

**CONSTRUCTION OF A GENE KNOCKOUT
CASSETTE FOR *Leucosporidium antarcticum***

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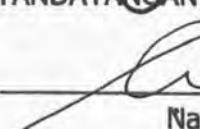
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ABSTRACT

CONSTRUCTION OF A GENE KNOCKOUT CASSETTE FOR *Leucosporidium antarcticum*

Leucosporidium antarcticum, a psychrophilic yeast was isolated from Antarctica. It grew optimally at 12°C, and adapted well to the cold temperature in Antarctic. The whole genome of the *L. antarcticum* has been sequenced using the 454 system. It is ideal to conduct systematic knockout of genes to determine its gene function especially genes that are responsible for the adaption to the cold. Hence, there is a need to construct a knock-out system for *L. antarcticum*. The gene knockout constructs were built by fusing an antibiotic (*Geneticin-G418*) resistance gene, *KanMX* used for gene knock out in *Saccharomyces cerevisiae* to a series of *L. antarcticum* gene's promoters. The gene promoters used belonged to the *ACT*, *SNF* and *Cyclophilic* genes of *L. antarcticum*. Gene fusion was conducted using the polymerase chain reaction (PCR). One set of primer was designed to amplify the genes' promoter region with an overhang that was homologous to the second set of primer used to amplify the *KanMX* gene. The promoter that was fused to the *KanMX* gene was cloned onto a CloneJet Vector (Fermentas) and subsequently used as the template to prepare the gene knock-out DNA fragment for *L. antarcticum*. The significances of the study involved the verification of the putative promoters obtained from *L. antarcticum* in the expression of the *KanMX* gene, secondly, the constructed gene knockout cassette can be used to knockout the targeted gene in order to reveal the function of the gene, and lastly, to demonstrate that gene fusion can be established seamlessly using PCR. Preliminary transformation of *L. antarcticum* using knockout cassette constructed was tested by using electroporation and heat-shock transformation systems applied in *S. cerevisiae*. The transformation of *L. antarcticum* was not successful. *L. antarcticum*'s colonies were found after electroporation transformation but did not grow onto the medium containing geneticin (G418). No colony was found after heat-shock transformation.

ABSTRAK

Leucosporidium antarcticum merupakan sejenis ragi psikrofilli terpencil dari Antartika. *L. antarcticum* bertumbuh dengan optimum pada suhu 12°C, dan juga menyesuai dengan baik pada suhu rendah di Antartika. Genom *L. antarcticum* telah dihimpun dengan sistem jujukan DNA 454. KO (Knock-out) gen-gen *L. antarcticum* dengan sistematik boleh menentukan fungsi gen terutamanya gen yang terlibat dalam adaptasi terhadap suhu yang rendah. Oleh demikian, pembinaan sistem KO adalah amat penting. Pembinaan kaset KO terlibat gabungan gen kanMX (ringtangan geneticin G418) dan gen promoter yang dipilih daripada gen ACT, SNF dan CYCLOPHILIN, gen kanMX adalah gen yang digunakan sebagai gen KO pada *S. cerevisiae*. Pengabungan gen-gen yang tersebut berlangsung dengan cara PCR. Satu set primer direka untuk bahagian promoter gen dengan pengubahsuai pada hujung 5' secara penambahan homologi gen kanMX. Pengabungan gen akan diklon ke dalam CloneJet (Fermentas) dan digunakan sebagai templat untuk menyiapkan gen KO serpihan DNA untuk *L. antarcticum*. Kepentingan kajian ini termasuk pecubaan gen promoter yang terpilih ke atas ungkapan gen kanMX. Kepentingan kedua ialah pembinaan kaset KO boleh digunakan untuk menyelidik ungkapan fungsi-fungsi gen yang terpilih. Kepentingan terakhir ialah untuk menunjukkan gabungan gen-gen boleh dilakukan dengan menggunakan PCR. Transformasi pecubaan telah dilakukan dengan menggunakan cara elektroporasi and cara kimia, kedua-dua cara tersebut telah dilaksanakan dalam *S. cerevisiae*. Transformasi pecubaan tersebut tidak berjaya dicapai. Koloni-koloni *L. antarcticum* telah dijumpai selepas transformasi dengan cara elektroporasi tetapi koloni-koloni tersebut tidak dapat menumbuh ke atas media yang mengandungi antibiotik geneticin (G418). Tiada koloni dijumpai selepas transformasi dengan cara kimia.

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LIST OF SYMBOLS AND ABBREVIATIONS

g	Gram
mg	Miligram
μg	Microgram
$^{\circ}\text{C}$	Degree Celsius
l	Liter
ml	mililiter
μl	Microliter
Amp	Ampicillin
<i>Kan</i>	Geneticin Resistance Gene
Bp	Basepair
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
<i>et al.</i>	<i>et alia</i> (and others)
i.e.	That is
kb	Kilobase pair
kV	Kilovolt
M	Molar
O.D	Optical density
PCR	Polymerase chain reaction
Rpm	Revolutions per minute
TBE	Tris borate EDTA
v/v	Volume per volume
w/v	Weight per volume
nm	Nanometer

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CHAPTER 1

INTRODUCTION

1.1 Preamble

Antarctica, a continent of 14 million square kilometers, surrounded by ice and snow, has been recognized as the coldest and driest habitat (Satyanarayana & Kunze, 2009). Antarctic is constantly exposed to solar radiation during the summer season. Extremely cold conditions are not favorable to most living organism. However, a significance number of life forms are found in the Antarctic, such as bacteria, yeast, fungi, lichens, small invertebrates, birds and mammals which have evolved to adapt to the environment with lower temperature, high salinity and high radiation (Satyanarayana & Kunze, 2009).

Many of the microorganisms isolated from cold environment are either psychrophilic or psychrotolerant (Ricardo *et al.*, 2002). Psychrophilic yeast have an optimal growth temperature of 15°C or lower (Fatimah *et al.*, 2010), but does not exceed temperature of 25°C (Satyanarayana & Kunze, 2009). Psychrotolerant organisms are able to grow at 0°C, but they tend to grow faster above 20°C (Ricardo *et al.*, 2002).

Yeasts are eukaryotic microorganisms which are heterogeneous in their nutritional abilities (Satyanarayana & Kunze, 2009), found in different geographical locations such as sea water, on moist, odd surface, dry substrates containing concentrated salt and sugar (Satyanarayana & Kunze, 2009). According to Satyanarayana & Kunze (2009), there are many types of yeasts in Antarctica that have been reported, such as *Bullera*, *Candida*, *Cryptococcus*, *Cystofilobasidium*, *Debaryomyces*, *Kondoa*, *Leucosporidium*, *Metschnikowia*, *Mrakia*, *Pseudozyma*, *Rhodotorula*, *Sakaguchia*, *Sporopachydermia*, *Sympodiomyces* and *Trichosporon*.

Among all the psychrophilic yeasts mentioned, *Leucosporidium antarcticum* was found in sea water near to the Casey station in year 2002 by the research team from Universiti Sains Malaysia (USM). *L. antarcticum* was interesting due to its ability to secrete lipase; catalyzing the hydrolysis of triacylglycerol into free fatty acids, glycerol (Fatimah *et al.*, 2010) and protease, and was also capable of fixing nitrogen. The genome of *L. antarcticum* has been fully sequenced by researchers from Universiti Sains Malaysia (USM) and Universiti Kebangsaan Malaysia (UKM). Genome portal has been set up in year 2009 for genome analysis.

Systematic gene knockout can reveal the function of genes (Guri *et al.*, 2002) in microorganisms such as *L. antarcticum*. Hence, there is a need to construct a gene knockout for *L. antarcticum*. This can be achieved by constructing a cassette containing an antibiotic resistance gene. This cassette is then fused to the homologous flanking region of the target gene to allow homologous recombination to knockout the desire gene at specific locus. The method is time effective, simple, and precise.

1.2 Significances of the Study

This study involved the verification of the putative promoters obtained from *Leucosporidium antarcticum* in the expression of the selectable marker (*kanMX* gene). Also, the construct gene knockout cassettes can be used to knockout targeted gene in order to reveal the function of the gene. Lastly, to demonstrate that gene fusion can be established seamlessly using polymerase chain reaction (PCR).

1.3 Objectives

1. To construct a gene knockout cassette for *Leucosporidium antarcticum*.
2. To attempt carry out gene knockout in *Leucosporidium antarcticum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Commercially Important Enzymes Found in Psychrophilic Yeast

The crucial feature that allows psychrophilic organisms to withstand the extremely cold environment is the ability to produce cold-adapted enzymes and to maintain high membrane fluidity (Ahmed *et al.*, 2001). The flexibility of the cold-adapted enzyme structure contributes in lower kinetic energy of reacting molecule, and according to Ricardo *et al.* (2002), the flexibility is formed by many structural properties including reduction in core hydrophobicity, decrease ionic and electrostatic interactions, increased charge of surface residue in order to increase solvent interaction as well as the additional surface loops (Ricardo *et al.*, 2002). Table 2.1 shows some of the enzymes isolated from psychrophilic yeast and its importance in commercial application.

Table 2.1: Enzymes isolated from psychrophilic yeast (Ricardo *et al.*, 2002)

Organism	Enzyme	Application	source
<i>Aspergillus nidulans</i> WG 312	Lipase	Food, detergent, Cosmetics.	Mayordomo <i>et al.</i> , 2000.
<i>Cryptococcus adeliae</i>	Xylanase	Dough fermentation, Protoplast formation, Wine and juice industry,	Petrescu <i>et al.</i> , 2000.
<i>Candida antarctica</i> CBS 6678	Glucoamylase	Starch hydrolysis.	Demot & Verachtert 1987.



<i>Leucosporidium antarcticum</i>	Serine proteinase, Lipase, α -glucosidase, Acid phosphatase, Alkaline phosphate, Beta- fructofuranosidase.	Diagnostic use. Detergent. Diagnostic use. Diagnostic use. Diagnostic use. Sugar hydrolysis.	Satyanarayana & Kunze, 2009.
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2.2 Genomic Data of *L. antarcticum*

Ever since the *Haemophilus influenzae* genome were sequenced in 1995, systematic gene knockouts have been used to study the function of the genes (Jenks, 1998). The genome of Antarctica yeast, *L. antarcticum* has been fully sequenced by researchers from Universiti Sains Malaysia (USM) and Universiti Kebangsaan Malaysia (UKM). The genome size is 2, 285, 101 base pairs and are assigned into 20 scaffolds. Each scaffold contains hundreds of Open Reading Frames (ORF), and the total number of estimated ORF is 7, 866 (personal communication, UKM).

Table 2.2: List of Total ORFs in Each Scaffold (personal communication, UKM)

Scaffold	Total number of ORF
1	382
2	278
3	771
4	584
5	546
6	383
7	281
8	445
9	292
10	471
11	527
12	567
13	537
14	382
15	370
16	879
17	165
18	2
19	3
20	1
Total	7 866

In addition, UKM has generated 34, 000 ESTs from the cDNA libraries based on three distinct parameters: temperature, different growth phases and different growth medium. To predict the putative genes, several gene prediction softwares such as Augustus, GlimmerHMM and GeneMark-ES were used. The predicted genes were aligned based on the protein annotated by National Center for Biotechnology Information (NCBI), Swissprot and Ontology.

2.3 Principles of Gene Knockout in Yeast

According to Michael *et al.* (1995), three general principles were involved in the construction of a gene knockout system for yeast, particularly for *S. cerevisiae*. First, the insert gene must be able to recombine at specific homologous sites in the genome. Secondly, a particular transformation condition to deliver DNA into the target cells and lastly, a selectable marker to identify the transformed cells. These

three principles are followed in this study. In which, 40 nucleotides homologous region at each 5' and 3' ends that flanked the *ADE1* and *ADE2* genes, is replaced by the *kanMX* gene through integration to the respected loci. Similar transformation methods are used in *S. cerevisiae*. The selectable marker used is the *kanMX* gene, a *geneticin* resistance gene. Systematic knockouts of genes will accurately reveal the function of these genes.

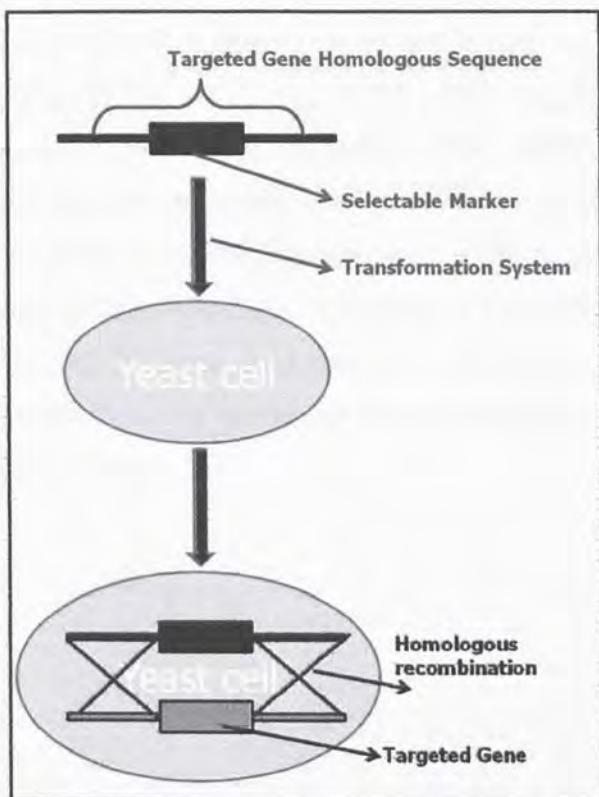


Figure 2.1: Schematic diagram of the gene knockout principles in yeast described Michael *et al.* (1995). The principles were homologous sequence, selectable marker and transformation system. The gene knockout occurred naturally through homologous recombination when homologous sequence from the knockout cassette complimented to the homologous sequence from the yeast genome, targeted gene was knocked out and was replaced by the selectable marker.

2.4 Auxotroph and *KanMX* Base Gene Knockout System

Auxotrophs, which are defined as organisms that have lost the ability to synthesize certain substances required for its growth and metabolism as the result of mutational change. It is also known as a useful marker in yeast, they will only be grown when the respective organic compound is added.

In general, the auxotrophic marker genes that are commonly used in yeast are genes that encode a specific enzyme in a metabolic pathway. Among the genes are *HIS3*, *LEU2*, *TRP1*, *MET15*, *ADE1* and *ADE2*, which encode for L-histidine, L-Leucine, L-trptophan and L-methionine respectively (Jack, 2002). One of the genes, *ADE1*, encodes for the enzyme *N*-succinyl-5-aminoimidazole-4-carboxamide ribotide (SAICAR) synthetase, which is required for *de novo* purine biosynthesis (Gerardo *et al.*, 1995) is the target gene in this study. According to Gerardo *et al.* (1995), yeast strains lacking of the SAICAR gene produced a red phenotype, making it an ideal reporter in gene knockouts where screening can be based on colour of the yeast colony after the transformation.

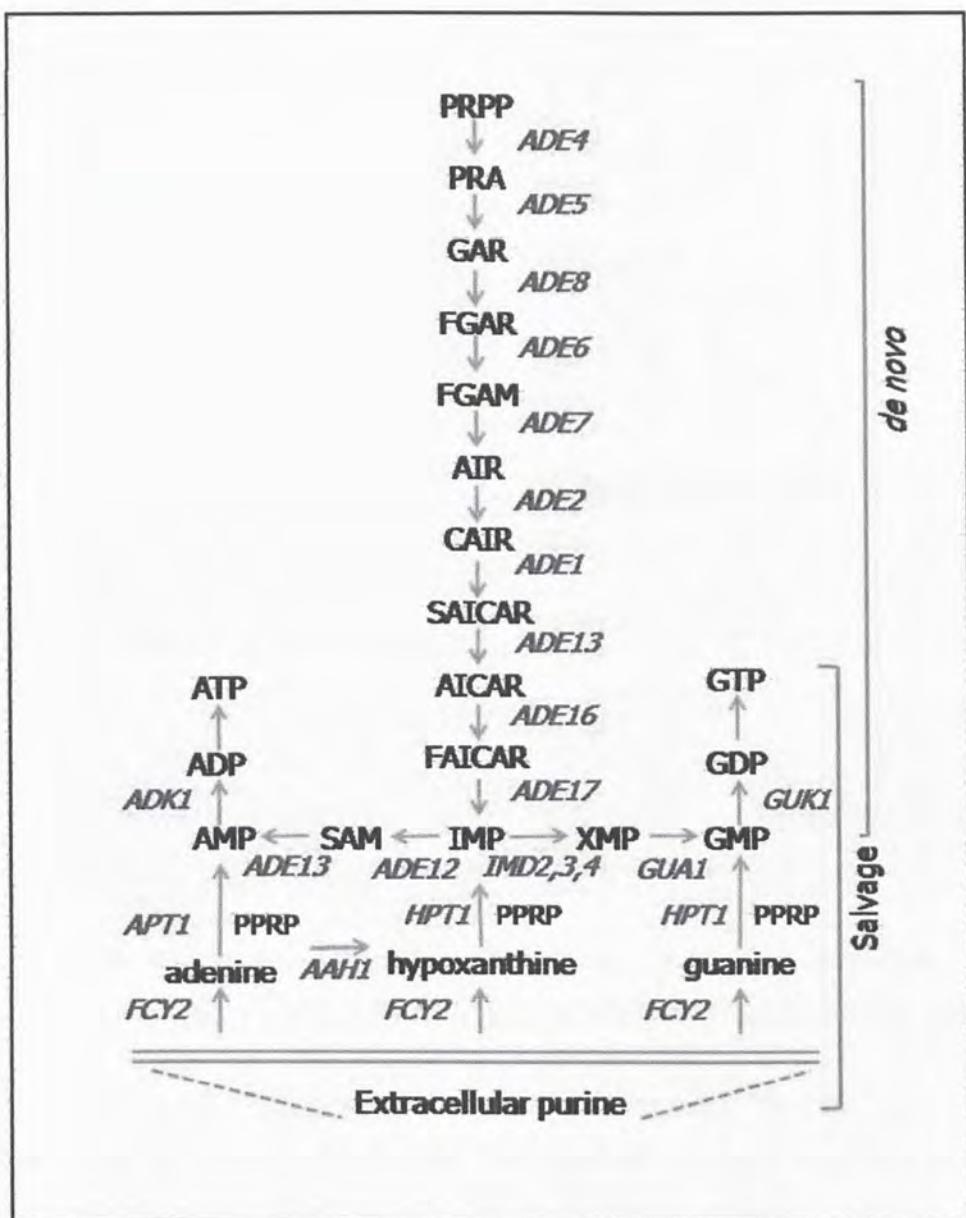


Figure 2.2: Schematic diagram of purine metabolism in *S. cerevisiae* (Rolfes, 2006; David *et al.*, 2008).

Besides of colour colony, the knockout gene also can be verified by observing the alteration in *L. antarcticum*. According to Guri *et al.* (2002) *S. cerevisiae*, have seven types of phenotypic references for gene deletion (Figure 2.3).

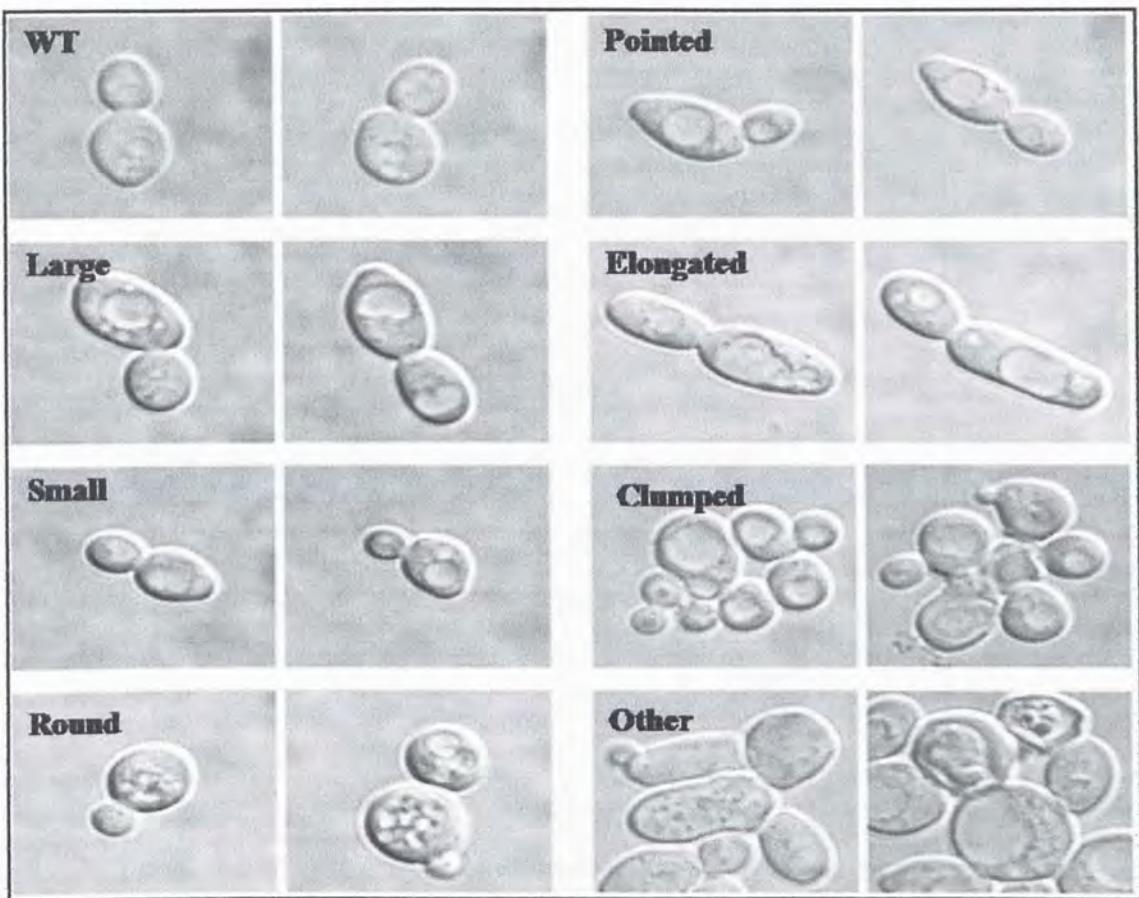


Figure 2.3: Phenotypic categories of deletion mutant morphologies in *S. cerevisiae*, WT, Wild type (Source obtained from Guri *et al.*, 2002)

In Yuriy *et al.* (2007), a gene knockout system using the *URA3* as a marker was developed for *Pichia guilliermondii*. The auxotrophic marker was used to stably maintain the expression vectors and also to introduce knockout mutation (Jack, 2002). On the other hand, selectable markers such as antibiotics are commonly used in cloning and gene knockout. Geneticin resistance gene, or *Karf* marker gene, has been reported as the important marker gene used in gene disruption experiment in *S. cerevisiae*. The *Karf* gene from *E. coli* transposon Tn903 when expressed in yeast render the transformants resistant to the aminoglycoside antibiotic G418 (Ulrich *et al.*, 1996; Anna & Dieter, 2000). *KanMX* gene is a useful gene used in gene knockout as many of the microorganisms are not resistance to geneticin. *KanMX* gene is embedded within the plasmid pFA6a-kanMX6 (GenBank accession: EU689038.1).

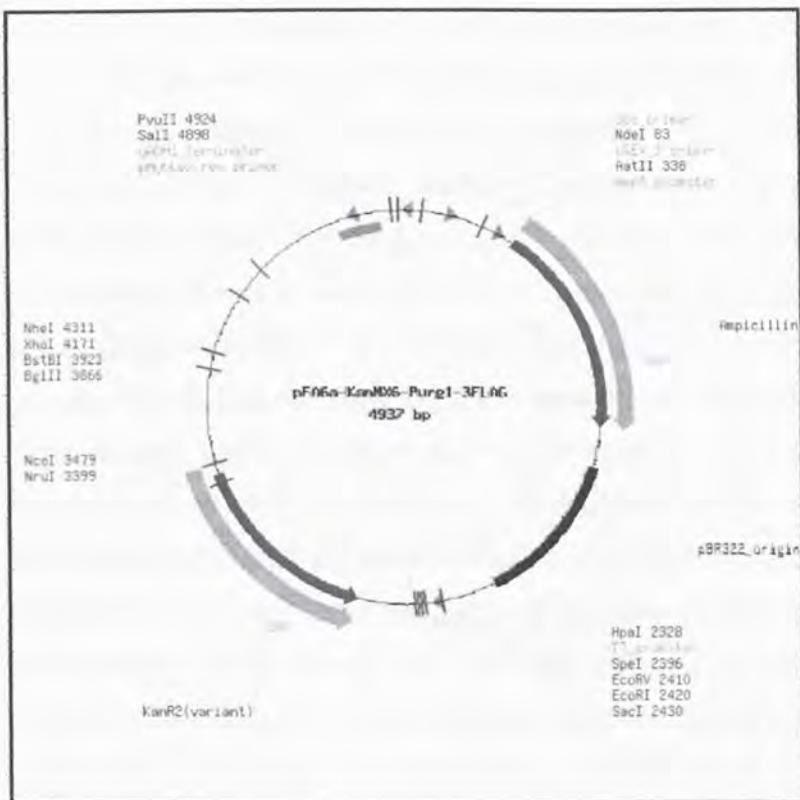


Figure 2.4: Schematic illustration of Plasmid pFA6a-kanMX6 (Source obtained from <http://www.addgene.org/pgvec1?f=c&cmd=genmap&plasmidid=19354&dim=600&mtime=1271372103>)

2.5 Transformation of Basidiomycetes

2.5.1 Transformation of *Coprinus cinereus*

Transformation method has been established to manipulate the basidiomycetes. Binninger *et al.* (1987) used a DNA-mediated transformation system to transform an agaric fungus, *Coprinus cinereus*. Two methods of competent cells preparation have been compared, the oidia (haploid asexual spores differentiate at the tips of monokaryotic mycelium) competent cell, and the other one is protoplast competent cell converted from oidia by using Onozuka R-10 cellulase and chitinase. The inserted gene was intergraded with pCc1001, containing the *TRP1* gene. The transformation method involved the treatment with lithium acetate treatment. In brief, the preparation of oidia competent cell, the harvested oidia cells, was resuspended in EDTA and TE buffer followed by lithium acetate and incubate. The

cells were harvested by centrifugation and later resuspension with lithium acetate before the addition of plasmid DNA in TE buffer following incubation, the cells were resuspended in lithium acetate containing PEG and incubated. The cells were washed and then plated on the growth medium. On the other hand, protoplast competent cells were prepared by treatment of oidia cells with Onozuka R-10 cellulase and chitinase. The oidia were cultured to obtain the mycelium. Then the mycelium was vortexed to release the protoplast. The separated protoplasts were washed twice with MM (0.5 M mannitol in 50 mM maleate pH 5.5) and once with MMC (MM with 50 mM CaCl₂) solution and resuspended in MMC solution and incubated. The protoplasts were harvested by centrifugation and washed with MM solution before resuspend in MM solution containing 20 mg cellulose and one mg chitinase for digestion. The protoplasts were then diluted and washed with MM and MMC solutions before plating. Comparing the two methods of competent cell preparations for transformation, the result showed that the protoplast competent cells yielded one transformant per 10⁷, which was 1 000-fold more than the oidia competent cell at one transformant per 10⁴ (Binninger *et al.*, 1987).

2.5.2 Transformation of *Lentinus edodes*

In *Lentinus edodes*, restriction enzyme-mediate of DNA integration (REMI) has been reported in Toshitsugu *et al.* (1998). This method successfully delivered DNA into host genome such as *Cochliobolus heterostrophus*, *Magnaporthe grisea*, *Saccharomyces cerevisiae* and *Dictyostelium discoideum*, with high transformation frequency and efficiency (Toshitsugu *et al.*, 1998). The method came with some simple principles.

First, the enzymes is allowed to penetrate the cell and nuclear membrane, followed by cleaving of the chromosomal DNA *in vivo* at specific restriction site and finally, the cleaved chromosomal DNA ends are used in ligation to the restriction enzyme-linearized plasmid DNA by the host cell enzyme (Tosshitsugu *et al.*, 1998).

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