Chapter V
Isolation of *Kluyveromyces lactis* killer strain
defective in growth on lactic acid

Killer *Kluyveromyces lactis* strain that cannot grow on lactic acid was constructed to use the silage additive to prevent silage from undergoing aerobic spoilage. I selected disruption of gene encoding phosphoenolpyruvate carboxykinase (PEPCK; ATP-dependent, EC4.1.1.49), the key enzyme of gluconeogenesis, by genetically modification. In this chapter, I present the cloning and sequencing of the gene coding PEPCK (*KlPCK1*) from *K. lactis*. The deduced aa sequence has been compared to those of other known PEPCKs. Furthermore, *K. lactis* PEPCK gene was disrupted by homologous recombination of original gene and disrupted counterparts. The *KlPCK1* defective strain could not grow on lactic acid medium as a sole carbon source. Other useful characters of this strain were able to preserve.

1) Isolation and nucleotide sequence of the gene
encoding phosphoenolpyruvate carboxykinase from *K. lactis*

The *KlPCK1* gene was cloned from *K. lactis* genom using a PCR amplicon from *S. cerevisiae PCK1* gene as a probe. A DNA fragment of about 4.8-kb containing *KlPCK1* complemented PEPCK activity of the mutant of *Saccharomyces cerevisiae* defective in PEPCK. The *KlPCK1* gene has an open reading frame of 1629 bp (543 aa). The *KlPCK1* nt sequence and deduced aa sequence showed 76% and 84% homologies to those of *S. cerevisiae PCK1*, respectively. Multiple alignment of ATP-dependent PEPCK genes shows that highly conserved regions. The nt sequences of *KlPCK1* has been submitted to the DDBJ/ Gen Bank/ EMBL Data Bank with accession number U88575.5.1.1

1.1)Cloning of *K. lactis* PEPCK

A 1.6-Kb of the PEPCK gene of *S. cerevisiae* (*PCK1*) was amplified by PCR with genomic DNA of *S. cerevisiae* YNN27 as a template and with primers 5'>ATAGGGCCCATGTCCCCTTCTAAATGAATGCTA<3' and 5'>CGCAAGCTTCTAACATGTTTCGTTTACTCGAATT<3' designed according to the nucleotide sequence of *PCK1*[45]. The *PCK1* gene was subcloned in the *Apal-Hind*III site of Bluescript II (BSII KS+) (Stratagene). *PCK1* was used for a probe to isolate the *K. lactis* PEPCK gene. Genomic DNA of *K. lactis* IFO1267 was digested with *Bam*HI, *Eco*RI and *Hind*III. This fragment hybridized as a single band to *K. lactis* genomic DNA

digested with *Eco*RI or *Hind*III in a Southern blot (Fig 5-1). The *PCK1* probe gave rather weak hybridization signals on *K. lactis* genomic digests in comparison with that of *S. cerevisiae*. The hybridized *Eco*RI-digested DNA fragments were eluted from the gel and subcloned in BSII. A clone containing a 4.8-kb insert (clone A3) was selected by colony hybridization from the subclone.

1.2) Complementation of *S. cerevisiae pck1*

To investigate the complementation of *S. cerevisiae PCK1*, plasmid YCp50 harboring the A3 fragment was transformed into a PEPCK-deficient mutant of *S. cerevisiae* CJM150 by using *URA3* for the selectable marker. A mutant of *S. cerevisiae* lacking *PCK1* is unable to grow on medium containing lactic acid as a sole carbon source for gluconeogenic sources[42]. On the other hand; the transformants could grow on SD medium containing 0.5% calcium lactate (Fig 5-2). PEPCK activity of the cell- free extracts harvested in ethanol media was measured. As shown in Table V-1a, the PEPCK activity was recovered in the transformant. These results showed that the A3 fragment is functionally equivalent to *PCK1* of *S. cerevisiae*, contains the PEPCK gene of *K. lactis* with a promoter, and that the *K. lactis* promoter can function in *S. cerevisiae*. I named the gene *KlPCK1*.

1.3) Mapping of the *KlPCK1* gene

The chromosomes of *K. lactis* IFO1267 were separated using CHEF. The A3 fragment hybridized only with a band corresponding to chromosome I in a Southern blot. The *KlPCK1* gene has been localized on the chromosome I (Fig. 5-8).

1.4) Sequence analysis of *KlPCK1*

The restriction pattern of the *K. lactis* A3 fragment was different from that of *S. cerevisiae* (Fig. 5-3). A Southern blot analysis showed that an approximately 2.6-kb region in the middle of the A3 fragment had high homology to *PCK1*. The nt sequence of the part of A3 fragment which showed high homology on hybridization were analyzed. The ORF sequence was 1629 bp, the deduced amino acid sequence was 543 aa (Fig. 5-4), and the molecular weight of the product calculated from the predicted aa sequence was 60325 Da.
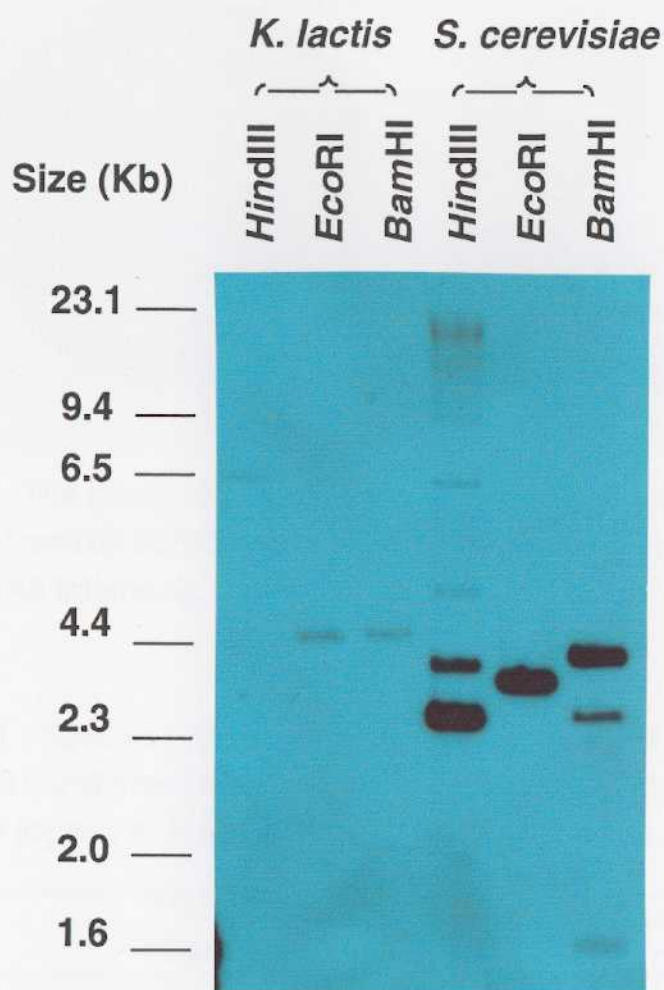
Fig. 5-1. Genomic Southern hybridization of the PEPCK gene from the strains
K. lactis IFO 1267 and S. cerevisiae YNN27.

The genomic DNAs were digested with HindIII, EcoRI or BamHI. Hybridiza-
tion was carried out using the PEPCK gene (PCK1) of S.cerevisiae as a probe.
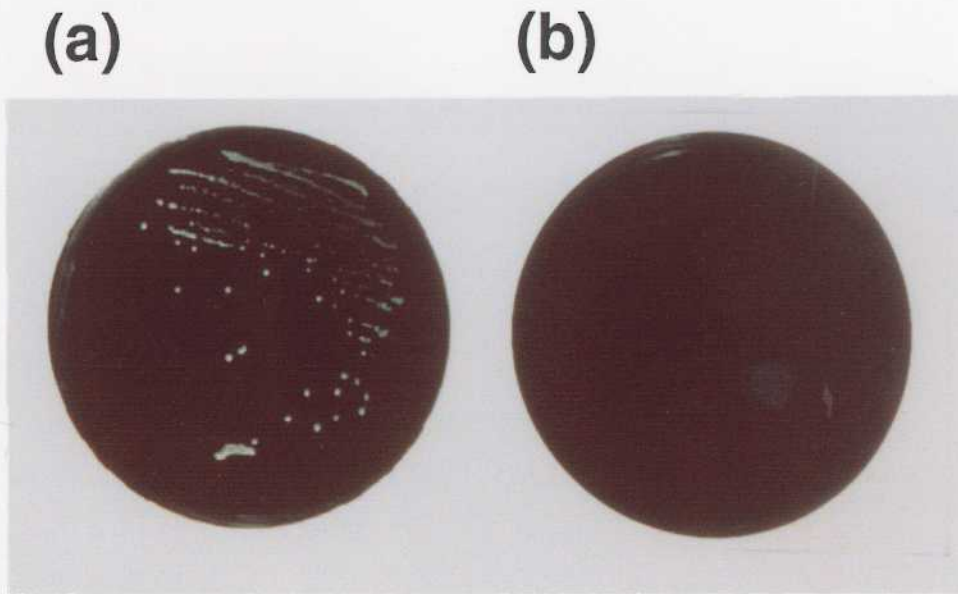
(a)   **(b)**

Fig. 5-2. The growth on lactic acid medium of the strain *S. cerevisiae* CJM150
(*pck1*) (b) and its transformant (a) harboring YCp50 containing a clone around
KlPCK1 (A3 fragment).

Table V-1. Specific PEPCK activities of *S. cerevisiae* CJM150 (PEPCK-defi-
cient) and transformed CJM150 cell free extract (a) , crude protein from *K. lactis*
IFO 1267 (b) and *K. lactis* IFO 1267 cell free extract (c).

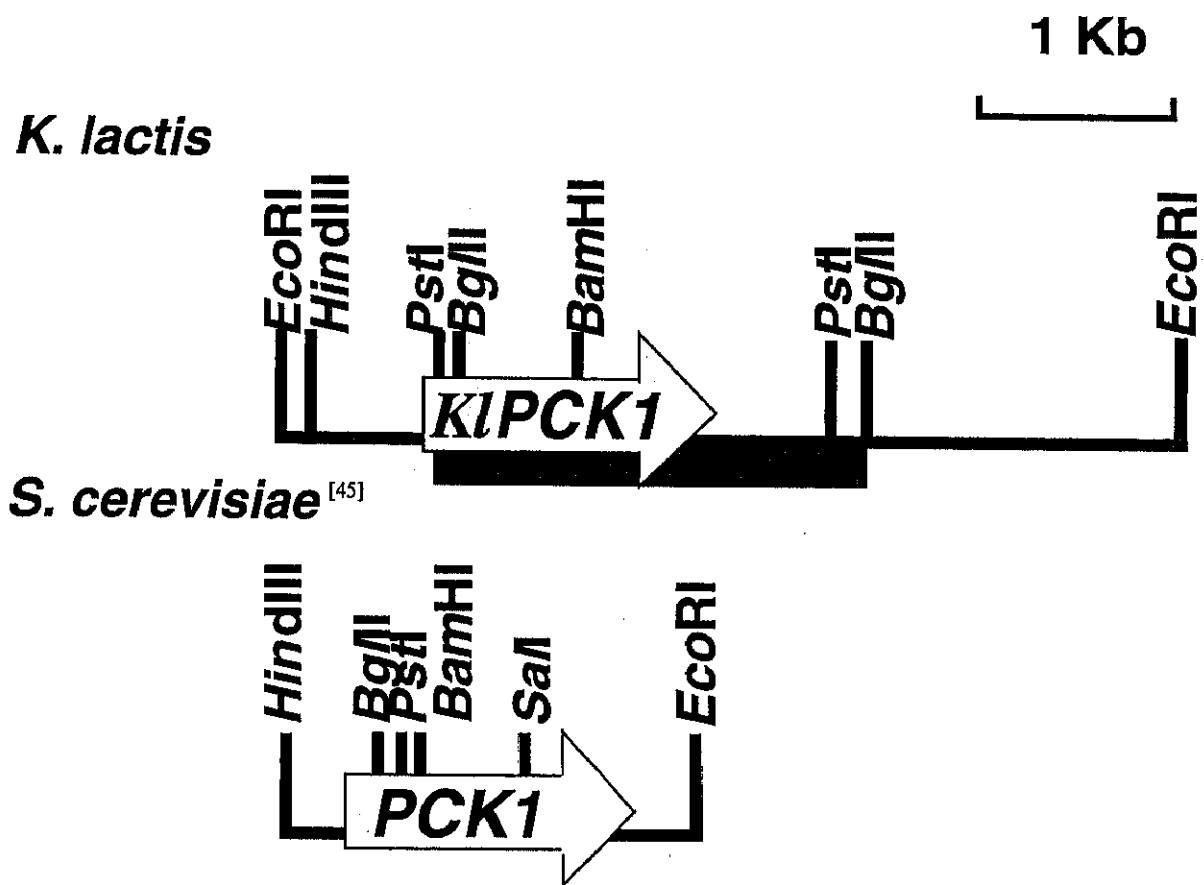|	Strain	Specific activity (nmol/min per mg protein)
a	*S. cerevisiae* CJM150 (PEPCK-deficient)	0.72
	S. cerevisiae CJM150 transformant	6.90
b	*K. lactis* with ADP	51.3
	K. lactis without ADP	5.4
c	*K. lactis* on glycerol	22.0
	K. lactis on glucose	1.8

Fig. 5-3. Restriction map of genomic clone around *KlPCK1* (A3 fragment).

The black box indicates the region that strongly hybridized with *S. cerevisiae* *PCK1*, and the arrow indicates the ORF.

-390   AATGAGTCAGATAACTCGCTGGAACATCAA
-360 TTATCCATCTAGTAATTAAGTGCAATTATTTATTCTCGTCCTGTCATCTCGTGAATTGATTAATATTCATCTTTTATGGTCATATATAAGGGCGATGGTTTTTCATGATTTAGTTTGATG
-240 GATACATTTCAAGTGTTGATAAGGTACAATAGTAGCTATATATTATCTCAGTTGTATTGCAATAAAAGTAAACCCAAAATTCAGTGATTCTACACATTGAAAGCACTTTAATAGTAAAGC
-120 CAAAATTCATACTATACGATAAATTGACTCTTCTCCATTTAAGAATACTCAATTAACCGTGTTATCGACTTTTCCTCTAGCGCGACTAAAAAGAAAGAAAATAAAACCTATTATCAAATA
   1 ATGTCACCAACTAAGACACAAAATTCTGCTGAAGAGAATATTAGGGCTGAGCTTGGCCTTTCTGCAGAAGTCGTCACCATCAGACGTAATGCTCCAGCTGCTTTGTTATATGAAGATGCT
   1 M S P T K T Q N S A E E N I R A E L G L S A E V V T I R R N A P A A L L Y E D A
 121 TTAAAGAAAGAGACACAGCCATTTCCAATGCTGGTGCCTTGATTGCCTACTCTGGTGATAAGACCGGAAGATCTCCACGTGATAAGCGTATCGTTGAAGAAGAAACTTCAAAGGATAAC
  41 L K E R D T A I S N A G A L I A Y S G D K T G R S P R D K R I V E E E T S K D N
 241 GTTTGGTGGGGTCCAGTAAATAAGCCATGTTCCGAAAGAACTTGGGAAATTAACAGAGAAAGAGCTGCTGATTACTTGAGAACTAGGGATCACATTTACATTGTTGATGCTTATGCTGGC
  81 V W W G P V N K P C S E R T W E I N R E R A A D Y L R T R D H I Y I V D A Y A G
 361 TGGGATCCTCGTTACAGAATCAAGGTCAGAGTGGTTTGTGCTAGAGCTTACCATGCCTTATTCATGACGAACATGTTGATCAGACCATCAAAGGAAGAACTAGAAAATTTCGGTGAACCT
 121 W D P R Y R I K V R V V C A R A Y H A L F M T N M L I R P S K E E L E N F G E P
 481 GACTTCACCGTCTGGAATGCTGGTCAATTCCCTGCTAACACACATACCTCCGGTATGACTTCTAAGACTACTGTGGAAATTAACTTCAAGCAAATGGAAATGGTCATCTTGGGTACCGAA
 161 D F T V W N A G Q F P A N T H T S G M T S K T T V E I N F K Q M E M V I L G T E
 601 TACGCTGGTGAAATGAAGAAGGGTATTTTCACTGTGATGTTCTACTTGATGCCTGTTAATCACAATGTCCTCACCTTACATTCTAGTGCCAACCAAGGTATTCAAGGTAATGACGTTACT
 201 Y A G E M K K G I F T V M F Y L M P V N H N V L T L H S S A N Q G I Q G N D V T
 721 TTGTTCTTCGGTTTGAGTGGTACTGGTAAGACTACCTTGTCCGGTGATCCTCATAGACTGTTGATCGGTGACGATGAACACTGTTGGTCCGATCATGGGGTTTTCAACATTGAAGGTGGC
 241 L F F G L S G T G K T T L S G D P H R L L I G D D E H C W S D H G V F N I E G G
 841 TGTTACGCTAAGTGTTTGGGATTGTCTGCCGAGAAGGAACCAGAAATTTTCAACGCTATCAAGTTCGGTTCTGTTTTGGAAAACATCATTTACGATCCTAACACTAGAGAAGTTGATTAC
 281 C Y A K C L G L S A E K E P E I F N A I K F G S V L E N I I Y D P N T R E V D Y
 961 GAAGATTCTACAATCACAGAAAACACTAGATGCGCTTACCCAATCGAATACATCCCAAGTGCTAAAATCCCATGTCTAGCTGATCACCATCCAAAGAACATTGTCCTGTTGACTTGTGAC
 321 E D S T I T E N T R C A Y P I E Y I P S A K I P C L A D H H P K N I V L L T C D
1081 GCTTCAGGTGTTTTGCCTCCAGTATCCAAGTTGACCCCTGACCAAGTTATGTACCACTTCATCTCTGGTTACACTTCCAAGATGGCTGGTACTGAACAAGGTGTCACTGAACCAGAAGCA
 361 A S G V L P P V S K L T P D Q V M Y H F I S G Y T S K M A G T E Q G V T E P E A
1201 ACTTTCTCCTCTTGTTTCGGTCAACCATTCTTGTCTCTACATCCAATGAAATATGCTACAATGTTGGCTGAAAAGATGGCCGAACATAACGCTAACGCTTGGTTAATCAACACTGGTTGG
 401 T F S S C F G Q P F L S L H P M K Y A T M L A E K M A E H N A N A W L I N T G W
1321 ACTGGTTCCTCTTACGTTGCTGGTGGTAAGCGTTGTCCTTTGAAGTACACCAGAGCTATCCTAGATGCCATCCACGATGGCTCTTTGGCTAAGGAAGAGTATGAAGTTTTGCCAATCTTC
 441 T G S S Y V A G G K R C P L K Y T R A I L D A I H D G S L A K E E Y E V L P I F
1441 AACTTGCAAATTCCAAAGGCTGTGGGTGACAAGGTTCCAGCTTCTCTACTAAATCCAATTAAGAACTGGGCTGAAGGTGAAGCTAATTACACTTCAAATGTCAAGTCATAGGCTAATTGG
 481 N L Q I P K A V G D K V P A S L L N P I K N W A E G E A N Y T S N V K S T A N W
1561 TTTGTAAAAAACTTCAAAACCTACCAAGATAAGGCTACCGAACAAGTCTTAGCTGCTGGTCCGCAATTATAATCACAATGTCCTCAACAGAAGTGCTATACCAAAATAACGAAAAGAGGC
 601 F V K N F K T Y Q D K A T E Q V L A A G P Q L *
1681 AGAGTGAAATATTATTGAGTAATATTTTCTCTTCAACATAGTTTCACTTTTTCCGTTACCTTCAACCAGACTATCTCAACCGTAGCCGGTTCATTATTATTAATATAAAATTCTGAGATT
1801 CATAAAAAACCAACTAACAATAATACACATACCATTGGCTCGGATTATTTGATTCTTGCATTTAGAAACTATCTTTTTCTCGACTACATATATAACAAGGAAATTCTACAGATATCTTGG
1921 GGTTTTTCTCATTCGTTTTTCTATTACTTTCGTCCTTTTTAATTTATCACTAACAGCCTTTAATCAATATGGTTATAGTACATACATACGCTTTAGTATCTTTTAATCTGGATATACTAT
2041 TTATTTATGGGAGGAATTAAATCATTAATATCATCCTTAAGCGAAGAATAAAAGCAGGTTAAATAGATCTTTTAATCTCATCCCATATATGCTGTGTTAACGTCTGTGTATAAACCTAGA
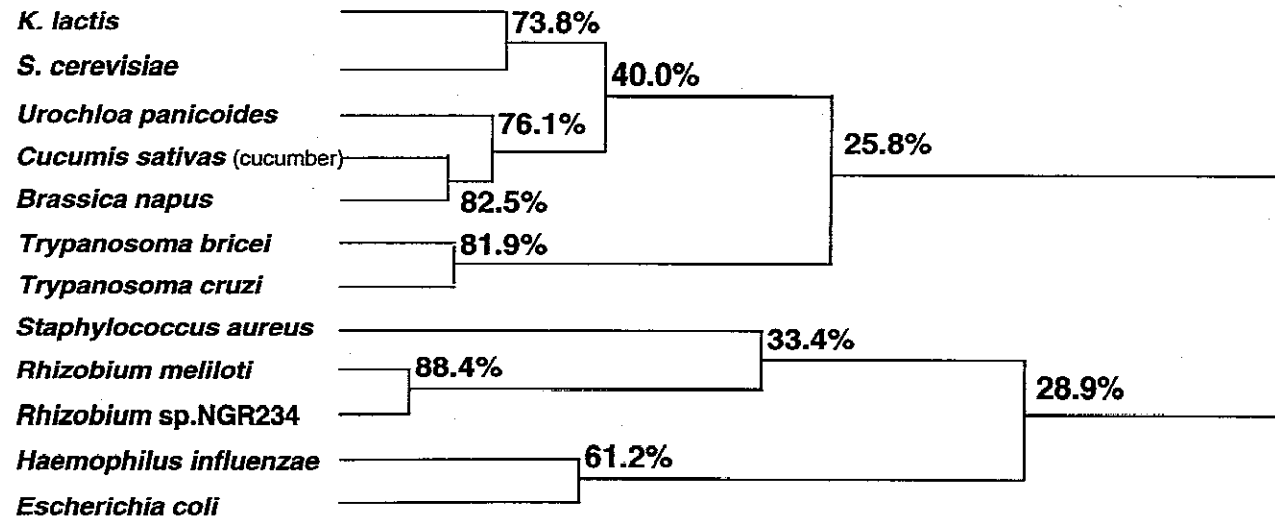2161 TGTGCTTGTACTCAAAGGTGGAGGCATTTTCCGTAGTAGCATTGTTTGCAGTCAAAAAATCAGCATCGTCGTTATTTT

Fig. 5-4. The nt sequence of *KlPCK1* and deduced amino acid sequence.

The residue of the first ATG codon in the ORF is numbered 1. The consensus sequences conserved in the ATP-dependent PEPCK[85] are underlined and labeled a-d. (a) unknown structural sequence; present in all, (b) phosphate-binding region in ATP-using proteins, (c) protein binding site for divalent or transition metal ion and (d) binding site for adenine in ATP-using proteins. The nt sequences of *KlPCK1* has been submitted to the DDBJ/ Gen Bank/ EMBL Data Bank with accession numbers U88575.

1.5) Comparison of deduced PEPCK amino acid sequences of
K. lactis, S. cerevisiae and other organisms

Sequences were analyzed using the MP search program (Intelli Genetics). The DNA sequence and deduced aa sequence showed 76% and 84% homology to those of *S. cerevisiae*, respectively. The PEPCKs found in plants and microorganisms are usually ATP-dependent, whereas those found in animals are almost exclusively GTP-dependent[84]. The deduced aa sequence of ATP-dependent PEPCKs of reported in Gen Bank was very similar to that of *KlPCK1*(Fig. 5-5). The ATP- dependent PEPCKs, phosphate and adenine-binding regions that are characteristic of ATP-using proteins are clearly identifiable[85]. The four consensus sequences in *KlPck1* (underlined segments in Fig. 5-4) were also highly conserved (Fig. 5-5). These data show that *KlPck1* is very similar to *Pck1*. Furthermore, *KlPck1* showed no significant homologies with GTP-dependent PEPCKs as described by Stucka et al.[44]. The PEPCK activity of the crude enzyme from *K. lactis* IFO 1267, harvested in glycerol, was very low in the absence of ADP (Table V-1b), indicating that it is the ATP-dependent type. This data coincide with the amino acid sequence homology data.

Occasionally the isolation of *K. lactis* homologues with transcriptional regulators by complementation of *S. cerevisiae* mutants could be cloned together[51]. I showed the functional complementation of *pck1* of *S. cerevisiae* by *KlPCK1* including promoter region. Although the promoter region of *S. cerevisiae PCK1* gene[86] and the nt sequence 390 bp upstream of the first ATG of the *KlPCK1* coding sequence shared no homology. The PEPCK activity of *K. lactis* on different carbon sources were measured (Table V-1c). The inactivation rate by glucose was 92%. It indicates that the PEPCK of *K. lactis* IFO1267 is repressed by glucose. This result supports the conclusion that the regulation mechanism of *KlPCK1* expression is different from that of *PCK1*.

Dendrogram:

- K. lactis ┐ 73.8%
- S. cerevisiae ┘
- 40.0%
- Urochloa panicoides ┐ 76.1%
- Cucumis sativas (cucumber) ┤
- Brassica napus ┘ 82.5%
- 25.8%
- Trypanosoma bricei ┐ 81.9%
- Trypanosoma cruzi ┘
- Staphylococcus aureus
- 33.4%
- Rhizobium meliloti ┐ 88.4%
- Rhizobium sp.NGR234 ┘
- 28.9%
- Haemophilus influenzae ┐ 61.2%
- Escherichia coli ┘

	No structural correlate known; present in all			Phosphate-binding region in ATP-using proteins			Protein binding site for divalent or transition metal ion			Binding site for adenine in ATP-using proteins		
K. lactis	197	ILGTEYAGEMKK	208...243	FFGLSGTGKT	252...274	GVFNIEG	280...354	IVLLTCD	360
S. cerevisiae	203	ILGTEYAGEMKK	214...248	FFGLSGTGKT	257...279	GVFNIEG	285...360	IILLTCD	266
Cucumis	222	ILGTQYAGEMKK	333...266	FFGLSGTGKT	275...397	GVSNIEG	403...478	VILLACD	484
Urochloa	276	ILGTQYAGEMKK	287...320	FFGLSGTGKT	329...351	GVSNIEG	357...432	VILLACD	438
Brassica	63	ILGTQYAGEMKK	74...108	EFGLSGTGKT	117...140	GVSNIEG	146...219	VILLACD	225
Trypanosoma	175	ILGTEYAGEMKK	186...219	FFGLSGTGKT	228...250	GVFNIEG	256...330	IIFLTND	336
Trypanosoma	175	ILGTEYAGEMKK	186...218	FFGLSGTGKT	227...250	GVFNIEG	256...330	VIFLTND	336
Staphylococcus	190	IGGTEYAGEMKK	201...234	FFGLSGTGKT	243...265	GVFNIEG	271...344	IIFLTAD	350
Rhizobium	190	IGGTSYAGEMKK	201...234	FFGLSGTGKT	243...265	GVFNEEG	271...344	IIMLTAD	351
Rhizobium sp.NGR234	190	IGGTSYAGEMKK	201...234	FFGLSGTGKT	243...265	GVFNFEG	271...345	IIMLTAD	351
Haemophilus	188	IGGTSYAGEMKK	199...232	FFGLSGTGKT	241...263	GIFNFEG	269...343	IIMLTAD	349
Escherichia	201	DCGTWYGGEMKK	212...245	FFGLSGNGKT	254...275	GVFNLKA	281...355	SLLLTAH	361

Fig. 5-5. Dendrogram grouping of ATP dependent PEPCK (4.1.1.49) and consensus sequences present in PEPCK.

2) Construction of *K. lactis* killer strains
## defective in growth on lactic acid by gene disruption

Killer strain of *K.lactis* defective in growth on lactic acid was constructed to prevent the aerobic spoilage of silage by disruption of *KlPCK1* gene encoding PEPCK. The disruptants are defective in their ability to grow on lactic acid as a sole carbon source. The PEPCK activity was also deficient in the disruptants. The growth rate on lactose medium and the killing activity were equal to those of the parental strain.

2.1) Selection of marker gene

The strongly expressed antibiotic G418 resistant marker, *ADH1-APT2* gene, was examined as a selection marker. The *ADH1-APT2* gene was constructed by insertion of bacterial aminoglycoside phosphotransferase II (*APT2*) gene from *E. coli* transposon Tn903 between a yeast alcohol dehydrogenase I (*ADH1*) gene promoter and cytochrome c1 (*CYC1*) gene terminator[55]. The *ADH1-APT2* gene was isolated and connected into the secretion vector pEPGK41 (Fig. 5-6) to confirm the possibility of its expression in *K. lactis*. The strain MW-98-8C MD1/2 transformed with this plasmid was resistant against 1000 ppm G418, while the *K. lactis* strain used in this research is sensitive to 50 ppm G418. I selected the *ADH1-APT2* gene as a marker.

2.2) Gene disruption of *KlPCK1*

A *Bam* HI site and two *Bgl* II sites were used to disrupt *KlPCK1*. Two integration vectors were created (Fig. 5-6); one, by insertion of the marker gene, *ADH1-APT2* into *Bam* HI site of the *KlPCK1* (pPCKI) and the other, by interchange of *Bgl* II-*Bgl* II fragment for the same marker gene (pPCKD). Both plasmids were digested with *Sal* I and *Xba*I to obtain the fragment for integration.
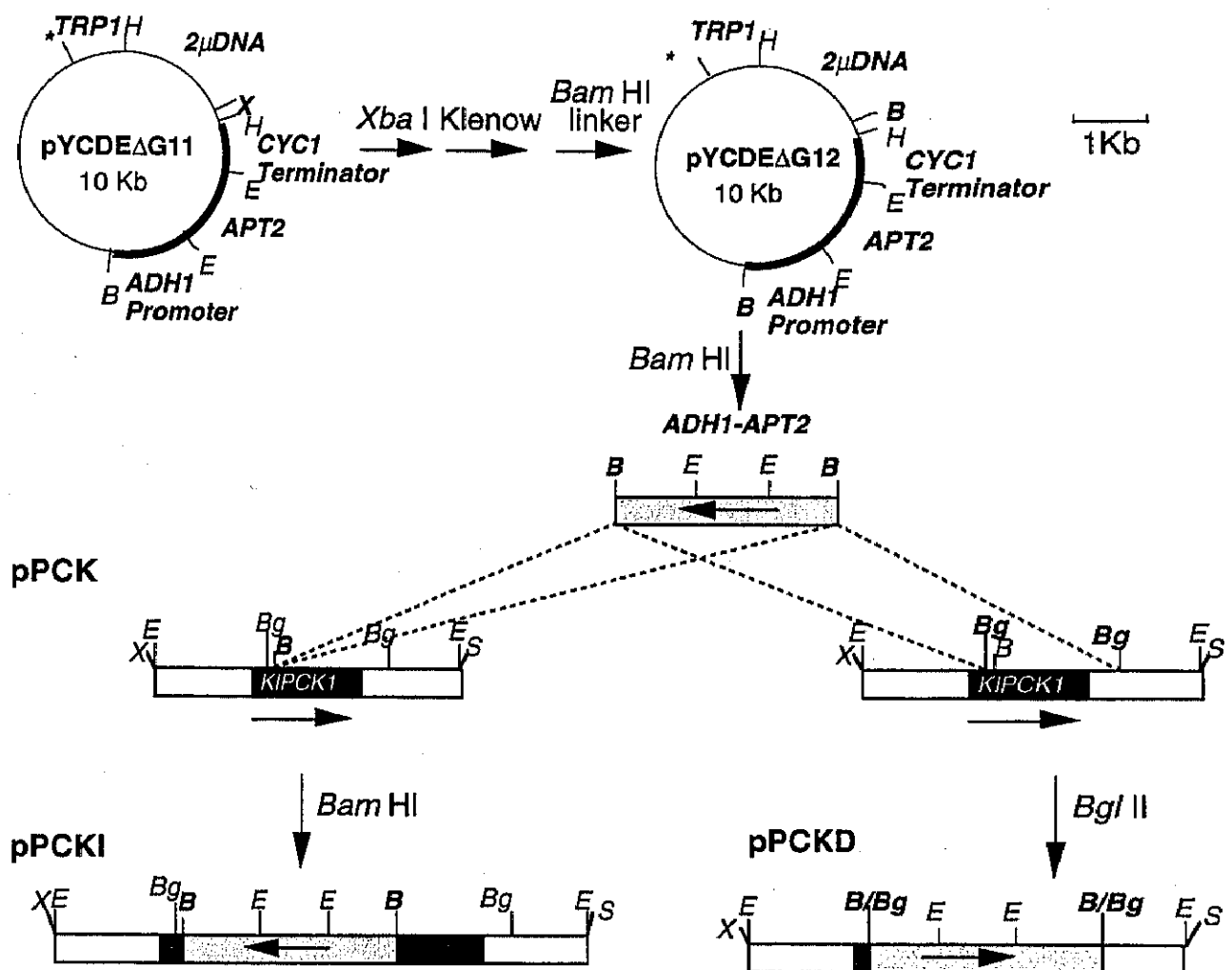
Plasmid pPCKI was constructed by insertion of the marker gene into *Bam* HI site of the *KlPCK1*. To obtain plasmids pPCKD, the fragment *Bgl* II-*Bgl* II was eliminated and subustituted by the marker gene. The plasmid pEPGK41::*APT2* was constructed to confirm the possibility of expression in *K.lactis* as a G418 resistant marker. The restriction sites for enzymes: B: *Bam*HI, Bg: *Bgl* II, E: *Eco* RI, H: *Hin* dIII, S: *Sal* I, X: *Xba* I.

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2.3) Isolation of the *KlPCK1* disruptant

Insertion and deletion disruptants with pPCKI and pPCKD were selected by 2 steps. Colonies grown on YPD plate containing 50 or 100 ppm G418 were transferred to SD plate containing 0.5% calcium-lactate as a sole carbon source (Lactic acid-SD). Some G418 resistant disruptants could not grow on Lactic acid-SD plate. The frequency of the strain lacking the ability to grow on Lactic acid-SD plate against G418 resistant was much lower in the wild strain IFO 1267 than in the mutant strains MW98-8C and MW98-8C1/2 (Table V-2).

Two putative *KlPCK1* disruptant clones, PCK27 and PCK50, selected from pPCKI and pPCKD disruptant, respectively, were used for further analysis to confirm the disruption of *KlPCK1*. No colony was observed from each strain on Lactic acid-SD plate after 20 generations in YPD liquid medium. Genomic Southern hybridization analysis was performed with the horseradish peroxidase labeled 4.8 kbp *Eco* RI-*Eco* RI fragment (A3) of pPCK as a probe (Fig. 5-7). Genomic DNA from both strain PCK27 and PCK50 showed two hybridization signals each, namely, 3.3 kbp and 3.8 kbp, and 1.9 kbp and 3.2 kbp, respectively. The size of each signal is the same as each plasmid used for transformation. On the other hand, the genomic DNA from the host strain showed only one signal (4.8 kbp). These data are in good agreement with the size of *KlPCK1* gene and the integration vectors.

2.4) Southern hybridization of chromosomes

KlPCK1 was localized on chromosome I, the smallest chromosome of six *K. lactis* chromosomes as described in chapter IV. Chromosomes of strains, PCK27, PCK50 and IFO1267 were isolated. The G418 resistant marker *APT2* was labeled and used as probe. The *APT2* hybridized to chromosome I of strain PCK27 and PCK50, but not to strain IFO1267 (Fig.5-8 B-b). The A3 fragment hybridized to chromosome I in all strains (Fig. 5-8 B-a).

2.5) Enzyme activity

The activities of enzymes assayed are shown in Table V-3. The disruptants lacked PEPCK. Lactic acid dehydrogenase, fructose-1,6-bisphosphatase and malate dehydrogenase were also determined because of their relation to gluconeogenesis. The wild strain and the disruptants have equal levels of enzyme activities.

Table V-2. Transformation efficiency of gene disruption of *K. lactis KlPCK1*.

Transformation Strain	Vector	Lactate growth (-) [a] / G418 resistant [b]	Efficiency (%) [c]
IFO1267	pPCKI	5/57	9
(wild type)	pPCKD	3/57	5
MW98-8C	pPCKI	10/15	67
	pPCKD	0/1	0
MD2/1	pPCKI	25/37	68
	pPCKD	16/29	55

a Not grown on lactic acid-SD plate.

b Grown on YPD plate containing G418.

c Rate of homologous recombination.
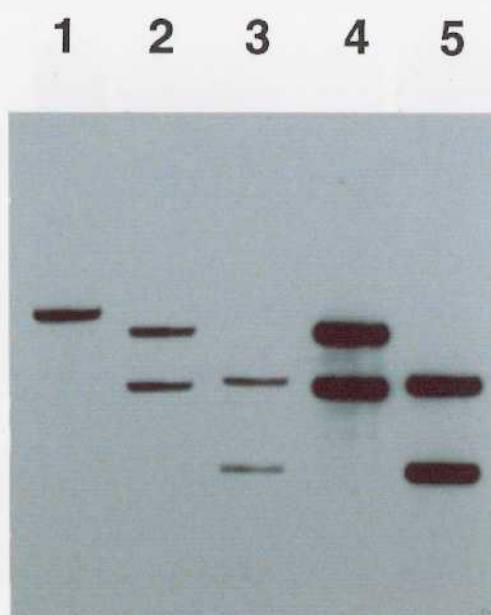
Fig. 5-7.   Genomic Southern-blot analysis of the wild strain
and disruptants of the chromosomal *KlPCK1* gene.

The genomic DNA and plasmids were digested with *Eco* RI. The probe used
was the 4.8 kbp *Eco* RI-*Eco* RI fragment from pPCK containing *KlPCK1* (see Fig.
5-6). Genomic DNA from IFO1267 (wild); (lane1), PCK27 carrying pPCKI; (lane2)
and PCK50 carrying pPCKD; (lane3). The plasmid DNA from pPCKI; (lane4) and
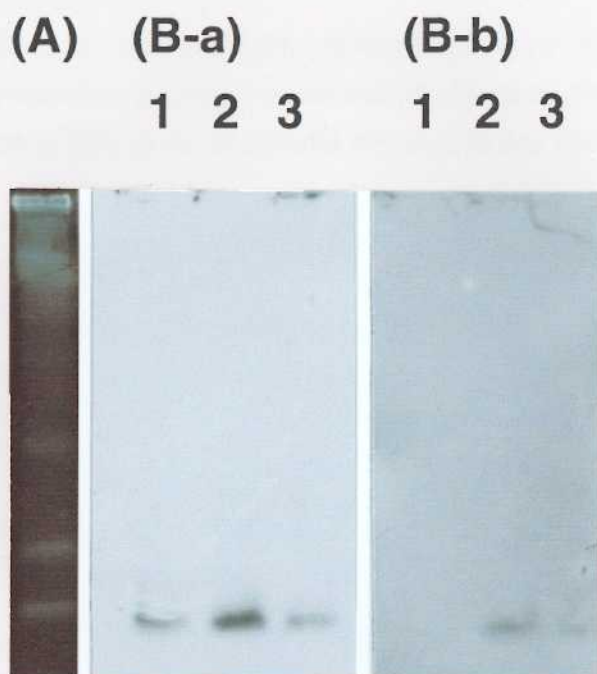pPCKD; (lane5).

(A) (B-a)   (B-b)

1 2 3   1 2 3

Fig. 5-8. Chromosomes of the *K. lactis* wild strain and the *KlPCK1* disruptants.

Chromosomes were separated by pulsed field gel electrophoresis. The gel was stained with ethidium bromide (A) ; *K. lactis* wild strain. Southern blot analysis (B). Chromosomes of the *K. lactis* wild strain (lane1), PCK27 carrying pPCKI; (lane2) and PCK50 carrying pPCKD; (lane3). The probe used was the 4.8 kbp *Eco* RI-*Eco* RI fragment from pPCK containing *KlPCK1* (A3 fragment(a), or *E. coli APT2* from pYCDEΔG11(b)).

Table V-3.　Specific activity of phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and fructose-1,6 - bisphosphatase (FBP) in *K. lactis* wild strain and the *KlPCK1* disruptants.

Strain (plasmids)	Carbon source	Specific activity (nmol/min per mg protein)			
		PEPCK	LDH	MDH	FBP
IFO1267 (wild type)	Glycerol	22.0	102.8	86.4	0.8
	Glucose	1.8	-	-	-
PCK27 (pPCKI)	Glycerol	2.4	61.5	80.5	0.4
PCK50 (pPCKD)	Glycerol	1.5	96.5	87.4	1.7

-: Not determined.

2.6) Growth curve of disruptants

Time course of growth of *K. lactis* wild strain and the disruptants was investigated on SD liquid medium containing lactic acid or lactose as a sole carbon source (Fig. 5-9). The disruptants failed to grow on Lactic acid-SD medium, while the wild strain showed good growth. The growth rates on Lactose-SD medium were all the same in these three strains.

2.7) Killing activity of disruptants

The killing activities of the host strain and the disruptants against the test strain, *S. cerevisiae* B511-4C, was assayed. The killing zone around the patch of disruptants are same as the host strain (Fig. 5-10). These data show that the disruptants have the same killing activity as a host strain.

2.8) Mating assay of *K. lactis* PCK27

To analysis mating efficiency of *K. lactis* PCK27 (a), I tried to cross with laboratory *K. lactis* strains of MW 98-8C (α) or strain PM6-7A (a). Diploid strain, isolated from cross of MW 98-8C and strain PM6-7A were grown on selection plate. However, I could not detect zygote formation under the microscope, nor isolate any zygote from cross of strain PCK27 and strain MW 98-8C, nor cross of strain PCK27 and strain PM6-7A on selection medium.
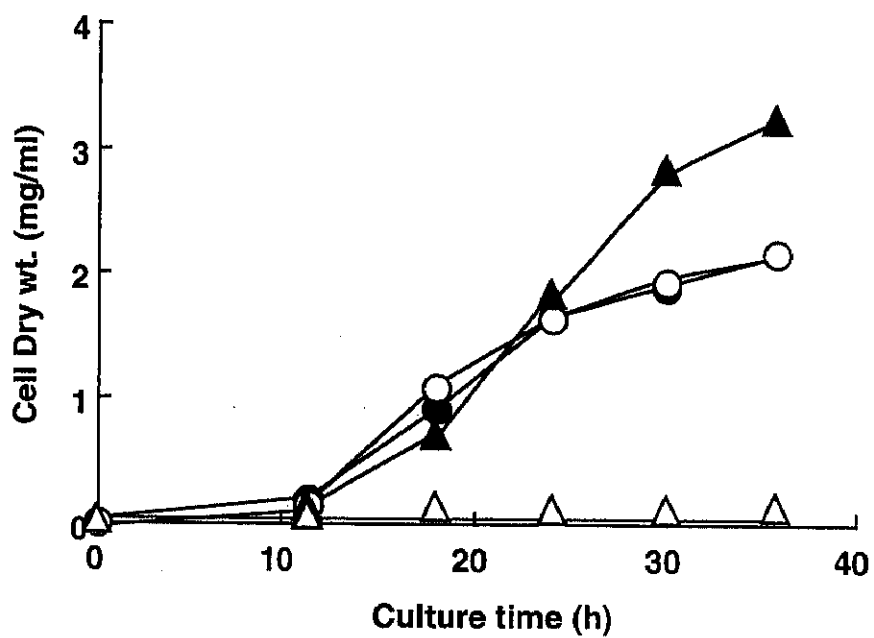
Fig. 5-9. Time course of growth of *K. lactis* wild strain and *KlPCK1* disruptants on lactic acid or lactose as a sole carbon source.

Cultures were grown in SD liquid medium at 30°C for 36h. △, PCK27 on lactic acid medium; O, PCK27 on lactose medium; ▲, wild yeast on lactic acid medium; ●, and wild yeast strain on lactose medium.
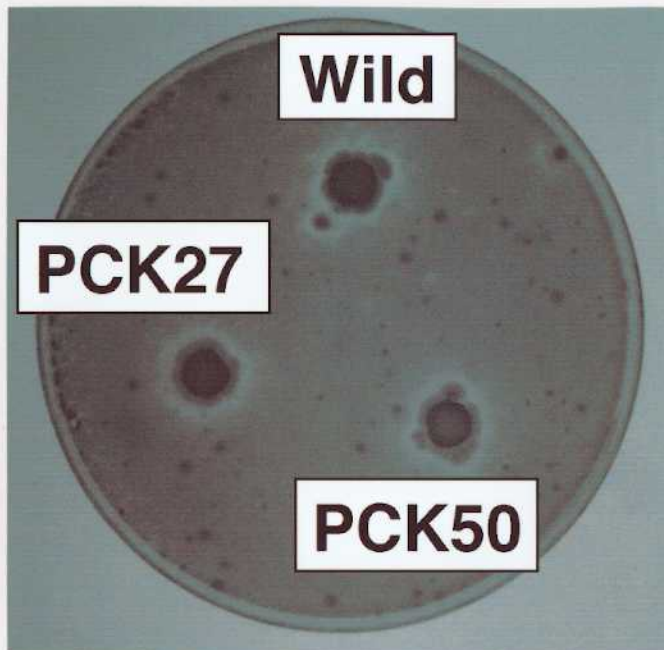
Fig.5-10. Agar plate assay for killer activity.

3) Discussion

To use *K. lactis* killer strain as a silage additive, I needed to isolate the killer strain that can not assimilate lactic acid. *K. lactis* IFO 1267 is a haploid strain which is suitable for breeding. Many of yeast can aerobically assimilate lactic acid after conversion to pyruvic acid by lactic acid dehydrogenase (LDH). In *K. lactis*, the D(L)-LDH are necessary for the stereospecific oxidation of D(L)-lactic acid to pyruvate[87]. I previously failed to isolate the mutant strain defective in assimilating lactic acid by UV irradiation with nystatin treatment. It was because of presence of isozyme and absent of positive selection procedure for such mutants. Furthermore, Niwa et al. reported 10 to 90% of *K. lactis* survived cell lost killer activity by UV irradiation, ethidium bromide treatment or by heat shock[83, 88].

I intend to use the genetically modification for disruption of the gene which is essential to grow on lactic acid because I want to avoid the possibility to lose the useful characters by random mutagenesis for the industrial usage, e. g. killer protein production and the good growth rate. Growth of microorganisms on lactic acid implies reversal of the glycolytic chain. Two enzymes are capitals in this reversal: fructose bisphosphatase and PEPCK. Perea, J. and Gancedo, C.[42] reported that the mutant of *S. cerevisiae* defective in PEPCK did not grow on non-carbohydrate carbon sources. Thus I intend to obtain the PEPCK defective killer strain.

The gene encoding *K. lactis* PEPCK was cloned and sequenced. *KlPCK1* and its promoter region complements the *S. cerevisiae pck1* mutation. The nt sequence and deduced amino acid sequence of *KlPCK1* showed 76% and 84% homology to *S. cerevisiae PCK1* respectively. PEPCK of *K. lactis* IFO1267 is repressed by glucose. However, the regulatory region of about 390 bp upstream of *KlPCK* coding sequence shared no homology. These data indicates the possibility of difference in regulation between *S. cerevisiae* and *K. lactis*.

The *KlPCK1* gene has been localized on the chromosome I, whereas the *PCK1* of *S. cerevisiae* was mapped on chromosome XI[89]. *K. lactis* has only six chromosomes. The sum of the molecular weights suggests that the genome size of *K. lactis* (12 M bp) is about the same as that of *S. cerevisiae*. *K. lactis* is one of the yeast which is closely related to *S. cerevisiae* in ribosomal DNA sequence revel. Wésolowski -Louvel and Fukuhara[49] showed the latest genetic and physical map of *K. lactis*. They revealed many rearrangements of chromosomal organization in functionally equivalent genes between *S. cerevisiae* and *K. lactis*, whereas local order of genes can often be similar in both strains. None of the *K. lactis* gene listed in the map has homology between any gene of chromosome XI of *S. cerevisiae*. I need further information of the localization of *K. lactis* gene on a map.

To disrupt the *KlPCK1* gene *in vitro*, I selected *ADH1-APT2* gene as a G418 resistant marker. This marker was strongly expressed even in the transformed *K. lactis* strain which has no selectable marker.

K. lactis IFO 1267 is a haploid strain. Homologous recombination was done on *K. lactis* IFO 1267 to exchange the original *KlPCK1* and disrupted counterparts. Since I was able to isolate the *KlPCK1* disruptants which cannot grow on Lactic acid-SD medium from among the G418 resistant strains transformed by both insertion and deletion vectors, I concluded that the PEPCK of *K. lactis* IFO 1267 is dependent on only one gene. This result well coincides with the data on *KlPCK1* gene mapping of chromosomes by hybridization (Fig.5-8). The activity of PEPCK in *S. cerevisiae* is induced when grown on non-carbohydrate carbon source and glycerol as a sole carbon source[42]. The activity of PEPCK in *K. lactis* wild strain was also induced by glycerol, and that the PEPCK activity of the disruptant which cannot grow on lactic acid was deficient as in *S. cerevisiae*. The gene disruption was stable in mutants transformed by both insertion and deletion vector (pPCKI and pPCKD, respectively). The growth rates of disruptants and the wild strain on lactose, as well as glycolytic carbon sources, are same. The disruptants also posses the killer activity as the parent. I believed that I was successful in breeding the killer *K. lactis* strain which is defective in the ability to grow on lactic acid and at the same time, the other useful characters were able to preserve of this strain.

The results presented in this work clearly show that *K. lactis* PEPCK gene was disrupted by homologous recombination. The *KlPCK1* defective strain could not grow on lactic acid medium as a sole carbon source due to lack of activity of gluconeogenesis key enzyme, PEPCK. The growth rate on sugar and the killer activity are the same as the wild strain. Further research should be carried out to explore the effect on prevention of silage from undergoing aerobic spoilage with this disruptant.