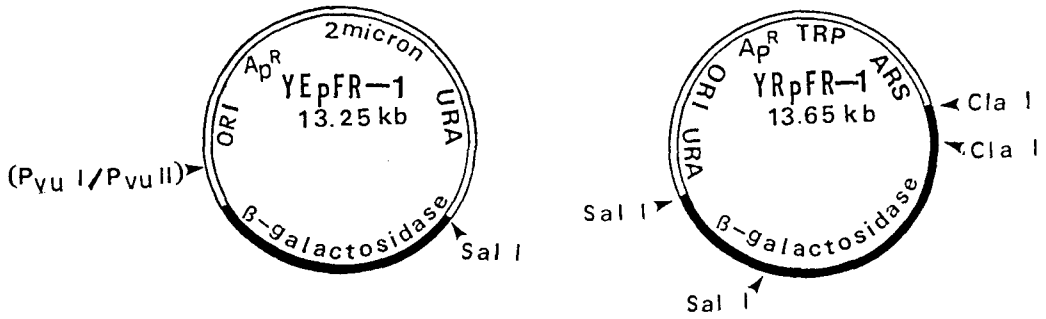

Fig. 4. The constucted *E. coli* - yeast shuttle vectors carrying the gene for *K. fragilis* β-galactosidase, YEpFR-1 and YRpFR-1.

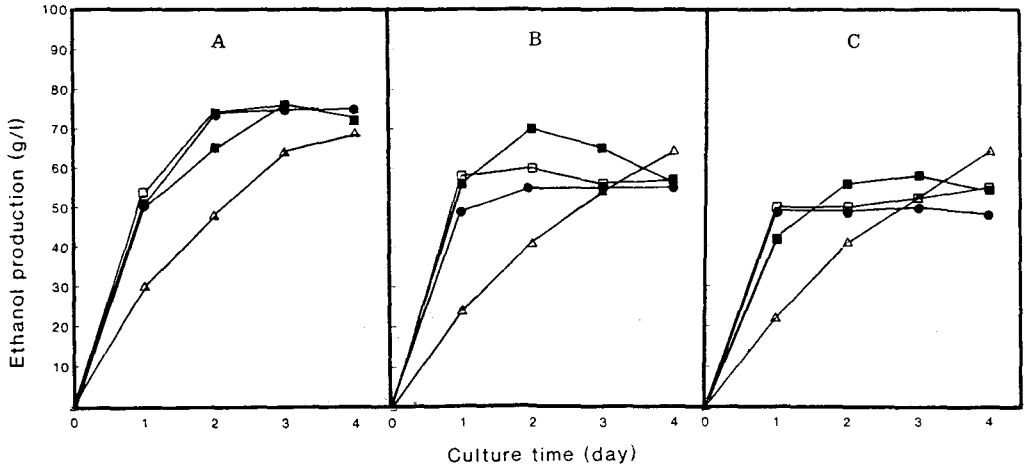

Fig. 5. The ethanol production profiles by *K. fragilis*, *S. cerevisiae*, and the recombinant *S. cerevisiae* cells harboring *K. fragilis* β-galactosidase.

The yeast strains were grown statically at 30°C on the medium containing (A); 15% glucose, (B); 12% glucose and 3% lactose, or (C) 10% glucose and 5% lactose. Symbols: ●–●; *S. cerevisiae* M1-2B, ■–■; *S. cerevisiae* M1-2B harboring YRpFR-1, □–□; *S. cerevisiae* YNN27 harboring YRpFR-1, △–△; *K. fragilis*.

harvested for large preparation of the plasmids. These amplified plasmids were then re-transformed into *S. cerevisiae* YNN27 or M1-2B, and the positive clones were selected on YPD medium containing X-Gal. In all cases blue yeast cells could be picked up and the plasmids, named YRpFR-1 constructed from YRp17 or YEpFR-1 from YEp24 vector, were isolated and identified (Fig. 4).

Expression of β-galactosidase Genes in the Recombinant *E. coli* and *S. cerevisiae*

To investigate the degree of the expression of *K. fragilis* β-galactosidase gene, the β-galactosidase enzyme activities produced by the recombinant *E. coli* and *S. cerevisiae* were measured. As shown in Table 2, in recombinant *E. coli* YMC9 and JM83 clones carrying pFR-1 plasmid, β-galactosidases were synthesized even in the absence of inducers such as lactose or IPTG. Furthermore, the recombinant strains did not grow well in lactose minimal medium and the synthesis of β-galactosidase did not increase much after the addition of inducers in the presence of glucose in culture medium. The same results were obtained for the recombinant *S. cerevisiae* YNN27 and M1-2B harboring YRpFR-1 or YEpFR-1 plasmid, even though the expression level of the gene was slightly higher than recombinant *E. coli*. In contrast, the gene in *K. fragilis* was greatly induced about 5 fold by supplementation of lactose to the medium compared to glucose, but the addition of IPTG did not affect the expression level of the gene. Its expression level in *K. fragilis* when induced by lactose was 3~6 times higher than that of recombinant *E. coli* or recombinant *S. cerevisiae* clones. These results indicate that the recombinant *E. coli* and *S. cerevisiae* strains could not utilize lactose as a sole carbon source, presumably due to the deficiency of lactose transport system although they synthesized the enzyme in relative amounts.

Fermentation Behavior of the Recombinant *S. cerevisiae* Cells

The ethanol fermentation by the recombinant yeasts was studied and compared with that of *K. fragilis* and *S. cerevisiae* host. Because YEpFR-1 plasmid was found to be somewhat unstable even though it gave slightly higher productivity of β-galactosidase than YRpFR-1 (data

not shown), *S. cerevisiae* clones harboring YRpFR-1 plasmid were studied on glucose medium supplemented with lactose (Fig. 5). No significant differences between the host and recombinant *S. cerevisiae* were observed when cultured on 15% glucose medium, but slightly higher ethanol productivity was achieved when lactose was added to the glucose medium. This shows that lactose could be consumed very slowly despite the lack of its uptake system.

DISCUSSION

In this study we report the cloning of the structural gene which codes for the β-galactosidase enzyme from *K. fragilis* UCD55-55. One colony was chosen as a positive clone based on the complementation of *lacZ* gene in host bacteria *E. coli* YMC9. The plasmid in the clone, pFR-1, had a 7.3 kb insert. Based on the report of Mahoney *et al.* (13), it could be assumed that the cloned gene is able to encode neary the full sequences of β-galactosidase enzyme.

In the DNA-DNA hybridization and immunochemical blotting studies, it was confirmed that the cloned gene of β-galactosidase comes from *K. fragilis* chromosomal DNA, not from the *E. coli* host. It was also of interest to know whether the cloned gene from *K. fragilis* is homologous with the β-galactosidase gene from *K. lactis* which has been cloned by Dickson's group (5). The hybridization experiment revealed that they cross-hybridize quite strongly, even though they have different restriction patterns.

The efficiencies of eukaryotic gene expression in the recombinant *E. coli* hosts were studied and compared with the case of *K. fragilis*. The production levels of β-galactosidase in *E. coli* YMC9 and JM83 were lower than that in *K. fragilis* when induced, but nearly the same when not induced. The similar profile of gene expression level was observed in recombinant *S. cerevisiae* hosts. This suggests that the eukaryotic gene is efficiently expressed in recombinant bacterial cells as well as in recombinant yeast cells. However, it is striking that the cloned gene lost the transcriptional and translational control by an inducer, lactose, in their host cells, even though any regulatory control of β-galactosidase gene (*Lac4*) expression by IPTG was not observed in *K. fragilis*, differently from *E. coli* gene (*LacZ*) (Table 2). Two possible

Table 2. The expression level of β-galactosidase genes.

Host	Plasmid	Inducer	Productivity (unit/cell)[a]	Specific Activity (unit/mg of protein)
Kluyveromyces fragilis UCD55-55		Glucose	54.8	79.1
		Glucose+IPTG	59.9	85.7
		Lactose	186.1	426.6
Escherichia coli YMC9	pFR-1	Glucose	49.9	103.1
		Glucose+IPTG	51.6	82.4
		Lactose	39.2	109.0
	YEpFR-1	Glucose	24.2	79.1
		Glucose+IPTG	21.5	50.5
		Lactose	22.3	75.9
Escherichia coli JM83	pFR-1	Glucose	40.8	79.1
		Glucose+IPTG	43.9	100.4
		Lactose	54.7	118.8
	YEpFR-1	Glucose	8.4	36.7
		Glucose+IPTG	8.5	31.1
		Lactose	10.6	26.5
Sacchromyces cerevisiae YNN27	YEpFR-1	Glucose	65.7	243.3
		Glucose+IPTG	68.2	227.3
		Lactose	63.8	ND[b]
	YRpFR-1	Glucose	70.8	202.5
		Glucose+IPTG	42.3	282.0
		Lactose	57.0	ND[b]
Sacchromyces cerevisiae M1-2B	YEpFR-1	Glucose	41.7	182.9
		Glucose+IPTG	40.9	184.1
		Lactose	20.9	ND[b]
	YRpFR-1	Glucose	32.8	115.1
		Glucose+IPTG	32.5	108.3
		Lactose	30.0	ND[b]

[a]Cell concentrations were measured in OD units at 600 nm. [b]ND; not determined. It was impossible to measure the amount of protein due to so poor growth of strains. The microbial cells were cultivated in minimal broth medium. The unit of enzyme activity was defined as the amount of o-nitrophenol (nmole) per minute when o-nitrophenyl-β-galactoside was used as a substrate. Each point represents an average value of duplicates.

explanations for why are that the promoter region of *K. fragilis* gene for this enzyme is not well bound with repressors in the presence of lactose, or that this gene for β-galactosidase was expressed from the *tet* promoter. The first possibility may be more reliable in the following viewpoints; 1) the repressor of *E. coli* has different binding properties from that of *K. fragilis* as shown in the case of IPTG, 2) there is no repressor for *Lac4* promoter in *S. cerevisiae*, and 3) the cloned β-galactosidase gene in *E. coli* does not show any remarkable differences in expression level compared to the case of *S. cerevisiae* or *K. fragilis*.

Another surprising fact is that the recombinant *S. cerevisiae* cells as well as *E. coli* cells are unable to grow on lactose media, even though they produce sufficient quantities of β-galactosidase. The fermentation results also show that the recombinant *S. cerevisiae* strains producing β-galactosidase do not improve ethanol productivity significantly when lactose is supplemented to culture broth. As reported by Sreekrishna *et al.* (23), it can be deduced that the recombinant strains might be lacking lactose transport system through cell membrane.

The enzyme β-galactosidase has been the focus of interest from both commercial and nutritional aspects. Especially, lactose has a low solubility which often causes problems in dairy products. One of the most promising solution for this problem is hydrolysis of lactose into its monosaccharide components, glucose and galactose. This suggests that scale-up production of this enzyme would be of interest to the dairy industry. A scale-up production of enzyme β-galactosidase may be further achieved by introducing the gene for *K. fragilis* lactose permease simultaneously with β-galactosidase gene in a ethanol-tolerant *S. cerevisiae* strain, and fermenting the constructed yeast in whey, the waste of cheese-industry.

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