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Cloning of the Alkaline Phosphatase Gene from *Kluyveromyces fragilis*

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In order to clone the gene coding for alkaline phosphatase in the yeast *Kluyveromyces fragilis*, a genomic library was constructed using the yeast-*E. coli* shuttle vector pHN114 as a cloning vector. From the genomic library, a clone carrying the gene was isolated and the plasmid was designated as pSKH101. A restriction enzyme map was made using this plasmid. Subcloning experiments and complementation studies showed that alkaline phosphatase was active only in the original 3.1 kb insert. Southern hybridization analysis confirmed that the cloned DNA fragment was derived from *K. fragilis* genomic DNA. Using a minicell experiment, the product of the cloned gene was identified as a protein with a molecular weight of 63 KDa. A 0.6 kb *Hin*dIII fragment, which showed promoter activity, was isolated using the *E. coli* promoter-probe vector pKO-1.

Phosphatases catalyze the hydrolytic cleavage of phosphoric acid esters. They are designated as either 'acid' or 'alkaline' phosphatases according to their pH optima. Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) occurs in various organisms and tissues.

It has been reported that the enzymatically active alkaline phosphatase (ALPase) of *E. coli* is composed of two identical polypeptides (21), coded by a single *phoA* structural gene. The *phoA* gene is located on 8.8 minutes region of the *E. coli* chromosome and encoding ALPase which, in mature form, is localized in the periplasmic space of the bacterium (13). The negative regulation of phosphate regulon involves the *pstA*(=*phoT*), *pstB*, and *pstU* genes, and all of them have been sequenced. The structural gene for the ALPases (ALPaseI and II) from *Bacillus licheniformis* has been cloned (6). The vegetative ALPase of *Bacillus subtilis* has been characterized (7). The ALPase structural gene(*phoA*) of *Pseudomonas aeruginosa* has also been cloned and sequenced (4).

In animals, ALPase activity is found in essentially all tissues. In almost all animals, tissue-nonspecific ALPase is the most common isozyme of ALPase, which is found in liver, kidney, bone, and most other tissues. A second isozyme is intestinal ALPase (IAP), which is found greatest abundance in the intestines of all mammals. A third isozyme is placental ALPase (PALP), which appears in placental tissue of humans and other higher primates. Recently, human placental and intestinal ALPase cDNAs have been cloned and sequenced (16).

Yeast cells have a number of hydrolytic enzymes, and the level of some of these enzymes depends on growth conditions, for example, under conditions of phosphate starvation. In *Saccharomyces cerevisiae*, the genetic regulating system of repressible acid phosphatase synthesis has been extensively studied, and many structural and regulatory genes have been characterized (19). Of the three regulated acid phosphatase genes (*PHO5*, *PHO10*, and *PHO11*) in *S. cerevisiae*, two have been cloned (*PHO5* and *PHO11*) and sequenced (24). Also, the *PHO 10* gene has been identified and characterized (24). Alkaline phosphatase is found in the vacuoles and cytoplasm in *S. cerevisiae* (25) and is known to be a glycoprotein of the Asn-GlcNac type. Two species of alkaline phosphatase have been found in cellular extracts of *S.*

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cerevisiae. One is a specific p-nitrophenyl phosphatase (approximately 90 KDa) encoded by *PHO13*, and the other, which is repressed by inorganic phosphate, is a nonspecific ALPase. Also, the particulate form of ALPase has been identified in *S. cerevisiae* (1). Expression of the *PHO8* gene, encoding nonspecific ALPase in *S. cerevisiae*, is regulated through a system consisting of products of the *PHO5, PHO80, PHO81*, and *PHO85* genes under the influence of inorganic phosphate in the medium (19). Recently, the nucleotide sequence of a 3,694 bp DNA fragment containing the *PHO8* gene was determined (9, 10). Yeast strains of the species *K. fragilis* have been studied extensively, and can be used commercially for the production of heterologous proteins. In contrast to *S. cerevisiae*, this yeast is capable of synthesis and secretion of a fully active enzyme (23). The regulatory mechanism of the ALPase of *K. fragilis* seems to be different from the ALPase of *S. cerevisiae*. Cloning the ALPase gene can provide tools for studying the function and regulation of the enzyme in the phosphate metabolism of this yeast. Furthermore, this enzyme can be a useful model enzyme for studies of protein transport (11). In this paper, we report the cloning and characterization of the gene coding for ALPase from *K. fragilis*. The cloned gene has its own promoter, which shows promoter activity in *E. coli*.

Table 1. List of strains and plasmids used in this study.

Strains	Genotypes and Phenotypes	Sources
Kluyveromyces fragilis		
Y610	wild type	Park *et al.* (1990)
Escherichia coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F'[traD36 proAB⁺ lacIq lacZΔM15]*	Yanisch-Perron *et al.* (1985)
YK537	*supE44 hsdR hsdM recA1 phoA1 leuB6* thi lacY rspL20 galK2 *ara-14 xyl-15*	Oka *et al.* (1985)
BD1854	*minA minB thi rspL his lac mtl man mal xyl tonA*	Diderischsen
Plasmids		
pHN114	Ap^r TRP1 ARS lacZ, shuttle vector	Park *et al.* (1990)
pUC18, 19	Ap^r lacZ	Yanisch-Perron *et al.* (1985)
pKO-1	Ap^r *galK*, expression vector	McKenney *et al.* (1981)
pSKH101	pHN114 containing the cloned fragment in *Bam*HI site	this study
pSKH201	pUC19 containing the cloned fragment in *Bam*HI site	this study

MATERIALS AND METHODS

Yeast, Bacteria, and Plasmids

The yeast, bacterial strains, and plasmids used in this study are listed in Table 1.

Media and Culture Conditions

K. fragilis Y610 was cultured in YEPD medium at 30℃ for 16 hours with shaking. The following media were used for cultivation of *E. coli*. LB medium was used for general propagation of *E. coli* cells. *E. coli* BD 1854 was grown in (A+B) minimal medium (2) containing A solution (1.85% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4, 0.5% NaCl, 1.0% NH_4Cl), and B solution (0.1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 1 μM $FeCl_3 \cdot 6H_2O$) with supplements (0.2% glucose, 0.4% vitamin free casamino acids (Difco), 5 μg/ml thiamine, 100 μg/ml L-leucine, 100 μg/ml L-histidine, and 50 μg/ml L-threonine). *E. coli* YK537 was grown in MOPS medium with a MOPS-buffered minimal salt solution (pH 7.4) and 0.2% glucose as a carbone source. The medium was supplemented with phosphate (as KH_2PO_4) either at a high (6.4×10^{-4} M) or low (6.4×10^{-5} M) concentration, and a 0.1% amino acid mixture (Difco). X-Gal agar plates (LB agar medium, 50 μg/ml ampicillin, 0.1 mM IPTG and 40 μg/ml X-Gal) were used for subcloning, with pUC series vectors, in *E. coli* JM109. X-P agar plates (MOPS medium with 40 μg/ml X-P and 50 μg/ml ampicillin) were used to select clones containing the ALPase gene. McConkey agar base (Difco) with 1% galactose and 50 μg/ml ampicillin was used for experiments with the pKO-1 promoter-probe vector (15). All *E. coli* cells were cultured aerobically at 37℃ for 16 hours.

Enzymes and Chemicals

All of the restriction endonucleases and T_4 DNA ligases were obtained from KOSCO Biotech or Takara Shuzo Co.. X-Gal, X-P, IPTG, MOPS, and Tricine were obtained from Sigma. [^{35}S]-methionine, α-[^{35}S]-dCTP, and Hyperfilm-^{3}H were obtained from Amersham. A random prime DNA labelling kit was obtained from Boeringer Mannheim. A Geneclean kit was obtained from Bio101 (La Jolla, USA). All other chemicals and enzymes were reagent grade and were obtained from commercial sources.

DNA Isolation

Chromosomal DNA was isolated from *K. fragilis* by the method of Cryer *et al.* (3). Large scale and small scale preparation of plasmid DNA was carried out by the SDS/alkaline lysis procedure of Birnboim and Doly (1).

Preparation of Cellular Crude Extracts and Enzyme Assay

E. coli cells grown for 16 hours were collected, washed with TM buffer (10 mM Tris-HCl (pH 8.6)+1 mM $MgCl_2$), and resuspended in the same buffer. The cell suspensions were sonicated in ice four times for 30 seconds at 1minute intervals. The sonicated extracts were centrifuged at 10,000×g for 10 minutes and used as enzyme solutions.

ALPase activity was determined by the published procedure of Onish *et al.* (18) with slight modifications. The standard assay mixture contained 0.8 m*l* of 0.1 M Tris-HCl buffer (pH 8.6), 0.1 m*l* of 10 mM p-nitrophenyl phosphate (disodium salt, Fluka), and 0.1 m*l* of enzyme solution. The reaction mixture was incubated at 50℃ and terminated by adding 0.5 m*l* of 1.0 M NaOH. The ALPase activity was determined by measuring optical density at 410nm using extinction coefficient of 1.73×10^4 $M^{-1}cm^{-1}$ in a spectrophotometer. A unit of activity was defined as the amount of enzyme which liberated 1 μM p-nitrophenol per minute at 50℃.

Electrophoresis

Agarose gel electrophoresis was performed as described by Maniatis *et al.* (14). SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (12).

Transformation

E. coli was transformed with plasmid DNA by the $CaCl_2$ method, as described by Maniatis *et al.* (14).

Cloning Procedure

Recombinant DNA techniques were performed as described by Maniatis *et al.* with some modifications (14). All enzymes were used as recommended by the manufacturers. Chromosomal DNA was partially digested with *Sau*3A1 to clone the structural gene coding for the ALPase of *K. fragilis*. Electrophoretic separation of partially digested DNA was performed in 0.8% agarose gel, and a gel piece with fragments over 2.0 kb was separated and purified using a hydroxylapatite column. The DNA was then ligated into the *Bam*HI site of yeast-*E. coli* shuttle vector pHN114 (20). Recombinant plasmids were transformed in *E. coli* JM109 and transformants were selected on X-Gal plates. About 5,000 white colonies were used for genomic library construction.

RESULTS AND DISCUSSION

Cloning of the Alkaline Phosphatase Gene

From the genomic library, plasmid DNAs were isolated and transformed in *E. coli* YK537, and clones were screened on X-P agar plates by complementation of *phoA* mutation. Positive complementation was indicated by the formation of blue colonies, in contrast to white colonies formed by *phoA* mutants. 47 blue colonies were selected, then small scale plasmid isolation was carried out. Isolated plasmid DNAs were analyzed by restriction enzyme digestion and one of them was identified as containing a 3.1 kb *Bam*HI fragment. The plasmid was designated as pSKH101 (Fig. 1).

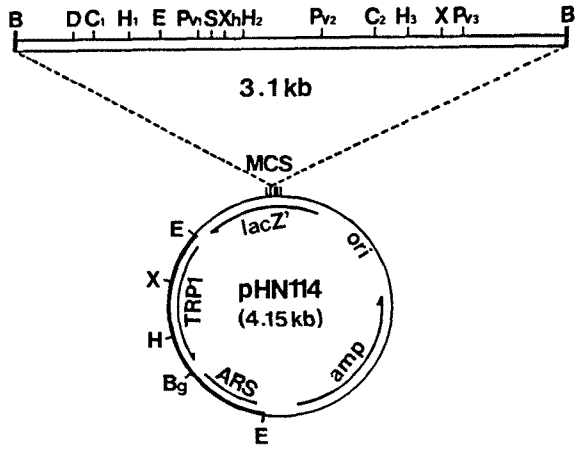

Fig. 1. Restriction map of pSKH101.
The top line shows the position of restriction enzyme sites in the 3.1 kb insert. The circular line represents the cloning vector pHN114. Abbreviations of restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*I; S, *Sst*I; X, *Xba*I; Xh, *Xho*I

From this result, it is clear that the cloned ALPase gene can be expressed in *E. coli*, and that the gene codes for ALPase, which cleaves the X-P monophosphoric acid ester.

Restriction Enzyme Mapping

Various restriction enzymes were used to map the restriction sites of pSKH101. The 3.1 kb pSKH101 insert contained single *Eco*RI, *Dra*I, *Xho*I, *Xba*I, and *Sst*I restriction sites, two *Cla*I sites, and three *Hin*dIII and *Pvu*II sites (Fig. 1).

Subcloning and Deletion Analysis

Subcloning was performed using the pUC vector series. Transformants were selected on X-Gal agar plates, or X-P agar plates. The location of the ALPase gene in the insert was determined by subcloning and complementation tests. As shown in Fig. 2, only the original 3.1 kb insert was necessary for complementing the *phoA* mutation of *E. coli* YK537.

pH Optimum

E. coli YK537 carrying pSKH201 was grown at 37℃ in MOPS medium (low phosphate) for 24 hours. The optimal pH of ALPase activity was 9.0 with p-nitrophenyl phosphate as a substrate (Fig. 3).

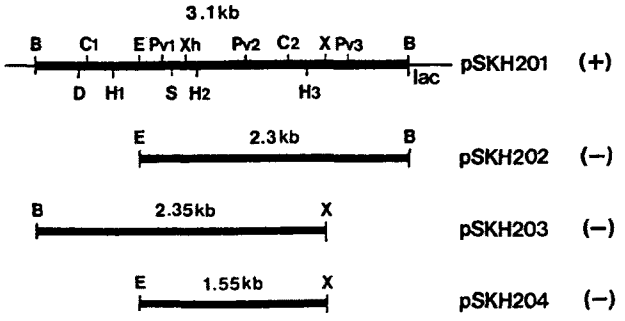

Fig. 2. Location of the alkaline phosphatase gene.
The horizontal thick lines represent derivatives of pSKH 101. All are hybrid plasmids derived from pUC19 and segments of the cloned fragement. The right side of the figure shows the complementation of *E. coli* YK537 *phoA* mutation.

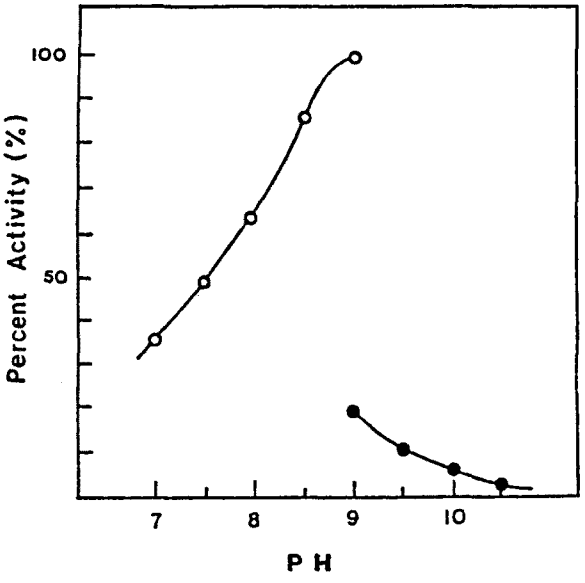

Fig. 3. Optimal pH of the *K. fragilis* ALPase.
The sonic extract was incubated at various pH values in the indicated buffers utilizing p-nitrophenyl phosphate as a substrate. The activity was expressed as percent of maximal activity. Buffer systems: ○−○, 0.1 M Tris-HCl buffer (pH 7.0~9.0); ●−●, 0.1 M glycine-KOH buffer (pH 9.0~10.5).

Southern Blot Analysis

K. fragilis chromosomal DNA and plasmid pSKH201 were digested with *Xba*I and *Dra*I, fractionated by 0.8% agarose gel electrophoresis, and then transfered to nitrocellulose membrane according to the method of Southern (22). The 1.9 kb probe fragments were prepared from pSKH201 by digestion with *Xba*I and *Dra*I, eluted with a Geneclean kit, and radiolabelled with α-[^{35}S]-dCTP by the random prime labelling method. As shown

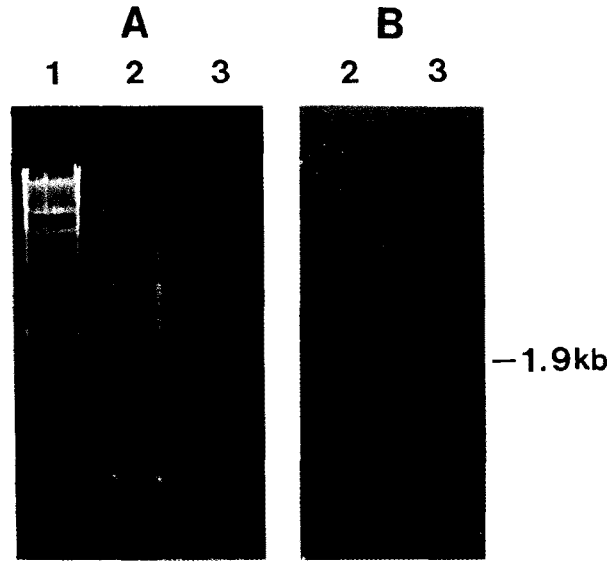

Fig. 4. Southern blot analysis of *K. fragilis* DNA.
Total genomic DNA from *K. fragilis* was digested with *Xba*I and *Dra*I, and hybridized to a 1.9 kb probe fragment, which was prepared from pSKH201 by digestion with the same enzymes. Photograph of DNA separation by electrophoresis (A) and by auto-radiography (B).
Lane 1: λDNA digested with *Hin*dIII, Lane 2: genomic DNA digested with *Dra*I and *Xba*I, Lane 3: pSKH101 digested with *Dra*I and *Xba*I

in Fig. 4, a distinct hybridization band, corresponded to a 1.9 kb size fragment is identified. This result confirmed that the cloned DNA fragment originated from *K. fragilis* genomic DNA. We thought that thinner bands below the main band (1.9 kb) in B were nonspecific background.

Minicell Preparation and Labelling of Proteins

A minicell experiment was carried out, as described by Jensen *et al.* (8), with slight modifications. Minicell strain *E. coli* BD1854 derivatives, containing the plasmid pUC19 and pSKH201, were cultured in (A+B) medium supplemented with 50 μg/ml ampicillin. The overnight cultures of minicells were recovered by centrifugation, then the pellets were mildly resuspended in BSG buffer (0.16% $Na_2HPO_4 \cdot 12H_2O$, 0.03% KH_2PO_4, 0.85% NaCl, 0.01% gelatin). The homogenates were layered over 30 ml of 20% sucrose gradient, then centrifuged. Minicell bands were collected and washed with 2 volumes of BSG buffer. The sucrose gradient layering and washing procedures were repeated and the resultant pellets were resuspended in appropriate volumes of (A+B) medium without amino acids, to a final optical denisity of 2.0 at 436 nm. Preincubation was performed at 37℃ for 1 hour to deplete endogenous mRNAs. To label the

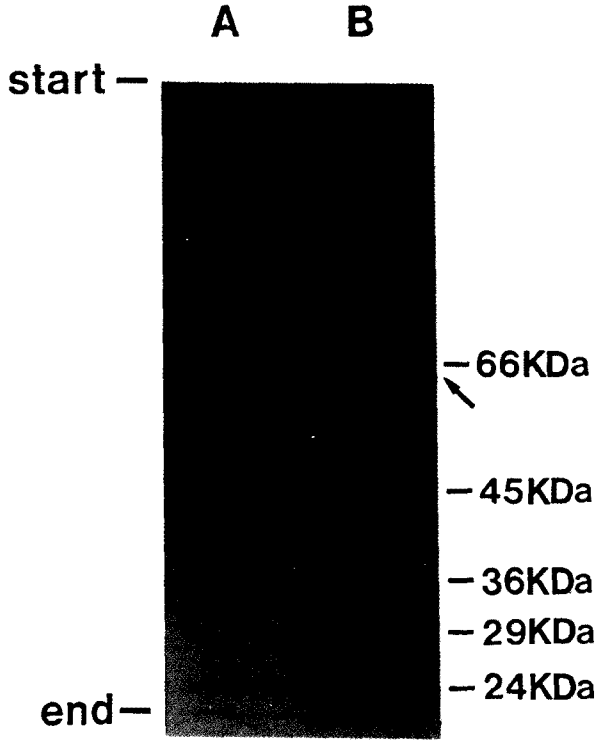

Fig. 5. Autoradiogram of [^{35}S]-methionine labeled proteins synthesized in plasmid-containing minicells.
Derivatives of *E. coli* carrying the plasmids pUC19 and pSKH201, were labeled with [^{35}S]-methionine. The crude extracts were prepared and separated in a 7.5% SDS-polyacrylamide gel electrophoresis.
Lane A: pUC19/BD1854, Lane B: pSKH201/BD1854
The arrow indicates the position of the polypeptide encoded by the cloned gene. The standard molecular weight proteins are trypsinogen (24 KDa), carbonic anhydrase (29 KDa), glyceraldehyde-3-phosphate dehydrogenase (36 KDa), egg albumin (45 KDa), and bovine albumin (66 KDa).

proteins encoded by the plasmids, each 300 μl-aliquot of minicell suspension was supplemented with 10 μl of 20% sucrose, 10 μl of thiamine-HCl (0.1 mg/ml), 10 μl of biotin(0.1 mg/ml), 20 μl of amino acid mixture (0.4 mg/ml, methionine-free), and [^{35}S]-methionine (10~20 μCi), then incubated 37℃ for 1 hour. After washing with (A+B) medium, the mixtures were subjected to 10% SDS-polyacrylamide gel electrophoresis. Several molecular weight markers were used to evaluate the sizes of proteins encoded by the plasmids. The gel was dried under vacuum and autoradiographed at −70 ℃ for 3 days(Fig. 5). This experiment showed that the subunit molecular weight of the proteins encoded by the cloned DNA fragment was approximately 63 KDa. The protein molecular weight was similiar to that of the

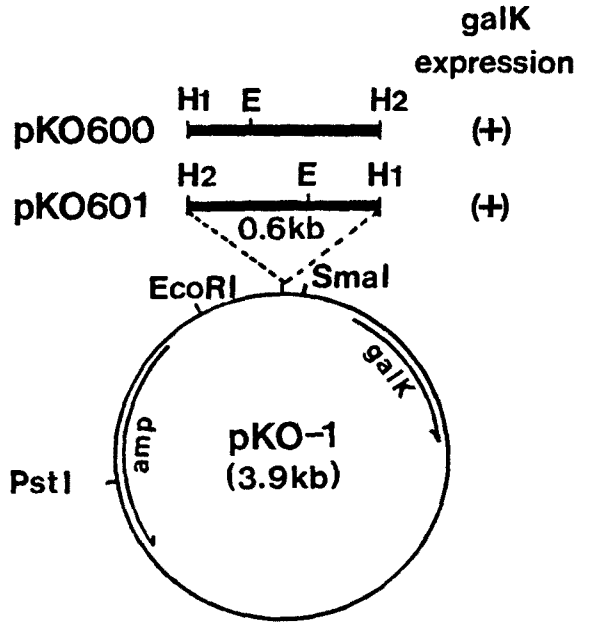

Fig. 6. Detection of the promoter region in the cloned fragments.
*Bam*HI digested segments (0.6 kb) of cloned fragments were introduced into promoter-probe vector pKO-1 and *galK*. Expression was examined on McConkey agar plates.

protein deduced from ORF2 of *PHO8* in *S. cerevisiae* (63,051 Da) (9).

In Vivo* Promoter Probing in *E. coli

In order to search the promoter region in the BamH insert of pSKH101, the plasmid pSKH101 was digested with *Hin*dIII. After electrophorsis in 0.8% agarose gel, the fragments from the insert were eluted and purified with a Geneclean kit. The *E. coli* promoter-probe vector pKO-1 was digested with the same restriction enzyme and ligated with the prepared fragments. The ligation mixtures were transformed in *E. coli* YK537(*galK2*). McConkey agar plates containing 50 μg/ml of ampicillin were used to select promoter-active clones. Among red colonies, 24 colonies were selected for small scale preparation of plasmids. The size of the fragments were analyzed by digestion with *Hin*dIII. All of the plasmids isolated contained 0.6 kb *Hin*dIII fragments. To identify the orientation of the promoter, restriction analysis with *Hin*dIII, *Eco*RI, and *Hin*dIII/*Eco*RI was performed. Physical maps indicated that both *Hin*dIII$_1$→*Hin*dIII$_2$(pKO 600), and *Hin*dIII$_2$→*Hin*dIII$_1$(pKO601) orientations exhibited promoter activities (Fig. 6). We thought that the ALPase gene of *K. fragilis* may be expressed by the *Hin*dIII$_1$→*Hin*dIII$_2$ orientation promoter from the result of the deletion analysis in Fig. 2. But further studies

on whether the expression of *K. fragilis* ALPase gene is done by its own promoter, is required by using the *K. fragilis* vectors.

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