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THESIS

CLONING, EXPRESSION AND PURIFICATION OF THE NOVEL ADENOSINE DEAMINASE FROM *STREPTOMYCES ANTIBIOTICUS*

ATTAWAN SUNANTAKARNKIJ

A Thesis Submitted in Partial Fulfillment of
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2'-Deoxycoformyin (2'-dCF) is a naturally occurring nucleoside antibiotic that had been isolated from *Streptomyces antibioticus*. 2'-dCF has been demonstrated to be one of the most potent inhibitors of adenosine deaminase. Thus, the ability of 2'-dCF producing *Streptomyces* to tolerate the inhibitory effect of this antibiotic is a pre-requisite for antibiotic production. A novel ADA, designated as ADA-II, had been proposed to be the key enzyme that confers *S. antibioticus* resistant to the inhibitory effect of 2'-dCF during biosynthesis of this antibiotic. Because antibiotic resistant gene and antibiotic biosynthetic genes are often clustered, cloning of the *ada*-II gene can possibly allow one to obtain the entire 2'-dCF biosynthetic genes.

Three different PCR-base methods were used to clone and determine the complete nucleotide sequence of the *ada*-II gene. The central region, 3'-downstream region, and 5'-upstream region of the *ada* gene was determined by normal PCR, inverse PCR, and single primer PCR, respectively. The complete ORF of the putative *ada*-II is 1041 bp in length and encodes for a protein of 346 amino acid residues (37.8 KDa). The deduced amino acid sequence of this *ada* gene shows 88%, 87% and 84% homology to ADA from *S. coelicolor* A3(2), *S. avermitilis* MA-4680 and *S. verginia*, respectively. When aligned to murine ADA (MuADA), seven of the eight catalytically active residues were also conserved in *S. antibiotics* ADA (StADA).

The putative *ada*-II gene was expressed in *E. coli* BL21 (DE3) as non his-tagged, his-tagged and fusion protein, using pET-26b, pET-28b and pYTB12, respectively. However, the protein formed inclusion bodies (IBs) in all cases upon induction with IPTG. Therefore, the protein in the IBs was unfolded and refolded with 2 M urea. After refolding and purification of the his-tagged protein, no deaminase activity was detected. However, because the calculated molecular weight of this putative ADA (37.8 KDa) is similar to that of ADA-II (38 KDa) purified from cell-free extract of *S. antibioticus*, therefore it is likely that this ORF is encoded for ADA-II. Base on multiple alignment of ADA from various organisms, a concerted addition-elimination type (SN2) mechanism is proposed for StADA.

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Student's signature Thesis Advisor's signature

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Attawan Sunantakarnkij

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LIST OF ABBREVIATIONS

ada	=	gene encoding for adenosine deaminase
ADA	=	adenosine deaminase
ADP	=	adenosine 5'-diphosphate
Ala	=	alanine
AMP	=	adenosine 5'-monophosphate
Ara-A	=	9-β-D-arabinofuranosyladenine
Asn	=	asparagine
Asp	=	aspartic Acid
ATP	=	adenosine 5'-triphosphate
bp	=	base pair
°C	=	degree Celsius
cAMP	=	adenosine 3',5'-cyclic monophosphate
CoF	=	Coformycin
dATP	=	deoxyadenosine 5'-triphosphate
2'-dCF	=	2'-deoxycoformycin
DNA	=	Deoxyribonucleic Acid
dNTPs	=	deoxynucleotide 5'-triphosphate
E. coli	=	*Escherichia coli*
EDTA	=	ethylenediaminetetraacetic acid disodium salt dihydrate
g	=	gram
Glu	=	glutamic acid
Gly	=	glycine
HDPR	=	6-Hydroxyl-1,6-Dihydropurine Ribonucleoside
His	=	histidine
IBs	=	inclusion bodies
IPCR	=	Inverse Polymerase Chain Reaction
IPTG	=	isopropyl-β-D-thiogalactopyranoside
kb	=	kilobase pair
kDa	=	kilo dalton

LIST OF ABBREVIATIONS (Continued)

K_i	=	inhibition constant
K_m	=	Michaelis-Menton constant
L	=	liter
LB	=	Luria-Bertani media
M	=	molar
Mb	=	megabase
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
MuADA	=	adenosine deaminase of murine
MW	=	molecular weight
NaCl	=	sodium chloride
ng	=	nanogram
nm	=	nanometer
OD	=	optical density
ORF	=	open reading frame
PCR	=	Polymerase Chain Reaction
PEG	=	polyethylene glycol
pH	=	logarithm of reciprocal of hydrogen (H) ion concentration
PMSF	=	phenylmethylsulfonyl fluoride
RNA	=	Ribonucleic Acid
rpm	=	revolutions per minute
SCID	=	Severe Combined Immunodeficiency Disease
SDS	=	sodium dodecyl sulfate
Sec	=	second
SP-PCR	=	Single Specific Primer Polymerase Chain Reaction
StADA	=	adenosine deaminase of *Streptomyces antibioticus*
TE	=	Tris EDTA

LIST OF ABBREVIATIONS (Continued)

T_m	=	melting temperature
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	unit
µg	=	microgram
µl	=	microliter
µM	=	micromolar
µmole	=	micromole
V_{max}	=	maximum velocity
YEME	=	Yeast Extract-Malt Extract media

CLONING, EXPRESSION AND PURIFICATION OF THE NOVEL ADENOSINE DEAMINASE FROM *STREPTOMYCES ANTIBIOTICUS*

INTRODUCTION

Adenosine deaminase (EC. 3.5.4.4) is a zinc metallo-enzyme, which exists in all organisms (Wilson *et al*., 1991). This enzyme is involved in purine catabolism by deamination of adenosine and 2'-deoxyadenosine to their respective inosine product and ammonia. This enzyme also plays an important role in the development of the immune system because deficiency of adenosine deaminase causes severe combined immunodeficiency syndrome (SCID). Patients with this disease lack of B and T-cell lymphocytes (Giblett *et al*., 1972). SCID is a fatal disease if not treated by gene therapy, bone marrow transplantation, or enzyme replacement therapy (Chan *et al*., 2005). In addition, the high expression level of adenosine deaminase causes various types of leukemia such as lymphoblastic leukemia, chronic lymphoblastic leukemia, cutaneous T-cell lymphoma, and hairy cell leukemia (Tung *et al*., 1976). For therapeutic, 2'-deoxycoformycin had been approved by Federal Drug Administration (FDA) in the United States in the treatments of various forms of leukemia. 2'-dCF is a potent inhibitor of adenosine deaminase with $K_i \sim 10^{-12}$ to 10^{-13}M. The tight binding of 2'-dCF to adenosine deaminase has been attributed to the fact that 2'-dCF mimics the structure of the tetrahedral transition state formed in the deamination reaction (Figure 1).

The 2'-dCF isolated from *Streptomyces antibioticus* is one of the naturally nucleoside antibiotics (Hanvey *et al*., 1984). In fact, *Streptomyces* species, gram-positive and soil-dwelling bacteria, possess ability to produce medically important antibiotics approximately 70% of all currently used (Hopwood *et al*., 1985). However, the biosynthesis of 2'-dCF has been studied by ^{14}C, ^{3}H, and ^{18}O isotopic labeling experiments. These studies have shown that adenosine is the precursor for the biosynthesis of 2'-dCF (Figure 3). During purification of enzyme, two forms of adenosine deaminase, designated as ADA-I and ADA-II, have been purified from *S.*

antibioticus, in the previous experiment. Although both of these deaminase exhibit similar kinetic properties, substrate specificity and zinc requirement, they are markedly differed in inhibition by 2'-dCF (Pornbanlualap, 1994). Similar to ADA from most species, ADA-I from *S. antibioticus* is potently inhibited by 2'-dCF, a transition state analog, with K_i of 10^{-9} M. ADA-II, however, is weakly inhibited by 2'-dCF with K_i of 10^{-3} M. This data suggested that ADA-II is a novel ADA that confers *S. antibioticus* the ability to be resistant to 2'-dCF during the biosynthesis of 2'-dCF. Because antibiotics are often toxic, the ability of the antibiotic producer to tolerate inhibitory effect of antibiotics is a prerequisite for antibiotic biosynthesis (Cundliffe, 2006).

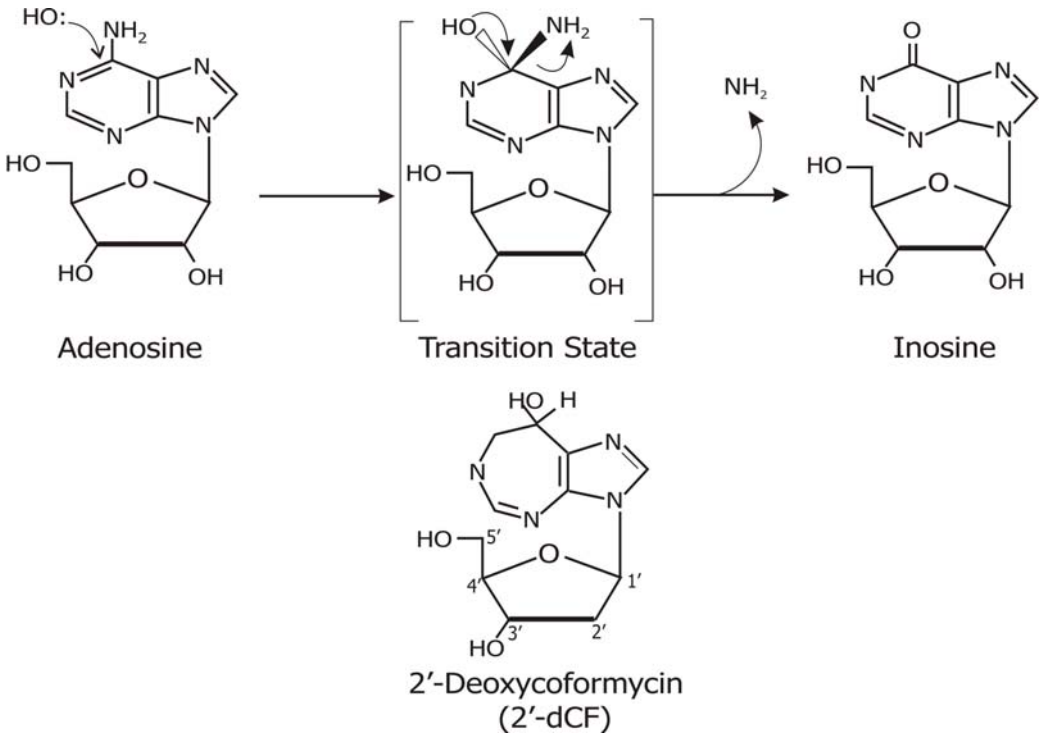


Figure 1 Catalytic mechanism of adenosine deaminase. The tetrahedral transition state or intermediate is formed by the addition of water molecule to the C6 position of purine ring. Elimination of the amino group from the tetrahedral intermediate results in the formation of inosine. Nucleoside antibiotic, 2'-deoxycoformycin (2'-dCF), which mimics the structure of the tetrahedral transition state formed in the deamination reaction, is a tight binding inhibitor of adenosine deaminase.

The gene encoding for adenosine deaminase from various sources had been sequenced. Alignment of amino acid sequence shows that amino acid residues involved in substrate binding and/or catalysis are highly conserved. Thus, we are interested in cloning the novel *ada* gene from *Streptomyces*. Although adenosine deaminase from *E. coli* has been cloned by the functional complementation approach (Chang *et al*., 1991), this approach is time-consuming. Therefore, inverse polymerase chain reaction (IPCR) and single primer polymerase chain reaction (SP-PCR), which are less time consuming, were used to clone adenosine deaminase from *Streptomyces antibioticus*.

OBJECTIVES

1. To clone the adenosine deaminase (*ada*) gene from *Streptomyces antibioticus* using inverse PCR (IPCR) and single primer PCR (SP-PCR).

2. To determine the nucleotide sequence of the *ada* gene from *S. antibioticus*.

3. To over-express, purify, and characterize its substrate/inhibitor specificity.

4. To unfold and refold the protein into biologically active form.

LITERATURE REVIEW

1. Adenosine Deaminase

1.1 Role of adenosine deaminase

Adenosine deaminase (EC. 3.5.4.4) is an important enzyme which presents in all organisms. This enzyme is involved in purine catabolism by catalyzing the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine to their respective inosine and ammonia. The products of the reaction, inosine and 2'-deoxyinosine, are subsequently degraded to uric acid, or recycled to the source of purine nucleotides (salvage pathway). Both adenosine and deoxyadenosine are biologically active purines that can effect the development of cellular physiology (Dolezal *et al*., 2005).

Genetic deficiency of adenosine deaminase (ADA) in human causes severe combined immunodeficiency disease (SCID), which is the first inherited disease reported (Giblett *et al*., 1972). This observation suggests that ADA is somehow involved in the development of the B- and T-lymphocytes of the immune systems in human. A lack of activity of ADA causes an increase in the level of adenosine and 2'-deoxyadenosine in the plasma. Accumulation of these toxic purine metabolites consequently leads to impairment of the development and functions of immune cells. The accumulated 2'-deoxyadenosine causes reducing production of lymphocytes because of pyrimidine starvation (Green and Chan, 1973) and causes neurological abnormalities. An increase in the concentration of 2'-deoxyadenosine has been reported to have an inhibitory effect on S-adenosylhomocysteine hydrolase (SAHH), an enzyme which is necessary for methylation reactions required for cell viability (Kredich and Martin, 1977). In addition, highly dATP inhibits ribonucleotide reductase, which is responsible for the reduction of purine and pyrimidine ribonucleotides to their deoxyribonucleotides, the precursors for DNA synthesis, and reduces ability to repair DNA (Ullman *et al*., 1978). Thus, ADA substrate accumulation induces apoptosis in lymphocytes. Affected patients with reduced levels

of ADA exhibit a defect of both cellular and immunity by fungal, viral, and bacterial infections resulting in death within a few months of life, if untreated (Aldrich *et al*., 2000). In human, there are two forms of ADA, ADA1 and ADA2. ADA1 is a majority of ADA activity and presents in all human tissues. However, ADA2 presents very small amounts and detects in the monocyte macrophage cell in human. These ADA might be also due to the breast cancer in women (Aghaei *et al*., 2005). In addition, the high expression level of adenosine deaminase causes various types of leukemia such as lymphoblastic leukemia, chronic lymphoblastic leukemia, cutaneous T-cell lymphoma, and hairy cell leukemia (Tung *et al*., 1976). Thus, for therapeutic, 2'-deoxycoformycin had been approved by Federal Drug Administration (FDA) in the United States in the treatments of various forms of leukemia. The 2'-deoxycoformycin (2'-dCF) is a potent inhibitor of adenosine deaminase.

Adenosine deaminase is an intracellular enzyme expressed in all cells and its activity is found highest in lymphoid tissues and lowest in erythrocytes (Hershfield and Mitchell, 1995). But in some tissue, ADA is also present on the surface of cell. ADAs that bound to CD26 and adenosine receptor (A1 and A2B) on the cell surface are called ecto-ADA and control the extracellular concentration of adenosine. Interaction between ecto-ADA and CD26 is needed for activation for blood T-cells. On the other hand, ecto-ADA on CD26 act as a co-stimulatory molecule in T cells (Franco *et al*., 1998). If the ecto-ADA is mutated, the elevated adenosine levels trigger adenosine receptors that regulate intracellular cAMP, cause the increase in the concentration of cAMP, and eventually lead to T-lymphocyte apoptosis (Kizaki *et al*., 1990).

1.2 Structure and mechanism of adenosine deaminase

Three-dimensional structure of murine adenosine deaminase has been determined by X-ray crystallography (Wilson *et al*., 1991). This enzyme is a zinc metallo-enzyme that contains a central $(\beta/\alpha)_8$ barrel and five additional helices. Alignment of adenosine deaminase from various sources, ranging from *E. coli* to human, shows that the catalytically essential residues at the active site are conserved.

In *E. coli* ADA, these conserved four amino acids are His214, Cys262, Asp295, and Asp296 (Chang *et al*., 1991). In murine ADA (MuADA), active site residues include Gly184, Glu217, His238, Asp295 and Asp296. In addition, the cofactor, zinc ion is coordinated by His15, His17, His214, and Asp295 in the active site of MuADA (Wilson *et al*., 1991). *In vitro* mutagenesis of active site residues (His17, His214, His238 and Glu217) eliminated ADA activity. In contrast, replacement of Cys262 retained at least 30-40% of ADA activity (Bhaumik *et al*., 1993). This data suggested that Cys262 is not a crucial role in the catalytic mechanism of adenosine deaminase. As observed with all of enzymes with TIM barrel fold, the active site of ADA was located and buried in β-face at C-terminal. From the sequence homology of *E. coli*, mouse and human adenosine deaminase, it indicates that the structures and functions of mammalian and bacterial enzymes are similar (Chang *et al*., 1991). Adenosine deaminase can be grouped in two major subfamilies, *bona fide* and ADGF/CECR1. The former is metabolic enzymes that have short N-terminal region and predominantly located in cytoplasm. The *bona fide* subfamily has been discovered in all organisms. The latter has an extended N-terminus which often contains a signal peptide for located in specific cell or secretion (Dolezelova *et al*., 2005).

Adenosine deaminases from different sources exhibit similar substrate specificity and kinetic constants. The catalytic efficiency of this enzyme is remarkable, with a rate enhancement of approximately 2×10^{12} (Frick *et al*., 1987). The catalytic mechanism of deamination has been proposed to occur by addition-elimination (SN2 mechanism), with formation of a tetrahedral transition state at the C-6 position of the purine ring (Kurz and Frieden, 1987). Evidences from the binding study of the enzyme to various transition-state analogues suggest that the presence of hydroxyl group at the tetrahedral carbon is essential for tight binding (Schramm and Baker, 1985). Evidences that support this SN2 mechanism include the facts that: (1) the nucleoside analogs such as 2'-dCF which mimic the structure of the tetrahedral transition state at the C-6 position of purine ring bind tightly to the enzyme, (2) ADA catalyzes a reversible hydration of pteridine, indicating the direct addition of water to the C-6 position of pteridine (Evans and Wolfenden, 1970), (3) the enzyme catalyzes elimination of leaving groups substituted at the C-6 position which is formed a

tetrahedral intermediate, generated by the attack of water of the C-6 position, (4) ADA releases the leaving group of C-6 of purine ring.

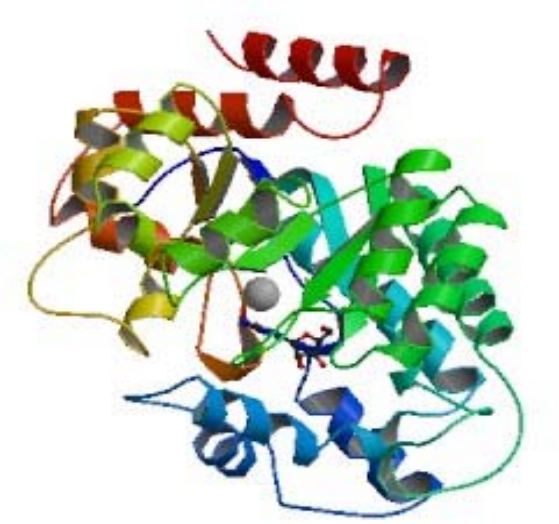


Figure 2 The ribbon structure of murine adenosine deaminase in complexed with purine riboside (orange ring), a transition state. The enzyme consists of 8 α-helices and 8 β -strands. The overall structure of the enzyme is similar to that of the TIM barrel. The catalytic site is in β-face of the barrel. Zinc ion which tightly bound to the enzyme and is required for catalytic activity is shown in white circle.

Source: Wilson *et al*. (1991)

2. Nucleoside Antibiotics

2.1 Importance of nucleoside antibiotics

Nucleoside antibiotics are derivative of purine and pyrimidine nucleosides that constitute an important group of microbial secondary metabolites which are effective agents against foreign microorganism, plant, and human diseases. These is interesting question that how the producing organisms defend themselves from the toxic effects of their products. Because antibiotic resistant and antibiotic biosynthetic genes are often clustered and regulated by the same promoter, the clustering of genes

allows simultaneous expression of antibiotic biosynthetic enzymes as well as the antibiotic resistant enzyme. Thus, the organisms which produce the antibiotic are tolerated the inhibitory effect by prerequisite synthesize antibiotic resistant (Cundliffe, 2006).

Nucleoside antibiotics can be classified into two groups: (1) the carbohydrate moieties modified (Farmer *et al*., 1972) and (2) the heterocyclic moieties modified (Hanvey *et al*., 1984). Tubercidin, formycin A, formycin B, and coformycin are examples of nucleoside antibiotics with their heterocyclic moieties modified. Ara-A, 2'-amino-2'-deoxyadenosine and cordycepin are examples of nucleoside antibiotics with their carbohydrate moieties modified. Most of these antibiotics are synthesized by *Streptomyces* as secondary metabolites. Estimated 8700 antibiotics discovered in *Streptomyces* by 2002, compared with 2900 and 4900 for bacteria and fungi, respectively (Hopwood, 2006).

2.2 Production and importance of 2'-dCF

2'-Deoxycoformycin (2'-dCF) is naturally nucleoside antibiotic that has been isolated from *Streptomyces antibioticus*. Adenosine has been demonstrated to be the direct precursor for the biosynthesis of 2'-dCF. The conversion of adenosine to 2'-dCF occurs by insertion of an "extra" carbon from C-1 of phosphoribosyl pyrophosphate (PRPP) between N-1 and C-6 of the purine ring (Hanvey *et al*., 1984) (Figure 3). 2'-dCF, commercially known as "pentostatin or nipent", is used medically as an immunosuppressing drug in the treatment of various forms of leukemia. It has been approved by the Federal Food and Drug Administration (FDA) in the United States to be used for T-cell lymphoblastic leukemia and hairy cell leukemia. 2'-dCF is a potent inhibitor of adenosine deaminase with $K_i \sim 10^{-12}$ to 10^{-13} M. The tight binding of 2'-dCF to adenosine deaminase has been attributed to the fact that 2'-dCF mimics the structure of the tetrahedral transition state formed in the deamination reaction.

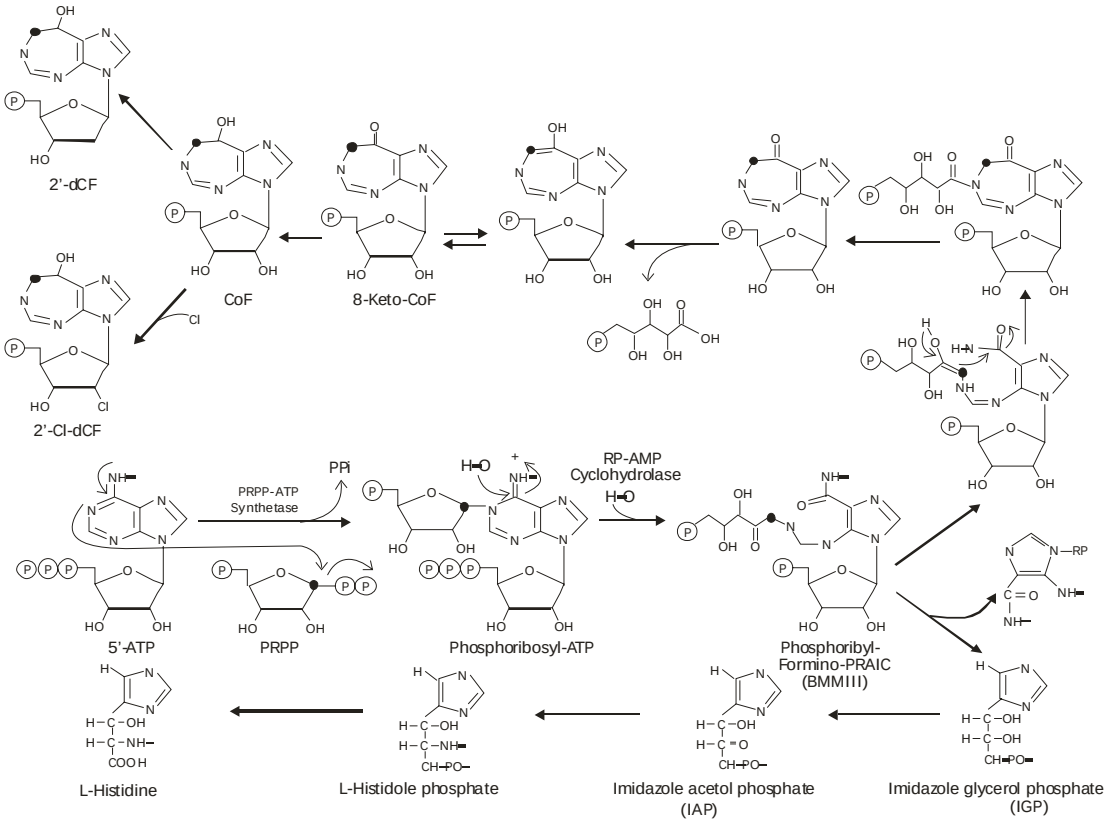


Figure 3 Proposed biosynthetic pathways for 2'-deoxycoformycin (2'-dCF). Adenosine has been proposed to be as a precursor of 2'-dCF. The "extra" carbon from C-1 (dark spot) of phosphoribosyl pyrophosphate (PRPP) interacted between N-1 and C-6 of the purine ring catalyzed by PRPP-ATP synthetase. The purine was opened by RP-AMP cyclohydratase to create phosphoribyl-formino-PRAIC (BMMIII). At this step, the pathway was branched to synthesize histidine and 2'-dCF. After that, the coformycin (CoF) was occurred by close the ring which is contained the "extra" carbon at C-7 position of the purine ring. The 2'-dCF was discovered by deletion of hydroxyl group at C-2' of ribose ring of CoF.

Source: Hanvey *et al*. (1984)

3. *Streptomyces antibioticus*

3.1 *Streptomyces sp*.

Streptomyces are beneficial microorganisms that are found in soils of tropical area such as Thailand. They are classified in actinomycetes Bacteria (Kalakoutski and Agre, 1976). Actinomycetes are gram-positive bacteria and have highly G+C content. However, the genome size of *Streptomyces* was discovered from genome project. It reveals that the chromosome of *Streptomyces coelicolor* A3(2) has a linear 8,667,507 bp with 7,825 predicted genes (Bentley *et al*., 2002). Furthermore, the linear DNA of *Streptomyces* approximately 8-9 Mb is carrying essential genes at a "core" and adaptive gene at "arms" (Hopwood, 2006). The biology of *Streptomyces* is of interest. Unlike other prokaryotes, *Streptomyces* have morphological differentiation in their life cycle. First, a resting spore produces young vegetative mycelium, germ tube, or form branching filaments that develop into mature mycelium. When nutrients become limited in the environment, they form spore-bearing aerial hyphae. At this time, they start to produce a wide variety of secondary metabolites (Hopwood, 2006).

Streptomyces, soil-dwelling bacteria, are particularly in the upper layers of the soil profile and withstand extremes of temperature and moisture (Loria *et al*., 2006). At this environment, *Streptomyces* possess the ability to produce and secrete numerous extracellular proteins such as lipases, proteases, amylases, and restriction endonucleases to degrade complex biological polymer including cellulose and chitin (Charter and Hopwood, 1989; Loria *et al*., 2006). In addition, *Streptomyces* also possess the ability to produce antibiotics approximately 70% of all antibiotics, for example, streptomycin, tetracycline, chloramphenicol, polyketide, actinomycin, erythromycin, puramycin, and 2'-deoxycoformycin. It is demonstrated that *Streptomyces* species is valuable as sources of pharmaceutically important antibiotics.

3.2 Adenosine deaminase of *Streptomyces antibioticus*

Two distinct forms of adenosine deaminase, designated as ADA-I and ADA-II, have been purified from *S. antibioticus* (Pornbanlualap, 1994). Although both of these enzymes have similar kinetic properties, substrate specificity, and zinc requirement, their inhibitions by 2'-dCF are markedly different. ADA-I, a molecular weight of 40 kDa, is potently inhibited by 2'-dCF with K_i of 1×10^{-9} M. On the other hand, ADA-II, a molecular weight of 38 kDa, is weakly inhibited by 2'-dCF with K_i approximately 10^6-10^7 fold lower than ADA from other sources. This data suggested that ADA-II is a novel ADA that confers *S. antibioticus* selectively resistant to 2'-dCF during the biosynthesis of 2'-dCF. Because antibiotic resistant gene and antibiotic biosynthetic gene are often clustered, gene that encoded for ADA-II (2'-dCF resistant gene) might be located nearly gene that encoded 2'-dCF.

4. Inverse Polymerase Chain Reaction (IPCR)

Inverse PCR (IPCR) is a rapid method for *in vitro* amplification of unknown DNA sequence that flanking the target known region in various organisms. This technique is developed by Ochman in 1980s base on the simple procedure of digestion of genomic DNA with the appropriate restriction enzymes and circularization of cleavage product before amplification. It has the primers that synthesized in the opposite orientations (Figure 4) (Ochman *et al.*, 1988). An inverse PCR strategy has widely many applications in molecular genetics such as the amplification and identification of transposable element, regulatory element, full length, chromosome walking, and DNA library screening.

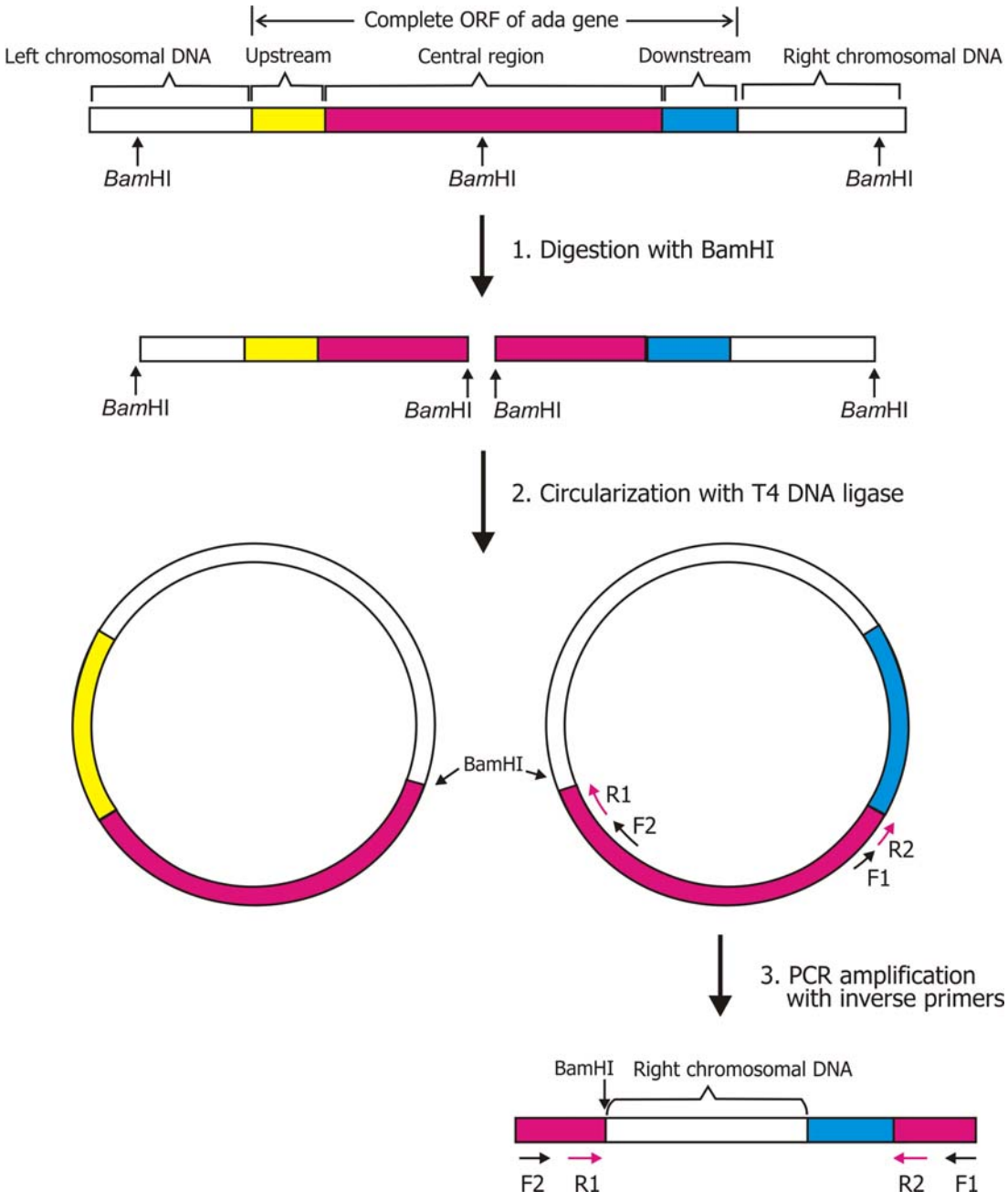


Figure 4 To determine the 3'-downstream region of the putative *ada* gene using inverse PCR. In step 1, genomic DNA (black line) is digested by a *Bam*HI restriction enzyme. It cleaves within the central region of *ada* gene (pink). In step 2, only upstream (yellow) or downstream (blue) flanking region of the *ada* gene can be obtained after recircularized DNA. Finally, two pairs of inverse primers are used to amplify the flanking region of the target gene. Thus, the 3'-downstream region of the *ada* gene will be obtained. This diagram is modified from Ochman *et al*., 1988.

5. Single Primer Polymerase Chain Reaction (SP-PCR)

SP-PCR approach is used to amplify the unknown region of the interested gene. In the PCR amplification, the reaction contains only one specific primer that acts as forward and reverse primers. This method is convenient because it requires no enzymatic modification of the template DNA (Parks *et al*., 1991). Annealing at low temperature, the primer was annealed to its specific region and to non-specific regions in the complementary strand (Figure 5) (Aquino and Figueiredo, 2004).

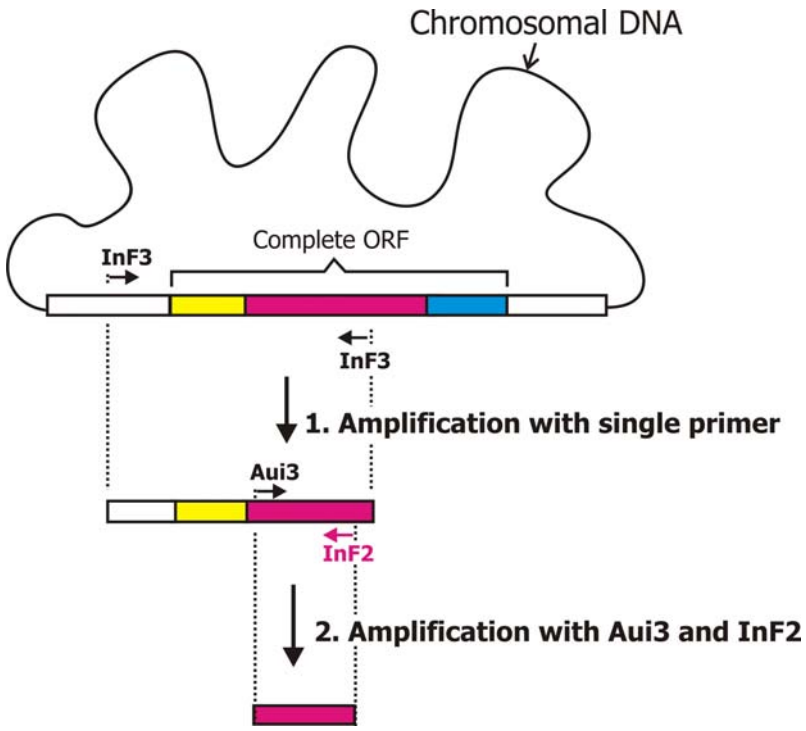


Figure 5 To determine the 5'-downstream region of the putative *ada* gene using single primer PCR (SP-PCR). The cORF of the putative *ada* gene contains upstream (yellow), central (pink), and downstream (blue) regions. The step of SP-PCR to determine the 5'-upstream region is proposed. In step 1, genomic DNA (black line) is used as the template for SP-PCR amplification using InF3 as forward and reverse primer. At low annealing temperature, the InF3 primer binds to specific and non-specific region. Then, the PCR product is used as the template in the nested-PCR which is performed by Aui3 and InF2.

MATERIALS AND METHODS

Materials

1. pH meter
2. SDS-PAGE electrophoresis
3. Agarose gel electrophoresis
4. Incubator shaker
5. Centrifuge machine
6. Autoclave
7. Hot oven
8. Spectrophotometer
9. PCR machine
10. Incubator oven
11. *Streptomyces antibioticus*
12. *Escherichia coli* strain DH5α
13. *E. coli* BL21 (DE3)
14. pGEM-T easy vector
15. pET26b, pET28b , and pTYB12
16. Restriction enzymes and T_4 DNA ligase
17. *Taq* DNA polymerase
18. Long and accurate DNA polymerase
19. Gel extraction kit
20. Plasmid isolation kit
21. Primers

Methods

1. Media and Growth Conditions

S. antibioticus grown in yeast extract-malt extract (YEME) medium (Hopwood *et al*., 1985) was vigorous shaking at 250 rpm for 48 hr at 30ºC before harvest for chromosomal DNA isolation. *E. coli* was grown on Luria-Bertani (LB) medium (Sambrook *et al*., 1989) with shaking at 250 rpm for 16 hr at 37 ºC before harvest. Both bacterial cells were harvested by centrifugation at 5,000 rpm for 20 min at 4ºC. Solid medium was prepared by adding 1.8% (w/v) of agar.

2. Isolation of Chromosomal DNA from *S. antibioticus*

Isolation of high molecular weight chromosomal DNA from *Streptomyces antibioticus* was performed as described (Hopwood *et al*., 1985). Briefly, two grams of *S. antibioticus* cells was resuspended in 5 ml of TE buffer containing 10 mg of lysozyme. After incubation at 30ºC for 1 hour, EDTA was added to a final concentration of 100 mM. After standing for 5 min at 30ºC, SDS was added to final concentration of 0.1% (v/v) and then incubated at 37ºC for 2 hours. The contaminated proteins in the mixture were removed by phenol-chloroform extraction. For removal of contaminated RNA, the reaction was incubated with RNaseA at a final concentration of 30 μg/ml at 37ºC for 1 hour. DNA was precipitated by addition of 0.25 volumes of 5 M NaCl and PEG to the final concentration of 10%. After dissolved DNA in 5 ml of TE buffer, DNA was precipitated again by sodium acetate and absolute ethanol. Chromosomal DNA was pooled and dissolved in TE buffer. The amount of DNA was determined by the absorption at 260 nm with spectrophotometer.

3. PCR Amplification of Central *ada* Gene from *S. antibioticus*

Amino acid sequences of ADA from various organisms were aligned by the ClustalW program. Degenerated primers were designed from conserved region (Figure 6). The PCR of central *ada* gene was performed in the reaction containing 200

ng of *S. antibioticus* chromosomal DNA as template, 1X *Taq* buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.2U *Taq* DNA polymerase (Egg Dragon Gene, Thailand), 0.3 μM Aui3 (5'-AGGCSSASCTSCACCTSCAC-3') and Aui4 (5'-CGSASGCCGTGGYCGA TSCG-3') primers (Table 1). The reaction was initiated at 94ºC for 5 min and subjected to 35 cycles of 1 min of denaturation at 94ºC, 50 sec of annealing at 65ºC and 1 min 30 sec of extension at 72ºC. After 35 cycles, the reaction was incubated at 72ºC for 10 min and cooled at 4ºC. PCR product was analyzed on 1% agarose gel electrophoresis.

4. Identification of Restriction Sites within Central *ada* Gene

After PCR amplification with Aui3 and Aui4 primers, 100 ng of PCR fragments was cleaved by various restriction enzymes including *Xho*I, *Psh*AI, *Hind*III, and *Bam*HI. Each reaction in total volume of 10 μl was incubated at 37ºC for 3 hours and then inactivated at 65ºC for 15 min. The digested PCR products were analyzed on 1% agarose gel electrophoresis.

5. Amplification of 3'-Downstream Region of *ada* Gene Using IPCR

Chromosomal DNA (2 μg) of *S. antibioticus* was digested into smaller fragments by various restriction enzymes including *Xho*I, *Psh*AI, and *Bam*HI, at 37ºC for 3 hours. After heat inactivated at 65ºC for 15 min, digested DNA fragments were analyzed on 0.8% agarose gel electophoresis. The digested DNA fragments were gel purified with gel extraction kit (Qiagen) and circularized using T_4 DNA ligase at final concentration 5 ng/μl. The reaction mixtures at a total volume of 200 μl were incubated at 16ºC for 16 hours and inactivated at 65ºC for 15 min. The circularized DNA was purified by nucleospin column. The concentration of DNA eluted from the nucleospin column was 10 ng/μl. The downstream region of *ada* gene was amplified by two pairs of specific inverse primers that had been designed based on known nucleotide sequence of central *ada* gene (Figure 6). The first round IPCR was performed in the mixture containing 50 ng of circle DNA as template, 1X Ex*Taq* buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5U Ex*Taq* polymerase (Takara, Kyoto), 0.5 μM InF2 (5'-CGGGTCCA CCTGGATCTCCAG-3') and InF1 (5'-TTCGGGCTCTCC

AACGACGAG-3') primers. The reaction was initiated at 94ºC for 5 min and subjected to 35 cycles, which consisted of 1 min of denaturation at 94ºC, 30 sec of annealing at 60ºC and 5 min of extension at 72ºC. After 35 cycles, the reaction was incubated at 72ºC for 10 min and cooled at 4ºC. The second round IPCR (nested-IPCR) was performed in the mixture containing 0.3 µl of first round PCR product as template, 1X Long PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1U Long PCR enzyme mix (Fermatus), 0.5 µM InR1 (5'-GGAGCGGCC CGCGTCGTACAG-3') and InR2 (5'-CTGGACAATCTGCACGCCTCC-3') primers. The nested-IPCR condition was carried out as first round IPCR. PCR product was analyzed on 1% agarose gel electrophoresis.

6. Amplification of 5'-Upstream Region of *ada* Gene Using SP-PCR

The upstream region of *ada* gene was amplified by using different set of specific primers (Table1). PCR amplification was performed in a reaction mixture containing 200 ng of *S. antibioticus* chromosomal DNA, 1X Ex*Taq* buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5U Ex*Taq* polymerase, 0.5 µM of specific primers (Aui3, Aui4, InF1, InF2, InF3, InR1, and InR2). The reaction was initiated at 94ºC for 5 min and followed by 35 cycles of denaturation for 1 min at 94ºC, annealing for 30 sec at 63ºC and extension for 5 min at 72ºC. The nested-PCR was performed to confirm which of PCR mixtures contained *ada* gene. Each of nested-PCR mixture containing 0.1 µl of first round PCR product as template, 1X Ex*Taq* buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5U Ex*Taq* polymerase, 0.5 µM forward and reverse primers that are suitable to each template (Table2). The nested-PCR condition was followed as first round SP-PCR amplification. PCR product was analyzed on 1% agarose gel electrophoresis.

7. DNA Sequencing

All interested PCR products were inserted into pGEM-T easy vector (Promega, Madison, WI). Plasmid DNA was prepared by plasmid isolation kit

(Fermatus). The nucleotide sequences were verified by Macrogen, Inc (Korea) or 1st BASE, Inc (Malasia).

Table 1 List of oligonucleotide primers

Oligonucleotide Primers	Sequence (5' ⟶3')	Length (nt)	T_m (°C)*
Primers for amplification of *ada* gene from *S. antibioticus*			
Forward primer			
AUI3	5'- AGGCSSASCTSCACCTSCAC -3'	20	68
ADA F	5'- CATATGGAGCGTGTACGTGATCTCTC -3'	26	70
Reverse primer			
AUI4	5'- CGSASGCCGTGGYCGATSCG -3'	20	71
ADA R	5'- AGATCTCTAGGCGGCCGGACGGGCCA-3'	26	72
Inverse primers for amplification flanking region using IPCR and SP-PCR methodology			
Forward primer			
InF1	5'- TTCGGGCTCTCCAACGACGAG -3'	21	68
InR2	5'- CTGGACAATCTGCACGCCTCC -3'	21	68
Reverse primer			
InR1	5'- GGAGCGGCCCGCGTCGTACAG -3'	21	74
InF2	5'- CGGGTCCACCTGGATCTCCAG -3'	21	70
InF3	5'- GTCCAGCGGGTGTTTCATGCG -3'	21	68

* Melting temperature (T_m) is calculated using the equation $T_m = 2(A+T) + 4(G+C)$

Degenerate primer Symbol Y = T or C, K = T or C, S = G or C, R = A or G and N = A, T, G or C

Red and blue lines represented recognition site of *Nde*I, and *Bgl*II, repectively.

Figure 6 Amino acid sequence alignment comparison of adenosine deaminase from *Streptomyces avermitilis* MA-4680 (NP826083), *S. virginiae* (BAA09298), *S. coelicolor* A3(2) No.1 (NP626784), *S. coelicolor* A3(2) No.2 (NP628805), *Pseudomonas fluorescens* PfO-1 (YP346404), and *Bordetella parapertussis* (NP883359). Arrows showed the regions, which designed primers for amplication of central *ada* gene (Aui3 and Aui4), IPCR (InF1, InF2, InR1 and InR2), and SP-PCR (InF3, InF2 and Aui3).

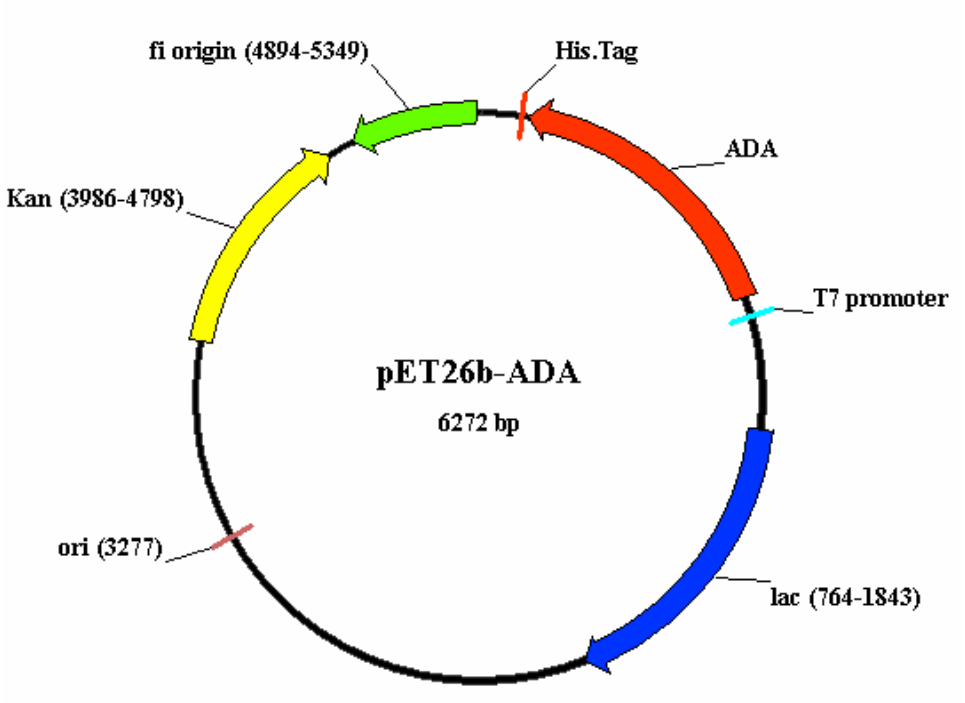


Figure 7 Construction of the recombinant plasmid pET26b-*ada*. The amplified novel *ada* gene from *S. antibioticus*, red box, was inserted into *Nde*I and *Bam*HI sites of pET26b expression vector. The recombinant protein, non his-tagged, was expressed in *E. coli* BL21 (DE3).

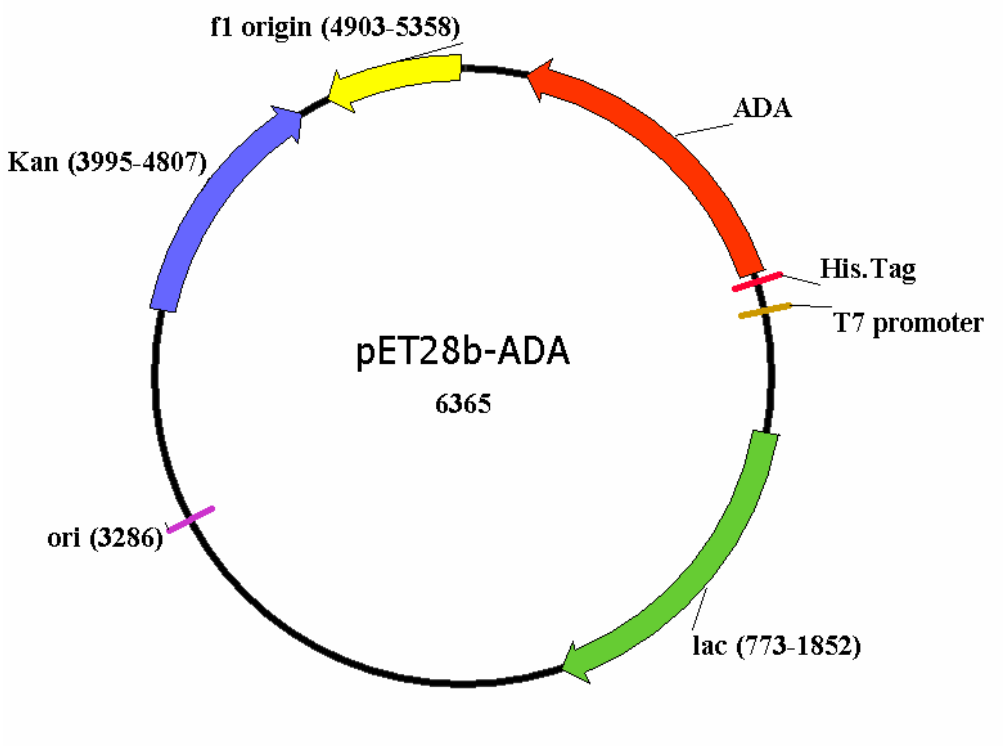
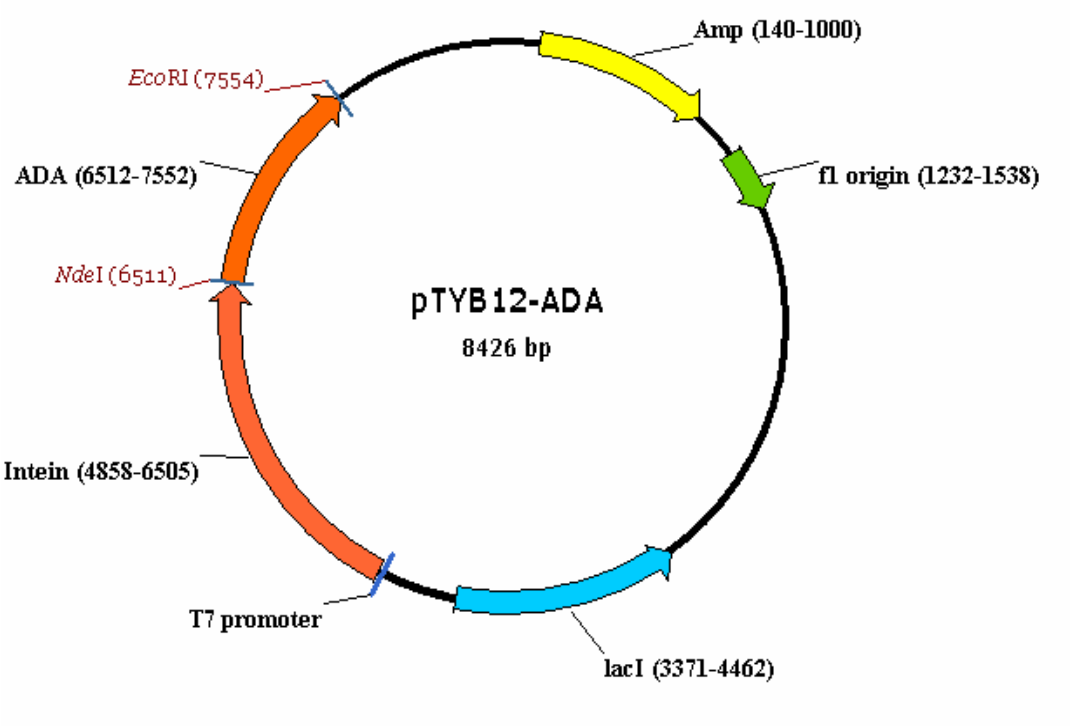
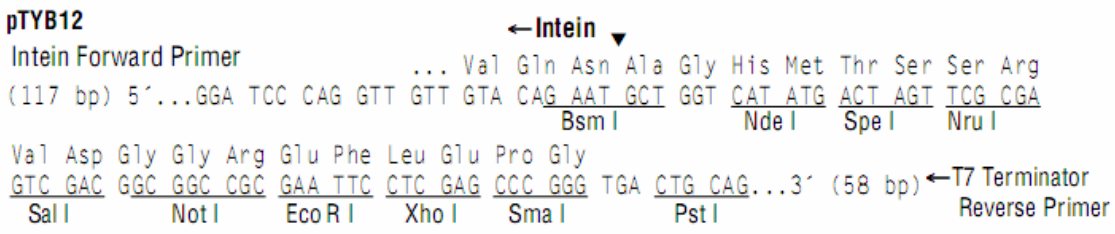


Figure 8 Construction of the recombinant plasmid pET28b-*ada*. The amplified novel *ada* gene from *S. antibioticus*, red box, was inserted into *Nde*I and *Bam*HI sites of pET28b expression vector. The recombinant protein, his-tagged at the N-terminal, was expressed in *E. coli* BL21 (DE3).





pTYB12 cloning/expression region

Figure 9 Construction of the recombinant plasmid pTYB12-*ada*. The amplified novel *ada* gene from *S. antibioticus*, red box, was inserted into *Nde*I and *Eco*RI sites of pTYB12 expression vector. The recombinant protein was fused with intein gene, red box, as fusion protein at N-terminal and expressed in *E. coli* BL21 (DE3). The cleavage site between two genes was indicated as ▼ .

Source: New England BioLabs (2006)

10. Cloning of the Putative *ada* Gene from *S. antibioticus*

The full length *ada* gene (1050 bp) was amplified by PCR in reaction mixture containing 200 ng of *S. antibioticus* chromosomal DNA as the template, 1X Ex*Taq* buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.5U Ex*Taq* polymerase, 0.3 μM ADA F (5'-CATATGGAGCGTGTACGTGATCTCTC-3') and ADA R (5'-AGATCTCTAGGC GG CCGGACGGGCCA-3') primers. The underlines are the added *Nde*I and *Bgl*II linker, respectively. PCR amplification was performed as described in central *ada* gene amplification. The PCR products were analyzed on 1% agarose gel electrophoresis and purified by nucleospin column. The purified *ada* fragments were ligated into pGEM-T easy vector using T_4 DNA ligase. The ligated mixture was transformed into competent *E. coli* DH5α and plated on LB supplement with ampicillin, IPTG, and X-Gal. Selected white colony that contained the pGEM-*ada* was grown on LB supplement with ampicillin overnight and recombinant plasmid was purified by alkali method. The pGEM-*ada* was doubling digested with *Nde*I and *Bgl*II to release the *ada* fragments which contained *Nde*I and *Bgl*II sites. The *ada* fragments were gel purified and ligated into pET-28b vector which had been previously digested with *Nde*I and *Bam*HI, to produce his-tagged protein at N-terminal. For production of non his-tagged protein, the *ada* fragments were ligated into pET-26b vector which had been previously double digested with *Nde*I and *Bam*HI. In addition, the pGEM-*ada* was doubling digested with *Nde*I and *Eco*RI. The digested *ada* fragments were inserted into pTYB12 vector, which has corresponding restriction sites, to produce intein fusion protein at N-terminal. The expression vectors, pET26-*ada*, pET28-*ada* and pTYB12-*ada* were transformed into *E. coli* DH5α and isolated by plasmid isolation kit (Fermatus).

11. Over-expression of the Putative ADA of *S. antibioticus*

To over-express the protein, pET26-*ada*, pET28-*ada* and pTYB12-*ada* constructs were transformed into BL21 (DE3) and platted on LB containing antibiotics: kanamycin for pET26-*ada* and pET28-*ada*, and ampicillin for pTYB12-*ada*. The obtained colonies carrying pET26-*ada* or pET28-*ada* was grown in 1 L of

LB medium supplemented with kanamycin at 37ºC. At OD_{600} nm of 0.5, the expression of the putative adenosine deaminase was induced by addition of 1 mM IPTG. Similarly, *E. coli* BL21 (DE3) carrying pTYB12-*ada* was grown in 1 L of LB medium supplemented with ampicillin at 37ºC. At OD_{600} nm of 0.5, fusion protein was induced by addition of 1 mM IPTG and grown at 16ºC. After 0, 1, 2, 3, 4 and 5 hours of induction, cells were harvested and the induced level of adenosine deaminase was determined on 15% SDS-PAGE electrophoresis.

12. Refolding and Purification of the Adenosine Deaminase from *S. antibioticus*

BL21 (DE3) carrying pET28-*ada* was grown in 1 L of LB supplemented with kanamycin. After 5 additional hours of induction with 1 mM IPTG at OD_{600} nm of 0.5, cells were harvested by centrifugation at 5,000 rpm for 15 min at 4ºC and suspended in lysis buffer [20 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM EDTA, 0.5% Tween 20, and 1 mM PMSF]. After breaking cell by sonication, the insoluble proteins (inclusion bodies, IBs) were washed with washing buffer [20 mM Tris-HCl (pH 8), 150 mM NaCl, 10% glycerol, and 1% Triton X-100] three times to remove the contaminant proteins. After stirring IBs on ices for 20 min, washed IBs were collected by centrifugation at 12,000 rpm for 30 min at 4ºC. To extract the protein from insoluble fraction, the extraction buffer [50 mM potassium phosphate buffer, 1% Triton X-100, 2 M urea (pH11)] was added at the ratio of 1 ml extraction buffer/1 g cell. After extraction by stirring on ice for 2 hours, the insoluble proteins were removed from supernatant by centrifugation at 10,000 rpm. After centrifugation, the IBs were extracted again with buffer S (50 mM Tris-HCl (pH 8), 1 mM EDTA, 10 mM β-mercaptoethanol, 8 M urea, and 0.5% TritonX-100) to calculate the total amount of protein. The supernatant fraction was dialyzed against 200 ml of 50 mM potassium phosphate buffer containing 0.5 M Urea and 50 mM glucose (pH 10.8). The 50 mM potassium phosphate (pH 7.25) was added with volume of 200 ml for 2 times. The buffer of dialyzed protein was drained in a half of volume (~300 ml). Again the 50 mM potassium phosphate (pH 7.25) was added with volume of 200 ml for 2 times. The added buffer was performed every 30 min. The aggregated proteins were removed by centrifugation at 12,000 rpm for 30 min at 4ºC. For purification of

putative adenosine deaminase from pET28-*ada*, the supernatant was added onto Ni^{2+}-NTA affinity column, which had been previously equilibrated with buffer B [20 mM Tris-HCl (pH 8), 500 mM KCl, and 0.1% Triton X-100]. After washing the column with 5 ml of buffer B, the polyhistidine-tagged enzyme was eluted by buffer B containing 20, 100, and 250 mM imidazole, respectively. The eluted enzyme was analyzed on 15% SDS-PAGE electrophoresis. Protein concentration was determined by the Bradford method, using bovine serum albumin as standard (Bradford, 1976).

13. Assay of Adenosine Deaminase Activity

The activity of purified adenosine deaminase was assayed by the decrease in absorbance at 265 nm on conversion of adenosine to inosine with spectrophotometer. One milliliter reaction containing 0.04-0.08 μmole of adenosine in 50 mM potassium phosphate (pH 7) was incubated for 1 min after the addition of enzyme. The amount of product formed was calculated using molar extinction coefficient 8.3 mM^{-1} (Cory and Suhadolnik, 1965). For determination of ara-A, 2'-deoxyadenosine, adenine, AMP, ADP, ATP, the same wavelength and molar extinction coefficient (8.3 mM^{-1}) was used.

RESULTS AND DISCUSSION

Results

Adenosine deaminase-II (ADA-II) had been proposed as the key enzyme that confers *S. antibioticus* resistant to the endogenous 2'-dCF during biosynthesis of this nucleoside antibiotic at the stationary phase of cell growth. Thus, cloning of the *ada*-II gene may allow one to obtain the genes involved in the biosynthesis of 2'-dCF in *S. antibioticus*. Previous attempt to clone the *ada*-II gene by functional complementation had been unsuccessful (Charopagorn, 2004). Therefore, PCR methodology was used in this thesis to obtain the complete ORF of the *ada* gene. Determination of the nucleotide sequence of *ada* gene is divided into three parts (Figure 10).

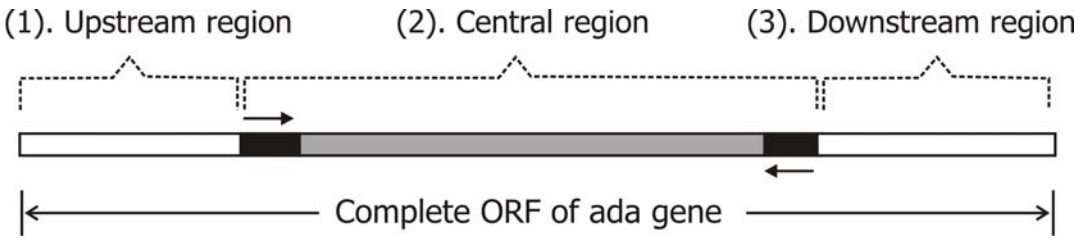


Figure 10 Outline of the strategy of determining the complete open reading frame of the *ada* gene. Diagram showing the complete ORF of *ada* gene is divided into the central region, 5'-upstream region, and 3'-downstream region. The central region of the gene can be obtained by PCR amplification of the *S. antibioticus* chromosomal DNA, using two specific primers (arrows) that complementary to the two highly conserved region (black square) of *ada* gene obtained by multiple alignment. Note that with this approach, either *ada*-I or *ada*-II gene may be obtained.

In the first part, the central region of the *ada* gene was amplified from the chromosomal DNA, using two specific primers. These primers were designed based on the two highly conserved regions of *ada* gene obtained by alignment of the amino acid sequences of ADA from various bacteria. In the second part, the downstream

region of the *ada* gene was determined by the IPCR. In the third part, the upstream region of the *ada* gene was obtained by SP-PCR. The complete ORF of the *ada* gene is finally obtained by combining the nucleotide sequence obtained by the three different PCR methodologies.

1. Determination of the *ada* Gene from *S. antibioticus* Using PCR Approaches

1.1 Determination of the central region of the putative *ada* gene from *S. antibioticus* DNA using normal PCR

PCR approach was used to amplify and determine the central region of the *ada* gene from *Streptomyces. antibioticus*. This approach requires two primers that can be hybridized specifically to the *ada* gene. Therefore, two specific degenerated primers (namely Aui 3 and Aui 4) were synthesized and used in the PCR amplification. The nucleotide sequences of these primers were designed based on the highly conserved regions of the ADA obtained through multiple amino acid alignments. From those of *S. avermitilis* MA-4680 (NP826083), *S. virginiae* (BAA09298), *S. coelicolor* A3(2) (NP626784), *S. coelicolor* A3(2) (NP628805), *Pseudomonas fluorescens* PfO-1 (YP346404), and *Bordetella parapertussis* (NP883359) deposited in the GeneBank database (Figure 6). After multiple alignments, degenerated forward primer (Aui3; 5'-AGGCSSASCTSCACCTSCAC-3') and reverse primer (Aui4; 5'-CGSASGCCGTGG YCGATSCG-3') were synthesized as shown in Table 1.

PCR amplification of the *S. antibioticus* chromosomal DNA with primers, Aui 3 and Aui 4, resulted in single band of 657 bp in length (Figure 11). This fragment was purified from the gel and its nucleotide sequence was determined. The nucleotide sequence of this 657 bp PCR fragment as shown in Figure 12 is 85% identical to those of the adenosine deaminase gene from *Streptomyces* sp., based on tblastx searches of the GeneBank. It should be noted that there is one *Bam*HI restriction site located within the central region of the *ada* gene (underline, Figure 12).

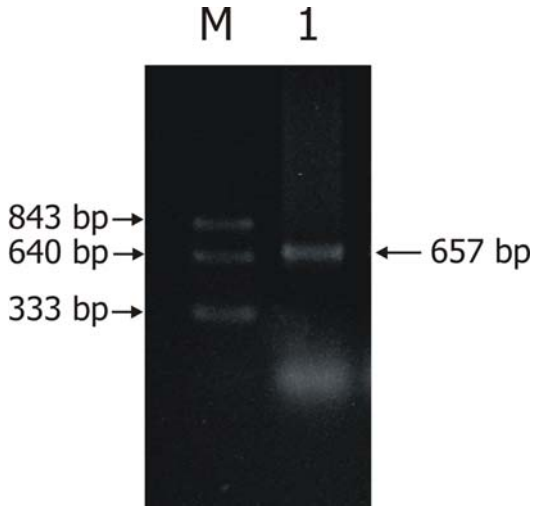


Figure 11 Analysis of the product from PCR amplification of the *ada* gene. Lane M, marker; lane 1, DNA product from amplification of the *ada* gene using Aui3 and Aui4 as primers.

```
GCCCATCTGCACCTGCACTTCACCGGGTCGATGCGACCAGGGACCGTGCTGGAGCTGGCCGACAAGTACGGC  72
 A  H  L  H  L  H  F  T  G  S  M  R  P  G  T  V  L  E  L  A  D  K  Y  G
GTACGGCTGCCCGACACGCTGCGGGACTCCCTCGTCAGCGGGGATCCGCCCCGGCTGCGCGCCACGGACGAG 144
 V  R  L  P  D  T  L  R  D  S  L  V  S  G  D  P  P  R  L  R  A  T  D  E
CGGGGCTGGTTCCGTTTCCAGCGGCTGTACGACGCGGCCCGCTCCTGCCTGCGTACCCCGGAGGACATCCAG 216
 R  G  W  F  R  F  Q  R  L  Y  D  A  A  R  S  C  L  R  T  P  E  D  I  Q
CGCCTGGTCCGGGAGGCCGCGGAGGAGGACCTGAGGGACGGCTCGGGCTGGCTGGAGATCCAGGTGGACCCG 288
 R  L  V  R  E  A  A  E  E  D  L  R  D  G  S  G  W  L  E  I  Q  V  D  P
ACGTCGTACGCGCCCCGGCTGGGCGGTCTGATCCCGGCGCTGGAGGTCATCCTGGACGCGGTCGACACGGCC 360
 T  S  Y  A  P  R  L  G  G  L  I  P  A  L  E  V  I  L  D  A  V  D  T  A
TCCCGGGACACCGGCCTCGGCATGCGCGTCCTGGTCGCCGCGAACCGCATGAAACACCCGCTGGACGCGCGC 432
 S  R  D  T  G  L  G  M  R  V  L  V  A  A  N  R  M  K  H  P  L  D  A  R
ACGCTGGCCCGGCTGGCGGTGCGCTACGCAGACCGGGGCGTGGTCGGCTTCGGGCTCTCCAACGACGAGCGG 504
 T  L  A  R  L  A  V  R  Y  A  D  R  G  V  V  G  F  G  L  S  N  D  E  R
CGGGGCCTGGCGCGGGACTTCGACCGGGCCTTCCACATCGCCCGCGAGGCGGGGCTGCTGTCGGCTCCGCAC 576
 R  G  L  A  R  D  F  D  R  A  F  H  I  A  R  E  A  G  L  L  S  A  P  H
GGCGGCGAGCTGGCCGGCCCGGCGTCGGTCCGGGACTGCCTGGACGATCTGCACGCCTCCCGGATCGGGCAC 648
 G  G  E  L  A  G  P  A  S  V  R  D  C  L  D  D  L  H  A  S  R  I  G  H
GGGGTGCGG                                                                 657
 G  V  R
```

Figure 12 The nucleotide sequence of 657 bp fragment from PCR amplification of the central region of the *ada* gene from *S. antibioticus*. Restriction site *Bam*HI is underline.

1.2 Identification of restriction sites within central region of the *ada* gene

To determine whether any unique restriction site is located in this central region of the *ada* gene, the PCR product of 657 bp was digested with restriction enzymes including *Xho*I, *Psh*AI, *Hind*III, and *Bam*HI. After incubation for 3 hours, the reaction mixture was analyzed on 1 % agarose gel (Figure 13). The result showed that *Bam*HI can cut once within the *ada* gene, generating two fragments of approximately 543 bp and 114 bp. This result indicated that the *Bam*HI restriction site is located within the *ada* gene. On the other hand, restriction enzymes *Xho*I, *Psh*AI and *Hind*III did not appear to cut within the *ada* gene. This result can be supported to determine the downstream region of the *ada* gene by the IPCR methodology.

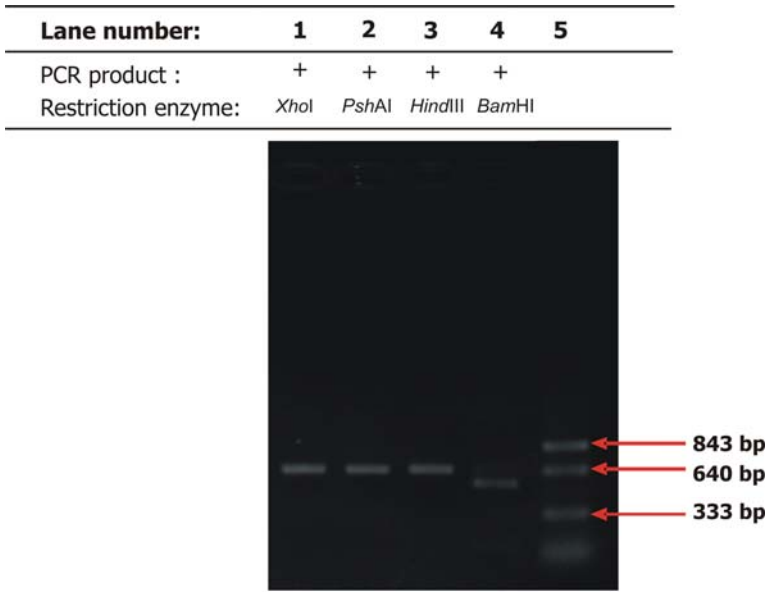


Figure 13 Identification of restrictions sites within central region of the *ada* gene in the PCR product. Lane 1-4: PCR product digested with *XhoI*, *Psh*AI, *Hind*III, and *Bam*HI as indicated; Lane 5, DNA markers. *Bam*HI can cut once within the *ada* gene, generating two fragments of 543 bp and 114 bp.

1.3 Partial digestion of *S. antibioticus* DNA

Once the sequence of the central region of the *ada* gene is known, the 3' downstream region from the gene was determined by the IPCR. High molecular

weight chromosomal DNA (~ 1mg) was isolated from two grams of *S. antibioticus* cells and digested into smaller fragments with various restriction enzymes. Because previous result showed that *Bam*HI can cleave within the *ada* gene, therefore this enzyme as well as *Xho*I and *Psh*AI were used that are not cleave within the *ada* gene. These DNA fragments were circularized by incubation with T_4 DNA ligase. The circular DNAs obtained were subsequently used as template in IPCR. The size of the circular DNA used as template for IPCR should be no longer than 3 kb (Ochman *et al*., 1988). This is because *Taq* DNA polymerase can not amplify DNA template that is larger than 3-5 kb in length. Thus high molecular weight chromosomal DNA was partially digested with several restriction enzymes into 3-5 kb which can be used for preparation of suitable circular DNA. *S. antibioticus* DNA fragments was digested with *Xho*I, *Psh*AI, and *Bam*HI (Figure 14). After 3 hours of digestion, the result showed that *Bam*HI is the best enzyme for digesting chromosomal DNA into suitable fragments (1-20 kb) comparing to other enzymes.

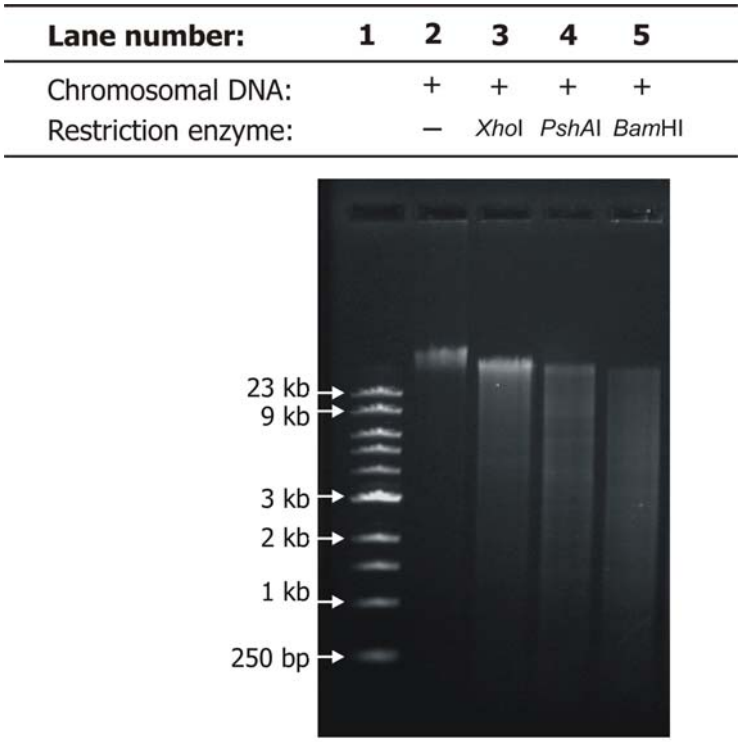


Figure 14 Partial digestion of *S. antibioticus* chromosomal DNA. Lane 1, marker; lane 2, chromosomal DNA of *S. antibioticus*; lane 3-5, digested DNA with *Xho*I, *Psh*AI, and *Bam*HI, respectively, for 3 hours at 37ºC.

1.4 Amplification of 3'-downstream region of *ada* gene using IPCR

After digestion with restriction enzyme *Xho*I, *Psh*AI, and *Bam*HI, the digested DNAs from *S. antibioticus* were clean up by gel extraction kit, circularized with T_4 DNA ligase, and used as template for IPCR. When the *Bam*HI digested DNA was self-ligated with T_4 DNA ligase, DNA fragments containing 5'-upstream and 3'-downstream regions of *ada* gene appeared into separated circle (Figure 6). In contrast, when *Xho*I or *Psh*AI-digested DNAs were circularized with T_4 DNA ligase, both 5'-upstream and 3'-downstream regions of *ada* gene were appeared into one circular DNA (Ochman *et al*., 1988). Based on the known nucleotide sequence of the central region of the *ada* gene, specific inverse primers were synthesized as showed in Figure 6 and Table 1. The designed primers, InF1 and InF2, were used as inverse primers in the first round IPCR, using circular DNA as template. Regardless of the origin of circular DNA that used as template, first round IPCR amplification resulted in several bands less than 700 bp in length (Figure 15). However, when *Xho*I-digested circular DNAs were used as template, PCR amplification showed weak band of ~1.5 kb in size (lane 1). When *Bam*HI-digested circular DNAs were used as template, two bands approximately 1.4 and 1.8 kb in size were observed (lane 2). However, when *Psh*AI-digested circular DNAs were used as template, all PCR products obtained appeared to be smaller than 700 bp (lane 3). When the undigested genomic DNA was used as template (the control) to determine if non-specific amplification occurred, one 2 kb, one 1.4 kb and several fragment that is smaller than 700 bp were observed (lane 4). These results indicated that 1.4 kb observed in the amplfication using *Bam*HI-digested circular DNA is probably a non-specific PCR product. Thus, the 1.5 kb and 1.8 kb PCR products obtained using *Xho*I- and *Bam*HI-circular DNA as the template were used in the subsequent experiment.

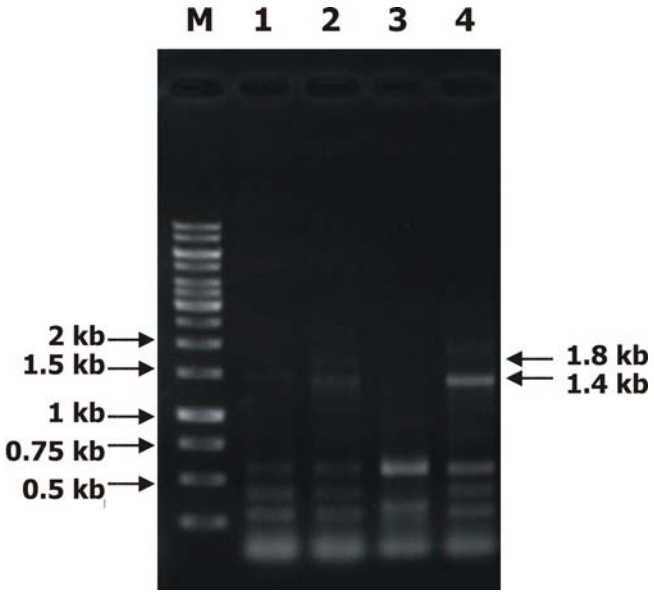


Figure 15 Analysis of first round IPCR product on 1% agarose gel electrophoresis. M, Marker; lane 1-3, circle DNA of *Xho*I, *Bam*HI, and *Psh*AI-digested fragments as the template, respectively; lane 4, undigested genomic DNA of *S. antibioticus* used as the control.

To confirm which PCR product, 1.5 kb or 1.8 kb, containing the *ada* gene, second round of IPCR (nested-IPCR) was performed. In the second round IPCR, InR1 and InR2 were used as the inverse primers and the PCR products obtained from the first round IPCR were used as DNA template. These primers were designed specifically for *ada* sequence. The result of nested-IPCR was analyzed. From second round of IPCR, the 1.5 kb fragment was obtained when *Bam*HI-circular DNA were used as the template (Figure 16A). In contrast, there is no PCR product obtained from the sample of *Xho*I-, and *Psh*AI-circular DNA as template (data not shown). Therefore, the 1.5 kb fragment from second round IPCR product was cloned and sequenced. The schematic of IPCR amplification was elucidated in Figure 16B.

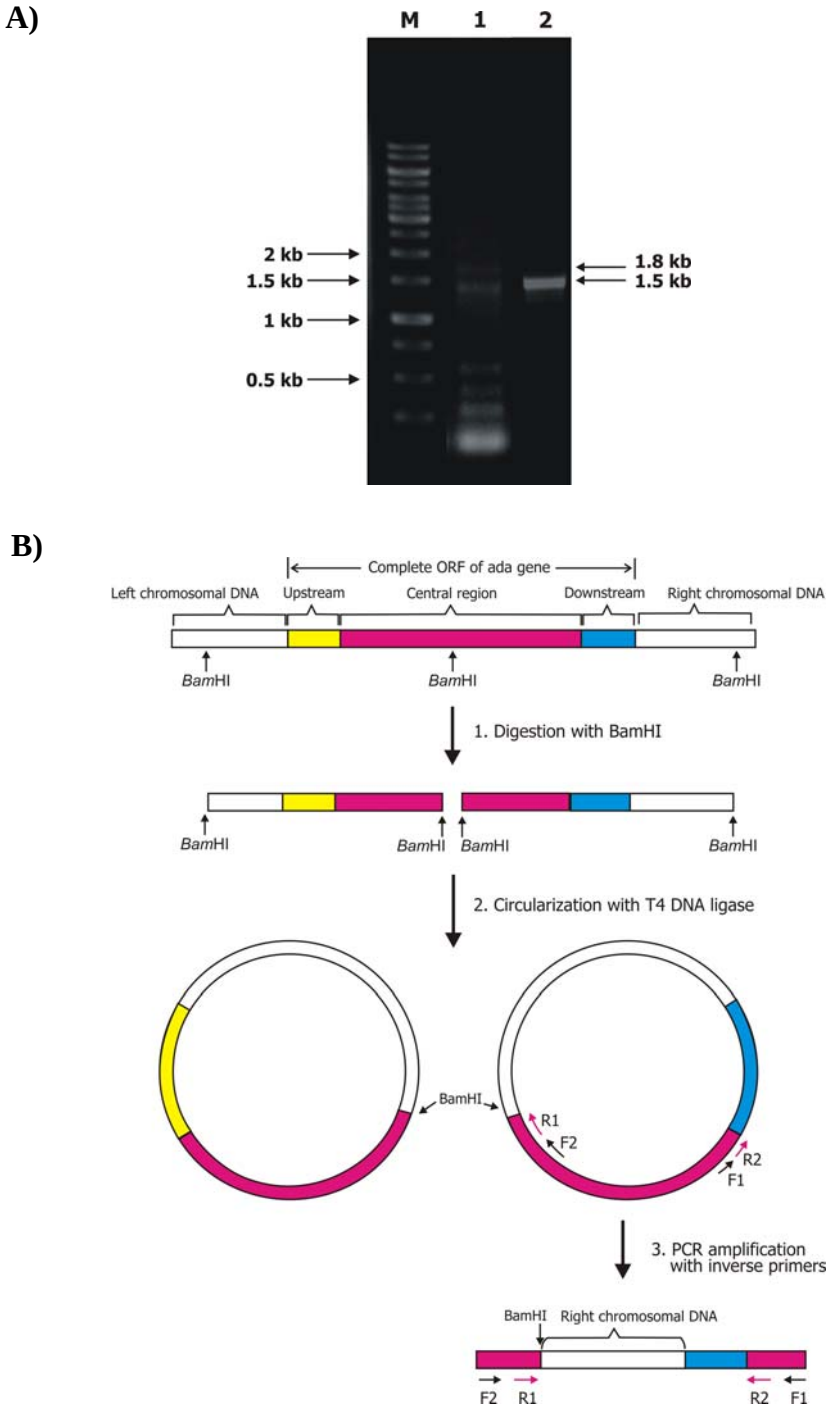


Figure 16 Analysis of product from IPCR using *Bam*HI-digested circular DNAs as template on 1% agarose gel electrophoresis. Fragment contained downstream region from the central *ada* gene and right chromosomal DNA region of *S. antibioticus*. (A) Lane M, marker ; lane 1, first round IPCR product; and lane 2, nested-IPCR product. (B) The schematic of IPCR; (1) after ligation of *Bam*HI-digested DNA, the circular DNA was used as the template in first round IPCR using InF1 and InF2 as primers; (2) the PCR product was used as the template in nested-IPCR using InR1 and InR2 as primers.

The nucleotide sequence of 1.5 kb fragment from SP6 universal primer direction contained the downstream region of *ada* gene (414 bp) and right chromosomal DNA region of *ada* gene (10 bp) (Figure 17). In addition, the sequence from T7 promoter primer direction showed the part of central *ada* gene (77 bp) and right chromosomal DNA region (575 bp) which is a partial part of probably transcriptional regulator (TetR family) with 74% identity to that of *S. griseus* subsp. *griseus* (BAG19708.1). The TetR family is a common class of transcriptional regulator which is a "repressor" of the gene transcription including antibiotic production, efflux pumps, osmotic stress, amino acid metabolism, and development of morphology (Hillerich and Westpheling, 2008).

1.5 Amplification of 5'-upstream region of *ada* gene using SP-PCR

Once the central region and downstream region of *ada* gene from *S. antibioticus* has been obtained by PCR and IPCR, the next step is to obtain the upstream region of the *ada* gene using single primer polymerase chain reaction (SP-PCR). In principle, one can also use IPCR to determine the upstream region of the *ada* gene. However, the attempt to obtain upstream region by IPCR methodology was unsuccessful. Thus, the single primer polymerase chain reaction (SP-PCR) was performed to obtain the upstream region of *ada* gene. The advantage of SP-PCR amplification is that it used only one primer that acts as both forward and reverse primers. In our experiment, seven primers (Aui3, Aui4, InF1, InF2, InF3, InR1, and InR2) were designed from *ada* gene and used independently in first round SP-PCR. At 63°C of annealing, the PCR products were obtained (Figure 18A). The PCR products using independently primers of InF1, InF2, Aui3, Aui4, and InR1 (lane 1-5) showed several sizes of fragments (250 bp to 4 kb). However, the single band of 2.8 kb fragment was obtained from InF3 amplification.

```
GGCCCGGCGTCGGTCCGGGACTGCCTGGACGATCTGCACGCCTCCCGGATCGGGCACGGGGTGCGGGCCGCG   72
 G  P  A  S  V  R  D  C  L  D  D  L  H  A  S  R  I  G  H  G  V  R  A  A
GAGGACCCGCGGCTGCTGAAGCGGCTGGCCGACCGGGGCGTGACCTGCGAGGTGTGCCCGGCCTCGAACGTG  144
 E  D  P  R  L  L  K  R  L  A  D  R  G  V  T  C  E  V  C  P  A  S  N  V
GCGCTCGGCGTGTACGAGAAGCCGCAGGACGTCCCGCTGCGCACCCTCTTCGAGGCGGGCGTGCCGATGGCG  216
 A  L  G  V  Y  E  K  P  Q  D  V  P  L  R  T  L  F  E  A  G  V  P  M  A
CTGGGCGCGGACGACCCGCTGCTGTTCGGCTCGCGGCTGGCCGCGCAGTACGAGATCGCCCGGCACGCCCAC  288
 L  G  A  D  D  P  L  L  F  G  S  R  L  A  A  Q  Y  E  I  A  R  H  A  H
GGCTTCTCGGACGAGGCGCTAGCGGAGCTGGCCCGGCAGTCGGTGCGGGGCTCGGCCGCGCCGAAGGAGGTC  360
 G  F  S  D  A  E  L  A  E  L  A  R  Q  S  V  R  G  S  A  A  P  K  E  V
AAGGCGCGGCTGCTGGCGGGCGTGGACGACTGGCTGGCCCGTCCGGCCGCCTAGTCCTGTCGTC……      424
 K  A  R  L  L  A  G  V  D  D  W  L  A  R  P  A  A  *
…(~500bp)……GCAGGGTGTCGTCGTGGCGCCGTTTGAGCTGGGTCTCGGCGTACAGGGACGCGGCGATCGG  909
            L  T  D  D  H  R  R  K  L  Q  T  E  A  Y  L  S  A  A  I  P
GAAGCTCTGCTCGTAGAAGAGCGCGGCCTGGCGGGCGATCTCGGTGAGGTTCTCCTCCAGGGTGGCCCGTCC  981
 F  S  Q  E  Y  F  L  A  A  Q  R  A  I  E  T  L  N  E  E  L  T  A  R  G
CGGTGCGGCGGAGAGGCTGTCGAGCAGCGGGGTGAGCCGGGGCAGCCGCTCGGTGAGGACCCGTACGAACAG 1053
 P  A  A  S  L  S  D  L  L  P  T  L  R  P  L  R  E  T  L  V  R  V  F  L
CTCTTCCTTGCTGTCGAAGTACTTGTAGAGGGCCGCTTCAGAGCAGCCGGCCGCGCGGGCGATCTCCTTGGT 1125
 E  E  K  S  D  F  Y  K  Y  L  A  A  E  S  C  G  A  A  R  A  I  E  K  T
GGTGGCGCGGGCGAGTCCGACGGTGAGCATCAGCTCGTGGGCGGCCTCGAGGATGCGCACACGCGCGGGCTT 1197
 T  A  R  A  L  G  V  T  L  M  L  E  H  A  A  E  L  I  R  V  R  A  P  K
CGTCTCCACGGGCTCCATGGAACTCCTAATCGGCCTTGACGCATCAGTAAGTGATTACTCACTCTAGGAGGC 1269
                ←TetR
 T  E  V  P  E  M
ACGTGGGTGAGTGAGCACTCACCCACCATGGACGCGCACGGGAATCGGGGAAGATCACCATGAAGCTCACTG 1341

TTTTCGGCGCCACCGGGGGGACCGGCCGTGAACTGGTCCGCCAGGCCCTGGACGCCGGTCACGACGTCACGG 1413

CGGTCGTGCGGGATCCGCCCCGGCTGCGCGCCACGGACGAGCGGGGCTGGTTCCGTTTCCAGCGGCTGTACG 1485

ACGCGGGCCGCTCCA                                                          1500
```

Figure 17 Nucleotide sequence of 1.5 kb fragments obtained from IPCR amplification. It contained part of *ada* gene and downstream region. The symbols in the asterisk, dash, and underline sequence indicated as stop codon (TAG), uncharacterized region (~500 bp) and the putative ribosomal binding site, respectively. The *tet*R coding region is shown as arrow. The *Bam*HI restriction site is indicates as highlight sequence.

Therefore, the second round of SP-PCR amplification, referred to as nested-PCR, was performed to reduce the number of non-specific products. In nested PCR, DNA products from first round PCR were used as the templates along with a suitable pair of primers. The expected size of nested-PCR product from each template was calculated based on *ada* sequence from GeneBank as shown in Table 2. After nested-PCR amplification, the product was analyzed (Figure 18B)

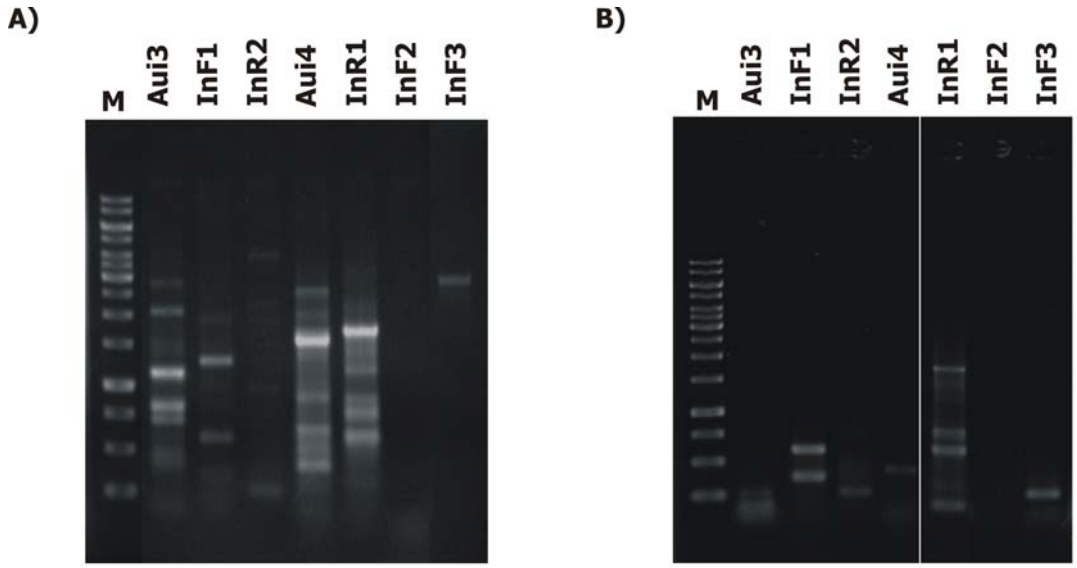


Figure 18 Analysis of PCR product from SP-PCR amplification. (A) first round SP-PCR and (B) nested-PCR. Primers for amplification were described in table 2.

As shown in Figure 18, nested-PCR amplification of the first round SP-PCR products with InF3 resulted in a 250 bp fragment, which is close to the expected size if the template contain *ada* gene (Table 2). In contrast, reactions from other nested-PCR did not give the corresponding size. Although, nested-PCR fragment obtained from Aui4 appeared correctly size of fragment (~ 408 bp) but in the first round of SP-PCR it contained several non-specific fragments and it is time-consuming to clone every fragment. Therefore, the 250 bp from InF3 was cloned and sequenced to confirm that this fragment is part of *ada* gene (Figure 19A). The nucleotide sequence of the 250 bp determined by sequencing was identified to a

region in the *ada* gene. The schematic diagram of SP-PCR amplification is shown Figure 19B.

The nucleotide sequence of 2.8 kb fragment from SP6 universal primer direction contained the part of upstream region of the central *ada* gene (463 bp) and left chromosomal DNA region of *ada* gene (48 bp) (Figure 20). The potential ribosomal binding site, GGAAG, was revealed at 27 to 31 upstream from start codon of the *ada* gene. In addition, the sequence from T7 promoter primer direction showed the left chromosomal DNA region (650 bp) which is a probably transcriptional antitermination protein NusG (Butanolide receptor) and translocase subunit secE with identities 88% and 95% of *S. griseus* subsp. *griseus* (BAG19704.1) and *S. galbus* (CAA65164.1), respectively. NusG protein is one factor required for N-antitermination in the transcription system of *E. coli*. SecE protein is integral membrane protein which is secreting the target protein to the cytoplasm through ATP driving force (Miyake *et al*., 1994).

Table 2 List of primers to amplify in second round of SP-PCR approach.

First round SP-PCR products from	Inner forward primer	Inner reverse primer	Expected size of PCR product (bp)
Aui3	InF1	Aui4	174
Aui4	Aui3	InF3	408
InF1	Aui3	InF1	477
InF3	Aui3	InF2	267
InR1	Aui3	InR2	165
InR2	Aui3	InR2	615

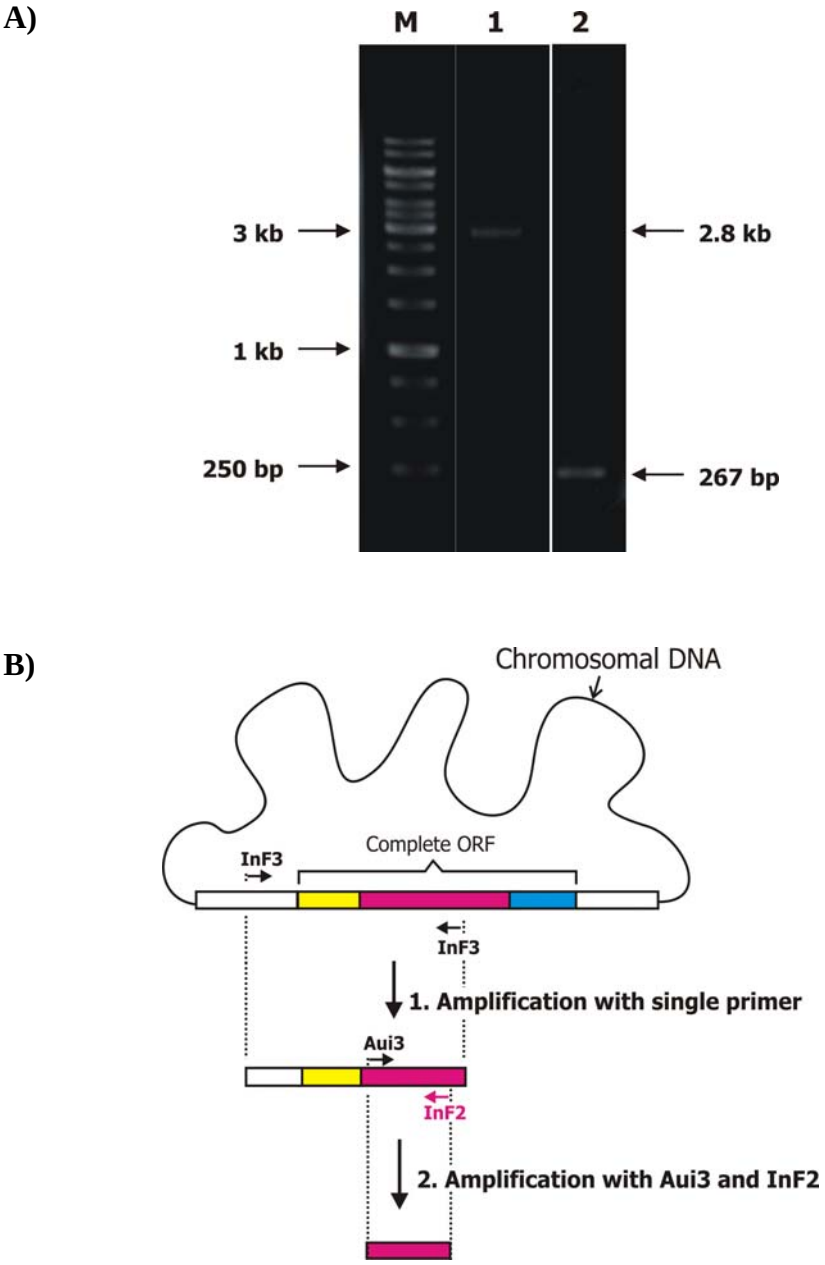


Figure 19 Analysis of SP-PCR product which containing left chromosomal DNA and upstream region of putative *ada* gene from *S. antibioticus* on 1% agarose gel electrophoresis; lane M, marker; lane 1, first round SP-PCR product; and lane 2, nested-PCR product (A). The process of SP-PCR; (1) first round SP-PCR amplification, InF3 primer was used to anneal genomic DNA of *S. antibioticus* at random and specific region; (2) the PCR product was used as the template in nested-PCR using Aui3 and InF2 that bind at specific region of *ada* gene as the primers (B).

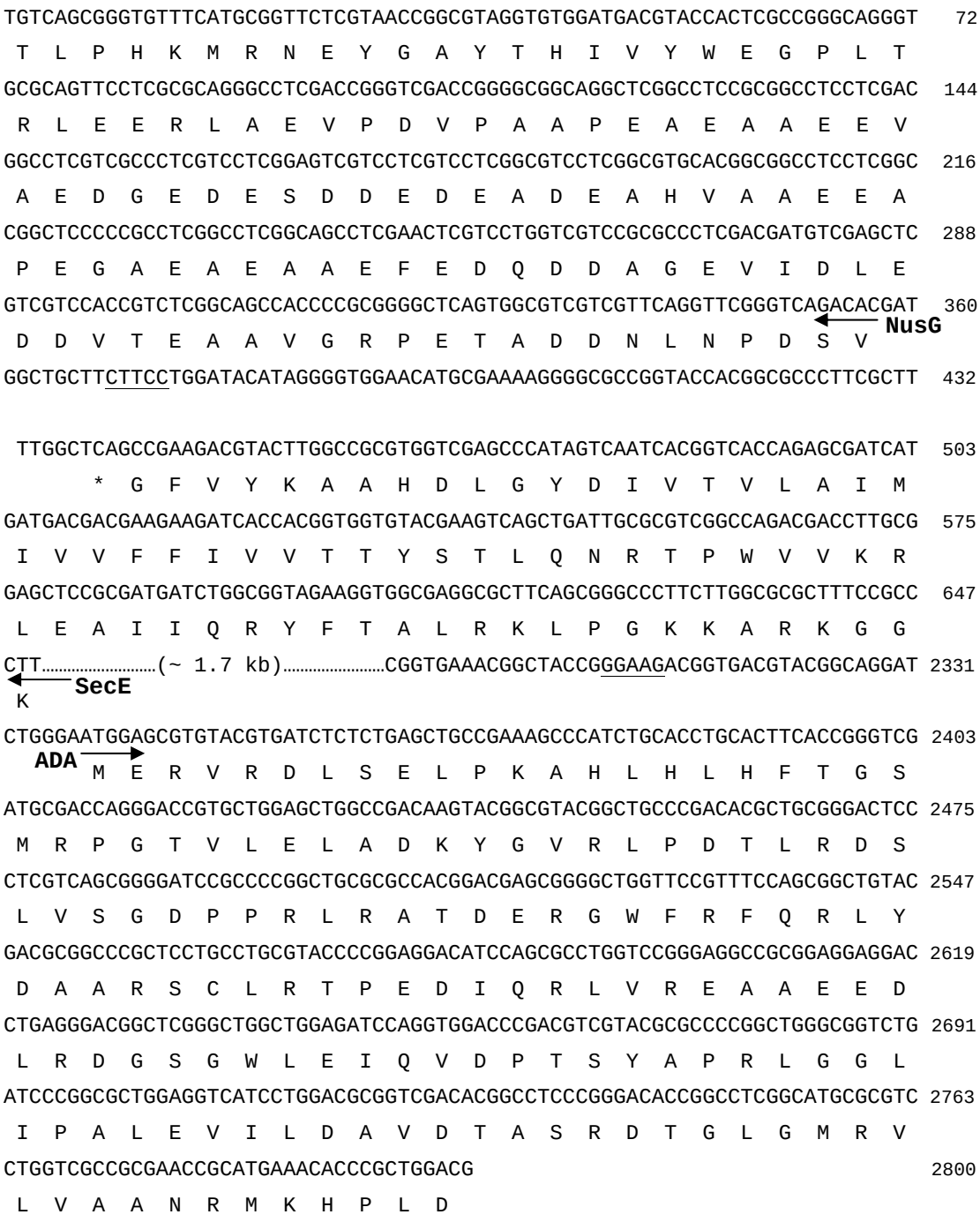


Figure 20 Nucleotide sequence of left chromosomal region and upstream region of *ada* gene from SP-PCR. This sequence contained left chromosomal DNA, uncharacterized region approximate 1.7 kb (dash) and upstream region of *ada* gene. The underline sequence is a putative ribosome binding site (rbs). The coding regions of *nus*G, *sec*E, and *ada* are shown as arrows.

1.6 Insertion of the PCR product of IPCR and SP-PCR into pGEM-T

The obtained PCR products of 1.5 kb and 2.8 kb fragments which obtained from IPCR and SP-PCR amplification, respectively, were ligated separately into the cloning vector pGEM-T. The reaction mixtures were transformed into *E. coli* DH5α. After blue-white selection, one white colony containing of recombinant pGEM-IPCR and of recombinant pGEM-SPPCR was grown to isolate recombinant plasmid DNA. Both of recombinant plasmids were isolated and digested with *Nde*I (Figure 21). The result showed that the 1.5 kb and 2.8 kb fragments had been successfully inserted into pGEM-T. The size of recombinant pGEM-IPCR was 4.5 kb (lane 3), and of recombinant pGEM-SPPCR was 5.8 kb (lane 6) which corresponds to the size of pGEM-T (3 kb) containing the insert 1.5 kb (lane 4) and 2.8 kb (lane 5) fragments. Both of recombinant DNA was sequenced as shown above in Figure 17 and 20.

Lane number:	1	2	3	4	5	6	7
PGEM-IPCR DNA:		+	+	−	−	−	−
PGEM-SPPCR DNA:		−	−	−	−	+	+
PCR fragment:		−	−	+	+	−	−
NdeI restriction enzyme:		−	+	−	−	+	−

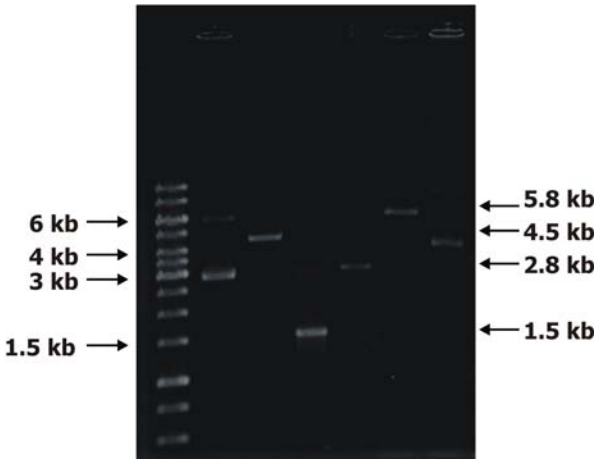


Figure 21 Detection of IPCR and SP-PCR product inserted into pGEM-T. Lane 1, marker; lane 2, pGEM-IPCR no digested; lane 3, pGEM-IPCR digested with *Nde*I; lane 4, 1.5 kb fragment from IPCR product; lane 5, 2.8 kb fragment from SP-PCR product; lane 6, pGEM-SPPCR digested with *Nde*I; lane 7, pGEM-SPPCR no digested.

1.7 Organization and Characteristic of Combined Sequence

The combined nucleotide sequences from normal-PCR, IPCR, and SP-PCR spanned a 4.5 kb DNA fragment (Figure 22). It contains the complete open reading frame (ORF) of putative *ada* gene sequence from *S. antibioticus*. The ORF of this gene is 1041 bp that contains a high G+C content of 74% and encodes for protein with 346 amino acid residues. The calculated molecular weight of this enzyme is 37.8 kDa. The deduced amino acid sequence is similar to ADA from *S. coelicolor* A3(2), *S. avermitilis* MA-4680, and *S. verginiae* with homology of 88%, 87%, and 84%, respectively. The gene starts with 'ATG' codon and terminates at position 1041 with 'TAG'. The potential ribosome-binding site (RBS) or shine-dalgarno sequence (SD), GGAAG, is present 27 to 31 bp upstream from the initiation codon.

In addition, the left chromosomal DNA region of the putative *ada* gene reveals the partial gene of *nus*G and *sec*E which is an antiterminator and a translocase protein, repectively. The right chromosomal DNA region of the putative *ada* gene reveals the partial gene of *Tet*R which is encoding for TetR repressor. These genes involved in the antibiotic biosynthetic genes which are normally presented in gram-positive bacteria (Miyake *et al*., 1994; Ramos *et al*., 2005). The potential ribosome-binding site (RBS) is presented as underlined (Figure 22).

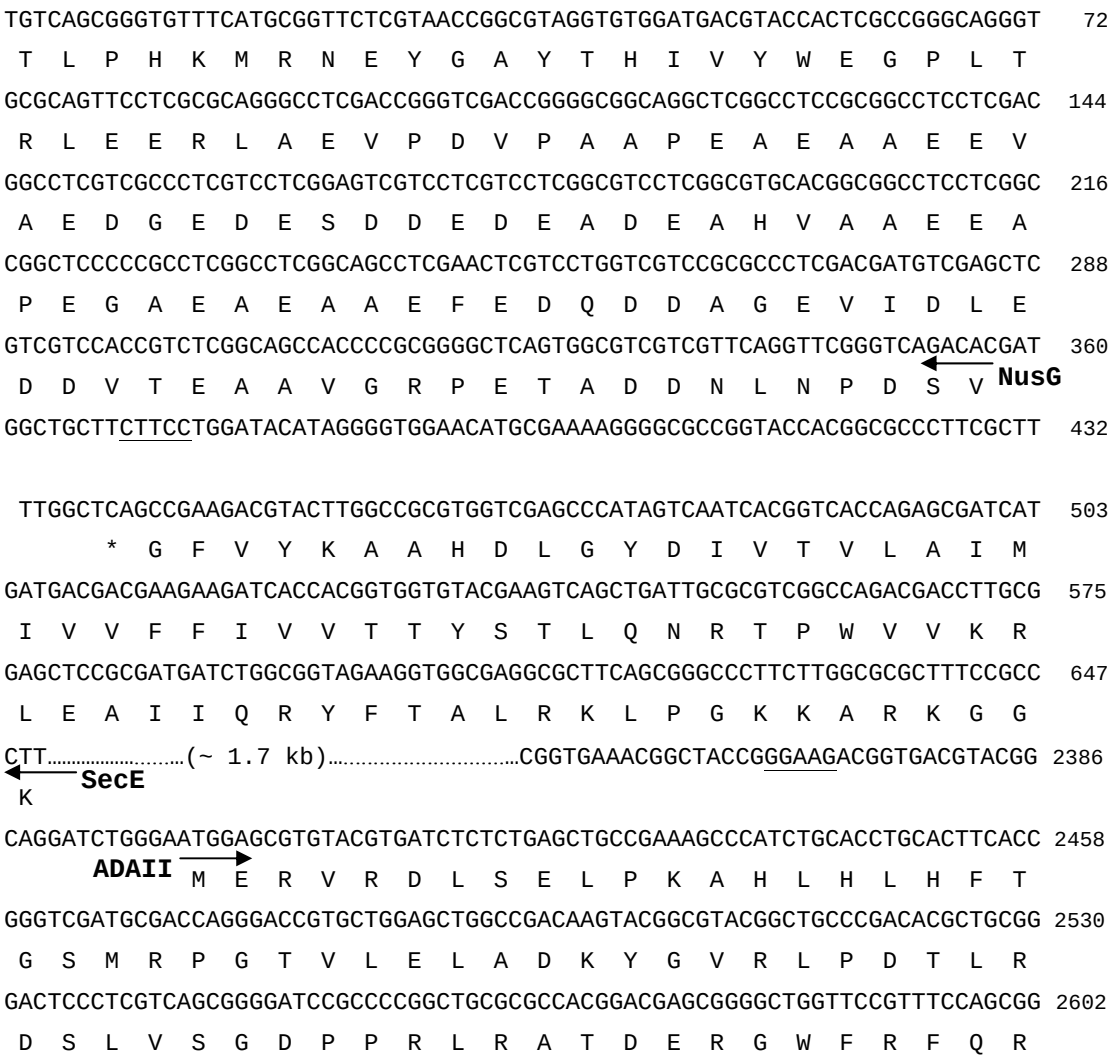


Figure 22 Nucleotide sequences and deduced amino acid sequences of the *nus*G, *sec*E, *ada*, and *tet*R of *S. antibioticus*. The combined nucleotide sequence of normal PCR, IPCR, and SP-PCR spanned the 4.5 kb of DNA fragment. The completed ORF reveals the putative *ada* gene with deduced polypeptide of 346 amino acids residues (~37.8 kDa). Furthermore, the right chromosomal DNA region of the putative *ada* gene contains partial *tet*R gene for encoding repressor protein. The left chromosomal DNA region of the putative *ada* gene contains partial *nus*G and *sec*E gene encoding for antiterminator protein and translocase protein, respectively. The underlines denote the putative ribosomal binding site (RBS). The asterisks and dashs indicated stop codon and uncharacterized regions.

```
CTGTACGACGCGGCCCGCTCCTGCCTGCGTACCCCGGAGGACATCCAGCGCCTGGTCCGGGAGGCCGCGGAG 2674
 L  Y  D  A  A  R  S  C  L  R  T  P  E  D  I  Q  R  L  V  R  E  A  A  E
GAGGACCTGAGGGACGGCTCGGGCTGGCTGGAGATCCAGGTGGACCCGACGTCGTACGCGCCCCGGCTGGGC 2746
 E  D  L  R  D  G  S  G  W  L  E  I  Q  V  D  P  T  S  Y  A  P  R  L  G
GGTCTGATCCCGGCGCTGGAGGTCATCCTGGACGCGGTCGACACGGCCTCCCGGGACACCGGCCTCGGCATG 2818
 G  L  I  P  A  L  E  V  I  L  D  A  V  D  T  A  S  R  D  T  G  L  G  M
CGCGTCCTGGTCGCCGCGAACCGCATGAAACACCCGCTGGACGCGCGCACGCTGGCCCGGCTGGCGGTGCGC 2890
 R  V  L  V  A  A  N  R  M  K  H  P  L  D  A  R  T  L  A  R  L  A  V  R
TACGCAGACCGGGGCGTGGTCGGCTTCGGGCTCTCCAACGACGAGCGGCGGGGCCTGGCGCGGGACTTCGAC 2962
 Y  A  D  R  G  V  V  G  F  G  L  S  N  D  E  R  R  G  L  A  R  D  F  D
CGGGCCTTCCACATCGCCCGCGAGGCGGGGCTGCTGTCGGCTCCGCACGGCGGCGAGCTGGCCGGCCCGGCG 3034
 R  A  F  H  I  A  R  E  A  G  L  L  S  A  P  H  G  G  E  L  A  G  P  A
TCGGTCCGGGACTGCCTGGACGATCTGCACGCCTCCCGGATCGGGCACGGGGTGCGGGCCGCGGAGGACCCG 3106
 S  V  R  D  C  L  D  D  L  H  A  S  R  I  G  H  G  V  R  A  A  E  D  P
CGGCTGCTGAAGCGGCTGGCCGACCGGGGCGTGACCTGCGAGGTGTGCCCGGCCTCGAACGTGGCGCTCGGC 3178
 R  L  L  K  R  L  A  D  R  G  V  T  C  E  V  C  P  A  S  N  V  A  L  G
GTGTACGAGAAGCCGCAGGACGTCCCGCTGCGCACCCTCTTCGAGGCGGGCGTGCCGATGGCGCTGGGCGCG 3250
 V  Y  E  K  P  Q  D  V  P  L  R  T  L  F  E  A  G  V  P  M  A  L  G  A
GACGACCCGCTGCTGTTCGGCTCGCGGCTGGCCGCGCAGTACGAGATCGCCCGGCACGCCCACGGCTTCTCG 3322
 D  D  P  L  L  F  G  S  R  L  A  A  Q  Y  E  I  A  R  H  A  H  G  F  S
GACGAGGCGCTAGCGGAGCTGGCCCGGCAGTCGGTGCGGGGCTCGGCCGCGCCGAAGGAGGTCAAGGCGCGG 3394
 D  A  E  L  A  E  L  A  R  Q  S  V  R  G  S  A  A  P  K  E  V  K  A  R
CTGCTGGCGGGCGTGGACGACTGGCTGGCCCGTCCGGCCGCCTAGTCCTGTCGTC……(~ 500 bp)……G 3950
 L  L  A  G  V  D  D  W  L  A  R  P  A  A  *
CAGGGTGTCGTCGTGGCGCCGTTTGAGCTGGGTCTCGGCGTACAGGGACGCGGCGATCGGGAAGCTCTGCTC 4022
 L  T  D  D  H  R  R  K  L  Q  T  E  A  Y  L  S  A  A  I  P  F  S  Q  E
GTAGAAGAGCGCGGCCTGGCGGGCGATCTCGGTGAGGTTCTCCTCCAGGGTGGCCCGTCCCGGTGCGGCGGA 4094
 Y  F  L  A  A  Q  R  A  I  E  T  L  N  E  E  L  T  A  R  G  P  A  A  S
GAGGCTGTCGAGCAGCGGGGTGAGCCGGGGCAGCCGCTCGGTGAGGACCCGTACGAACAGCTCTTCCTTGCT 4166
 L  S  D  L  L  P  T  L  R  P  L  R  E  T  L  V  R  V  F  L  E  E  K  S
GTCGAAGTACTTGTAGAGGGCCGCTTCAGAGCAGCCGGCCGCGCGGGCGATCTCCTTGGTGGTGGCGCGGGC 4238
 D  F  Y  K  Y  L  A  A  E  S  C  G  A  A  R  A  I  E  K  T  T  A  R  A
GAGTCCGACGGTGAGCATCAGCTCGTGGGCGGCCTCGAGGATGCGCACACGCGCGGGCTTCGTCTCCACGGG 4310
 L  G  V  T  L  M  L  E  H  A  A  E  L  I  R  V  R  A  P  K  T  E  V  P
CTCCATGGAACTCCTAATCGGCCTTGACGCATCAGTAAGTGATTACTCACTCTAGGAGGCACGTGGGTGAGT 4382
 ◄──TetR
 E  M
GAGCACTCACCCACCATGGACGCGCACGGGAATCGGGGAAGATCACCATGAAGCTCACTGTTTTCGGCGCCA 4454

CCGGGGGGACCGGCCGTGAACTGGTCCGCCAGGCCCTGGACGCCGGTCACGACGTCACGGCGGTCGTGCG   4524
```

Figure 22 (Continued)

1.8 Cloning of the putative *ada* gene from *S. antibioticus*

1.8.1 Amplification of the full length *ada* gene

To express the protein, the full length of putative *ada* gene was amplified by PCR using *S. antibioticus* chromosomal DNA as template. Two specific primers were designed to ensure that a complete nucleotide sequence of *ada* gene was amplified by the PCR reaction. Forward primer (ADA F) was designed to contain an added-on *Nde*I site whereas the reverse primer (ADA R) was designed to contain both a stop codon and an added-on *Bgl*II site. The amplified PCR product was analyzed on 1% agarose gel and purified by nucleospin column. Approximately 0.2 µg/µl of the PCR fragment of 1050 bp was obtained (Figure 23).

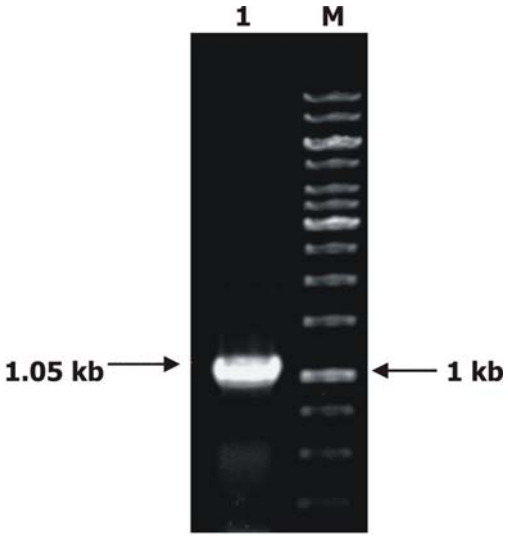


Figure 23 PCR amplification of the putative *ada* gene from *S. antibioticus*. The PCR reaction was performed using chromosomal DNA of *S. antibioticus* as the template and specific ADA F and ADA R as primers. Lane 1, specific PCR product; lane M, DNA ladder.

1.8.2 Insertion of the amplified *ada* gene into pGEM-T cloning vector

Because PCR product contains the added on restriction sites at both ends have been reported to digest inefficiently by the restriction enzymes, thus the amplified *ada* gene was first cloned into pGEM-T easy vector. After ligation, the reaction mixtures were transformed into *E. coli* DH5α and one positive colony was selected and analyzed for the presence of pGEM-*ada*, the recombinant plasmid carrying the *ada* gene. This clone was grown in LB supplemented with ampicillin and its recombinant plasmid was isolated and analyzed for the presence of *ada* gene by restriction digestions (Figure 24). When isolated plasmid was double digested with *Nde*I and *Bgl*II, a 1050 bp was detected. The size of recombinant pGEM-*ada* was approximately 4 kb (lane 5), which corresponds to the size of pGEM-T (3 kb) plus the insert fragment of 1.05 kb (lane 6). The pBluescript (3 kb) was used as the control to compare the size of recombinant plasmid. This result indicated that the *ada* gene had been successfully inserted into pGEM-T.

Lane number:	1	2	3	4	5	6	7
PBluescript DNA:		+	+	–	–	–	–
PGEM-ada DNA:		–	–	+	+	+	–
PCR fragment:		–	–	–	–	–	+
NdeI restriction enzyme:		–	+	–	+	+	–
BglII restriction enzyme:		–	–	–	–	+	–

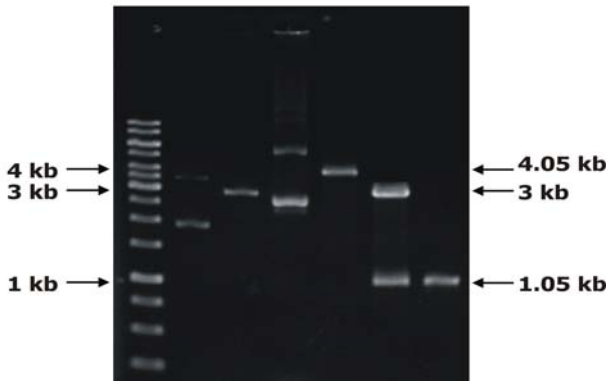


Figure 24 Analysis of pGEM-*ada*. Lane 1, 1 kb ladder; lane 2, supercoiled pBluescript; lane 3, linear pBluescript by digestion with *Nde*I (3 kb); lane 4, supercoiled pGEM-*ada*; lane 5, linear pGEM-*ada* by digestion with *Nde*I (~4 kb); lane 6, double digestion of pGEM-*ada* with *Nde*I and *Bgl*II; lane 7, *ada* fragment (1.05 kb).

2. Expression of the Putative *ada* Gene from *S. antibioticus* in *E. coli*

2.1 Subcloning of the putative *ada* gene into the expression vectors pET28b and pET26b

To subclone the *ada* gene into the expression vector, *ada* gene was recovered from the recombinant pGEM-*ada* by digested with *Nde*I and *Bgl*II. Fragment containing the *ada* gene was then gel purified and ligated into expression vectors. For expression of protein as N-terminal his-tagged protein, the *ada* gene was ligated into pET-28b. For expression of protein as non his-tagged protein, the *ada* gene was ligated into pET-26b. To prepare the expression vectors for insertion of the *ada* gene, vectors were first double digested with *Nde*I and *Bam*HI. It should be noted that the *Bam*HI site and *Bgl*II site are compatible and can be anneal to each other. Once the *ada* gene had been inserted into the expression vectors with T_4 DNA ligase, DNA in the reaction mixtures were transformed into *E. coli* DH5α. Two positive clones which carrying recombinant plasmid with *ada* gene, designated as pET28-*ada* or pET26-*ada*, was obtained and its plasmid DNA purified. The isolated plasmids were analyzed to confirm the presence of *ada* gene by restriction digestion (Figure 25). The result showed that the 1050 bp fragments had been successfully inserted into pET-28b expression vector. The size of recombinant pET28-*ada* was approximately 6.4 kb (Figure 25, lane 4) which corresponds to the size of pET-28b (5.4 kb) containing the inserted fragment of 1.05 kb (Figure 25, lane 5). The supercoil of pET-28b was used as the control. The result of restriction digestion of recombinant pET26-*ada* was showed the same result as presence in pET28-*ada* digestion because the size of both expression vectors is similar (data not shown). Then, the pET28-*ada* and pET26-*ada* were isolated and transformed into competent *E. coli* BL21 (DE3), the host used for expression of putative ADA protein.

Lane number:	1	2	3	4	5	6	7
PET-28b DNA:	+	+	–	–	–	–	
PET28-ada DNA:	–	–	+	+	+	–	
PCR fragment:	–	–	–	–	–	+	
NdeI restriction enzyme:	–	+	–	+	+	–	
EcoRI restriction enzyme:	–	–	–	–	+	–	

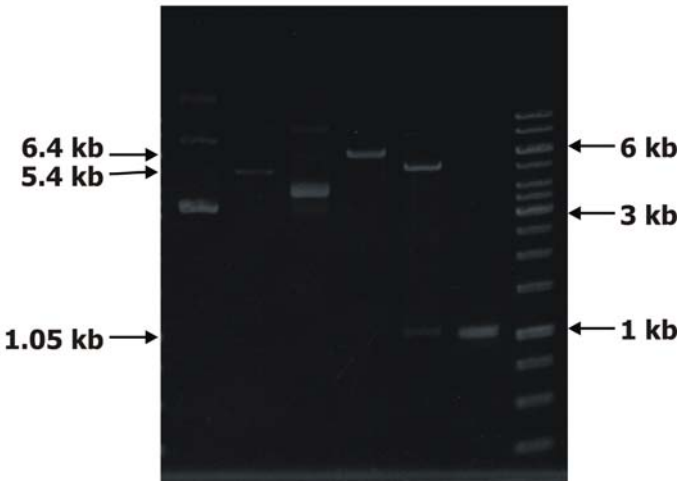


Figure 25 Determination of the presence of the putative *ada* gene in pET-28b. Lane 1, supercoil pET-28b; lane 2, linear pET-28b by digestion with *Nde*I (~5.4 kb); lane 3, supercoil pET28-*ada*; lane 4, linear pET28-*ada* by digestion with *Nde*I (~6.4 kb); lane 5, double digestion of pET28-*ada* with *Nde*I and *Eco*RI; lane 6, *ada* fragment (1.05 kb); lane 7, 1 kb DNA ladder.

2.2 Subcloning of the putative *ada* gene into pTYB12 vector

The recombinant pGEM-*ada* of 5 ug was double digested with *Nde*I and *Eco*RI to release *ada* fragments which contained *Nde*I and *Eco*RI sites. The DNA fragments of *ada* gene were gel purified. To reproduce the fusion protein of intein gene at N-terminal, the purified DNA fragments were ligated into pTYB12 vector which had been previously double digested with *Nde*I and *Eco*RI. The reaction mixtures were transformed into *E. coli* DH5α. The single clone of DH5α carrying recombinant DNA designated as pTYB12-*ada* was grown to isolate the recombinant plasmid. The presence of the *ada* gene (1050 bp) was detected by restriction digestion

(Figure 26). The size of recombinant pTYB12-*ada* was approximately 8.4 kb (Figure 26, lane 5) which corresponds to the size of pTYB12 (7.4 kb) containing the inserted fragment of 1.05 kb (Figure 26, lane 6). The supercoil of pTYB12 was used as the control. The result showed that the *ada* fragments had been successfully inserted into pTYB12 fusion vector. Then, the pTYB12-*ada* was isolated and transformed into expression host *E. coli* BL21 (DE3).

Lane number:	1	2	3	4	5	6	7
PTYB12 DNA:	+	+	–	–	–	–	
PTYB12-ada DNA:	–	–	+	+	+	–	
PCR fragment:	–	–	–	–	–	+	
NdeI restriction enzyme:	–	+	–	+	+	–	
EcoRI restriction enzyme:	–	–	–	–	+	–	

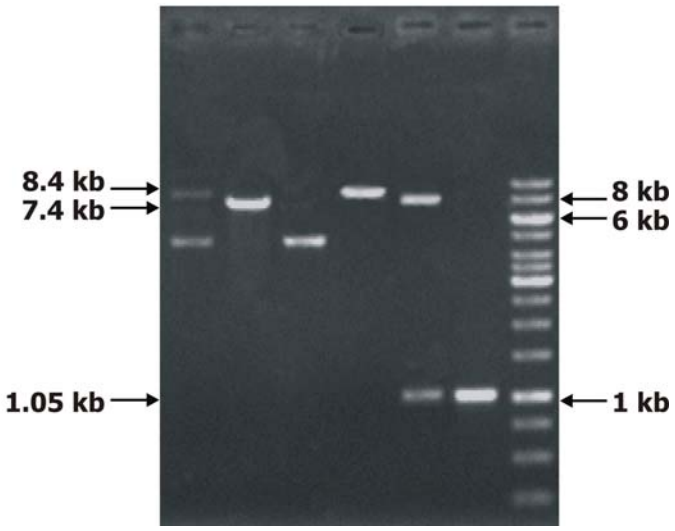


Figure 26 Determination of the presence of the putative *ada* gene in pTYB12. Lane 1, supercoil pTYB12; lane 2, linearized pTYB12 by digestion with *Nde*I (~7.4 kb); lane 3, supercoil pTYB12-*ada*; lane 4 linearized pTYB12-*ada* by digestion with *Nde*I (~8.4 kb); lane 5, double digestion of pTYB12-*ada* with *Nde*I and *Eco*RI; lane 6, *ada* fragment (1.05 kb); lane 7, DNA marker.

2.3 Expression of the putative ADA as non his-tagged protein

To express and characterize the non his-tagged protein, *E. coli* BL21 (DE3) carrying pET26-*ada* was grown in 200 ml of LB supplemented with kanamycin at 37°C to 0.5 of OD_{600} nm. At this time, the expression of non his-tagged protein was induced by addition of 1 mM IPTG. After 1 to 5 hours of induction, 1 ml of cells was collected by centrifugation and the level of expressed ADA was detected on 15% SDS-PAGE gel (Figure 27). After 2 hours of induction, a protein with molecular weight of 38 kDa which correspondent to that of the non his-tagged ADA was observed. The expression level of this protein continued to increase and reached maximum at 4 hours after induction. Although the level of protein expressed at 4 and 5 hours of induction is approximately the same, cells harvested at 5 hours of induction were able to grow a higher cell density.

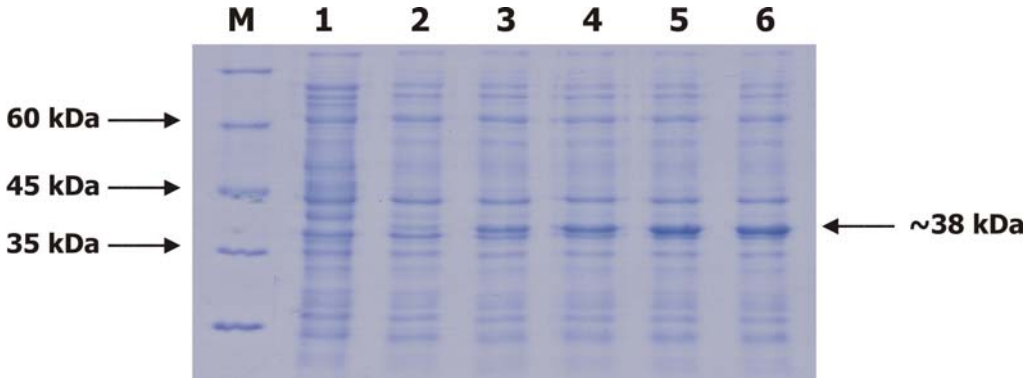


Figure 27 Determination of the expression level of BL21 (DE3) carrying pET26-*ada* which produced non his-tagged ADA with 1 mM IPTG. Lane M, protein marker; lane 1-6, total protein after induction at 0, 1, 2, 3, 4, and 5 hours, respectively.

2.4 Expression of the putative ADA as his-tagged protein

It is sometime desirable to express the target gene as his-tagged protein, either by his-tagging the protein at the N- or C-terminus. His-tagged protein can be easily purified in a single step with a Ni-NTA column. To express ADA as the N-

terminus tagged protein, the *ada* gene was subcloned into the pET-28b. *E. coli* BL21 (DE3) carrying pET28-*ada* which was grown in 1 L of LB supplemented with kanamycin at 37°C of OD_{600} nm. When OD reached to 0.5, the expression of recombinant N-terminus his-tagged protein was induced by addition of IPTG at 1 mM. After 1 to 5 hours of induction, 1 ml of cells was harvested by centrifugation every hour. The levels of expressed ADA were analyzed on 15% SDS-PAGE gel (Figure 28). The molecular mass approximate 40 kDa of his-tagged ADA was appeared after 1 hour of induction. The expression level of putative ADA was increased reach to 5 hours after induction. And then, cells were harvested and dissolved in lysis buffer in ratio 1 g cell per 4 ml lysis buffer. The lysed cells were disrupted by ultrasonication and centrifuged at 12,000 rpm. Unfortunately, the putative ADA was detected in the insoluble or inclusion bodies fraction (Figure 30).

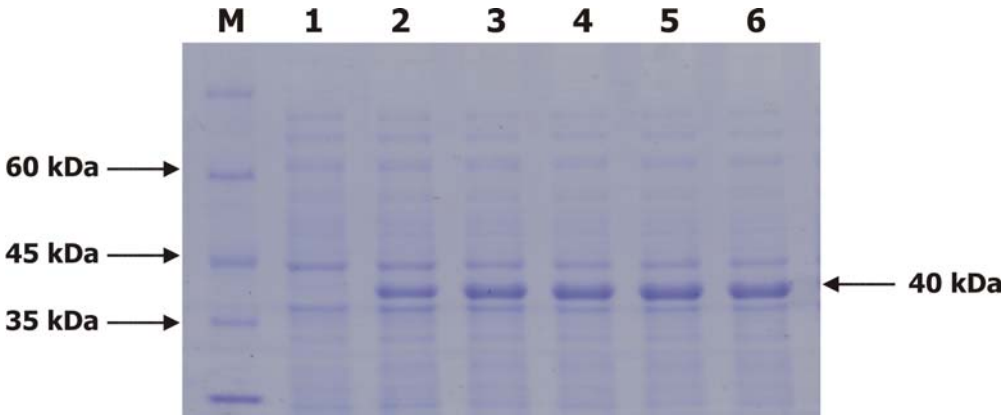


Figure 28 Determination of the expression level of BL21 (DE3) containing pET28-*ada* which produced his-tagged protein at N-terminal with 1 mM IPTG. Lane M, protein marker; lane 1-6, total protein after induction at 0, 1, 2, 3, 4, and 5 hours, respectively.

2.5 Expression of the putative ADA as an intein fusion protein

Because *ada* gene expressed using pET vectors, either pET-26b or pET-28b, formed inclusion bodies (IBs) and could not be refolded into the biologically active form, we decided to express *ada* gene in the form of an intein fusion protein. It

has been suggested that protein expressed intein fussion protein is more soluble and this may lead to an increase in the level of protein expression. Thus, the *ada* gene was subcloned into pTYB12 and the resulting plasmid is designated as pTYB12-*ada*. The BL21 (DE3) carrying pTYB12-*ada* was grown in 1 L of LB supplemented with ampicillin at 37°C of OD_{600} nm reach to 0.5. Cells were induced with 1 mM IPTG and grown at 16°C. The expression levels of recombinant putative ADA (40 kDa) that fused intein at N-terminal (55 kDa) were analyzed from 0 to 4 hours after induction in 15% SDS-PAGE gel (Figure 29). The result showed that no recombinant protein was observed at 0 hour (lane 1). The molecular mass of 55 kDa of intein protein was determined after 1 hour of induction (lane 2). In addition, the expression of intein-fused ADA, 95 kDa, was detected at 1 hour and increased reach to 4 hours after induction (lane 3-6). After 1 hour of induction, cells were harvested and dissolved in lysis buffer Cells were destroyed by ultrasonication and centrifuged at 12,000 rpm. Unfortunately, the intein-fused ADA was still formed the inclusion bodies (IBs). In contrast, the intein protein was detected in the soluble fraction.

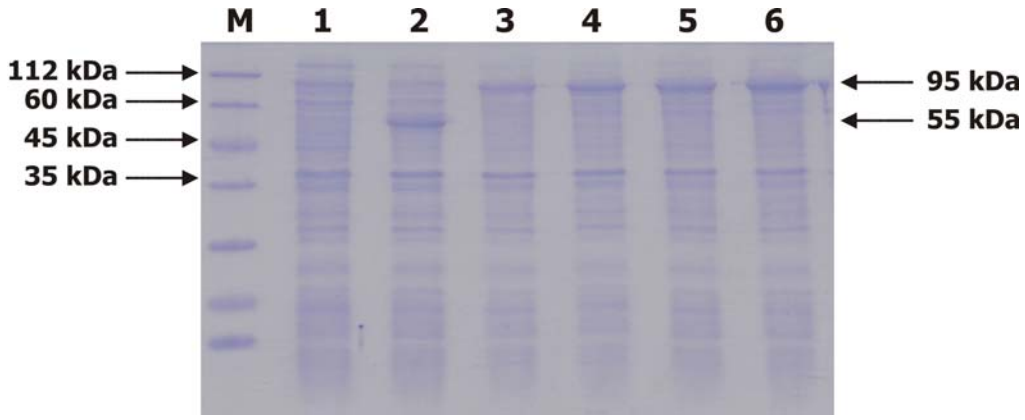


Figure 29 Protein analysis of putative ADA after over-expression of BL21 (DE3) carrying pTYB12-*ada* as a fusion protein with intein at N-terminal region. The 15% SDS-PAGE gel was strained with Coomassie Brilliant Blue. Lane M, standard protein marker; lane 1, no induction; lane 2, induced pTYB12 as a control; lane 3-6, total protein after induced with 1 mM IPTG of pTYB12-*ada* at 1, 2, 3, and 4 hours, respectively.

3. Refolding and Purification of the Adenosine Deaminase from *S. antibioticus*

After 5 hours of induction, BL21 (DE3) cells carrying pET28-*ada* from 1 L of culture were harvested, dissolved in lysis buffer and disrupted by sonication. After centrifugation to separate the soluble protein from cell debris, the his-tagged ADA was observed in pellet in the form of insoluble inclusion bodies (IBs) (Figure 30, lane 2). After extracting IBs with buffer containing 2 M urea, his-tagged ADA became soluble and accounted for more than 60% of total proteins. The extracted proteins were dialyzed against 50 mM potassium phosphate buffer (pH 7.25). After refolding by decreasing the concentration of urea from 2 M to 0 M by dialysis, ADA aggregated and precipitated. The aggregated proteins were removed from the supernatant by centrifugation at 12,000 rpm. Both the aggregated and soluble proteins were analyzed on 15% SDS-PAGE (Figure 30, lane 4-5). The result showed that the putative ADA formed in the soluble protein (lane 5). This data indicated that his-tagged ADA was successfully refolded. Then, the refolded his-tagged protein was purified by Ni^{2+}-NTA affinity column. After elution with buffer B containing imidazole, the putative ADA was presented in the fraction of 100 mM to 250 mM imidazole (Figure 30, lane 6-7). The eluted protein has a molecular mass of approximately 40 kDa with purity more than 95%.

The protein profile was shown in Figure 31. Non his-tagged proteins, fraction no.1, were passed through the Ni^{2+}-NTA affinity column. And then the contaminant proteins, fraction no.2-5, were eliminated with 4 volumes of buffer B containing 500 mM NaCl. After that, his-tagged proteins were eluted with buffer B containing 20, 100, 250 mM imidazole, respectively. The result showed that the putative ADA protein was obtained at highest amount in the fraction no. 11 when eluted with buffer B containing 100 mM imidazole. It was detected a little amount of ADA protein in the buffer B containing 250 mM imidazole fractions.

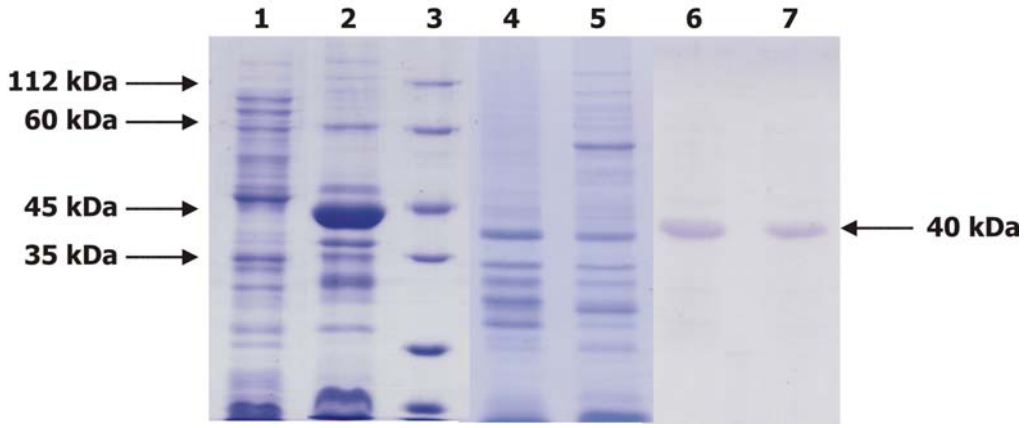


Figure 30 Analysis of the purified and refolded putative *S. antibioticus* adenosine deaminase on 15% SDS-PAGE gel. After sonication and centrifugation, the inclusion bodies in the pellet were extracted with 2 M urea and dialyzed against 50 mM potassium phosphate buffer (pH 7.25). Protein was purified by Ni-NTA column. Lane 1, cell-free extract; lane 2, pellet (IBs); lane 3, protein marker; lane 4, aggregated forms of protein after dialysis; lane 5, soluble forms of protein after dialysis; lane 6, protein eluted with buffer B contain 100 mM imidazole; lane 7, protein eluted with 250 mM imidazole.

The purification scheme of the protein was summarized in Table 3. Total amount of insoluble proteins in the pellet were combined from extracted IBs with 2 M and 8 M urea. It showed that the total protein was 18.6 mg. Because protein aggregates during refold using dialysis method, percent protein recovery dropped from 100% to 18%. After refolding, the his-tagged protein was purified by Ni^{2+}-NTA affinity column. This purification scheme resulted in 1.1 mg of approximately 95% pure protein, with 6% recovery. When the purified protein was assayed for activity, using adenosine, 2'-deoxyadenosine, adenine, AMP, ADP, and Ara-A as substrates, no activity was detected. The inability to detect ADA activity is probably due to protein misfolding during purification, which resulted in loss of the enzyme activity.

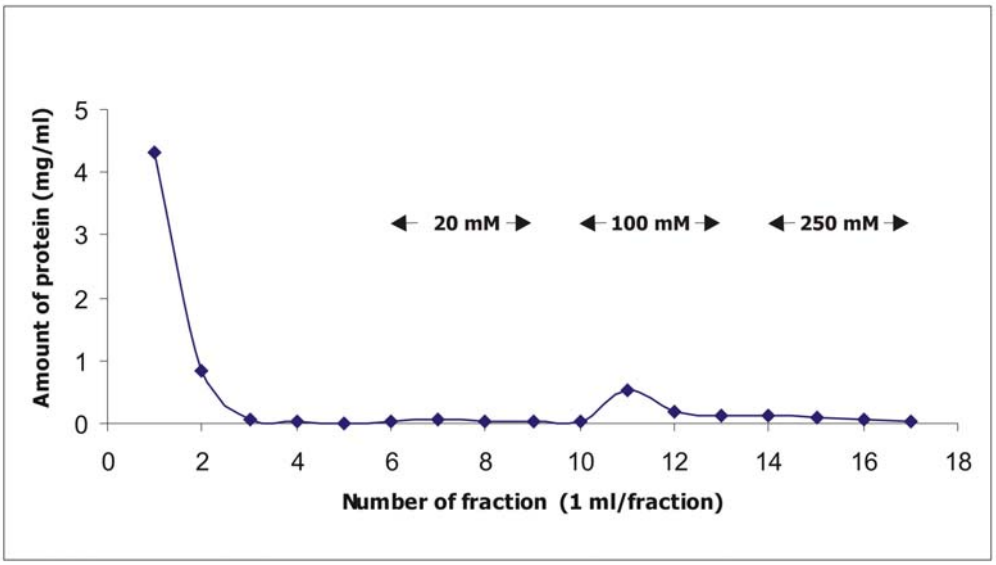


Figure 31 Protein profile of putative adenosine deaminase from *S. antibioticus* eluted with 20, 100, and 250 mM imidazole from 1 ml bed volume of Ni-NTA column.

Table 3 Summary of the purification of putative adenosine deaminase from *S. antibioticus*

Step	Total volume (ml)	Total protein (mg)	Activity (unit)	Recovery (%)
Extraction IBs	8	18.6	nd*	100
Dialysis	16	3.3	nd	18
Ni-NTA affinity	5	1.1	nd	6

nd* – Non detectable

4. Sequence Analysis

Amino acid sequences of ADA from various organisms were aligned using ClustalW (Figure 32). Based on the alignment result, all seven amino acids that had been suggested to involve in the catalysis in the crystal murine ADA were conserved in *S. antibioticus* ADA. In *S. antibioticus* ADA, these amino acids are located in the equivalent position as His16, His18, His204, Glu207, His228, Asp285, and Asp286.

This result strongly suggested that the expressed putative protein from *S. antibioticus* is likely to be ADA (StADA). The deduced amino acid sequence of ADA showed 88% identity to *S. coelicolor* 2 ADA, 87% identity to *S. avermitilis* ADA, and 84% identity to *S. verginiae* ADA, 25% identity to human ADA, 27% identify to mouse ADA (MuADA), 27% identity to *E. coli* ADA, and 25% identity to *S. coelicolor* 3 *ADA* (NP629054).

```
S.antibioticus      ------------------MERVRDLSELPKAHLHLHFTGSMRPGTVLELADKYGVRLPD    41
S.coelicolor2       ------------------MERVRDVSELPKAHLHLHFTGSMRPTTLLELADKHGVRLPE    41
S.virginiae         ------------------MEHARDLTLLPKAHLHLHFTGSMRPSTLLELADKYGVRLPD    41
S.avermitilis       ------------------MEHVRDVSELPKAHLHLHFTGSMRPSTLLELADKYGVRLPD    41
Homo sapiens        -------------------MAQTPAFDKPKVELHVHLDGSIKPETILYYGRRRGIALPA    40
Mus musculus        -------------------MAQTPAFNKPKVELHVHLDGAIKPETILYFGKKRGIALPA    40
E.coli              ----------------------MIDTTLPLTDIHRHLDGNIRPQTILELGRQYNISLPA    37
S.coelicolor3       MTSRSTEKSAAANPAAVSKTPSPDRIRRAPKVLLHDHLDGGLRPGTIVELARETGYGDLP    60

S.antibioticus      TLRDSLVSGDPPRLRATDERGWFRFQRLYDAARSCLR-TPEDIQRLVREAAEEDLRDGSG   100
S.coelicolor2       TLTEALGRGESPKLRATDERGWFRFQRLYDAARSCLQ-TPEDIQRLVREAAEEDLRDGSG   100
S.virginiae         ----ALTAGEPPKLRATDERGWFRFQRLYDAARSCLR-EPDDIRRLVREAAEEDVRDGSG    96
S.avermitilis       ----ALTSAEPPKLRATDERGWFRFQRLYDAARSCLR-EPEDIRRLVREAAEEDIKDGSG    96
Homo sapiens        N---TAEGLL-NVIGMDKPLTLPDFLAKFDYYMPAIAGCREAIKRIAYEFVEMKAKEGVV    96
Mus musculus        D---TVEELR-NIIGMDKPLSLPGFLAKFDYYMPVIAGCREAIKRIAYEFVEMKAKEGVV    96
E.coli              Q---SLETLIPHVQVIANEPDLVSFLTKLDWGVKVLA-SLDACRRVAFENIEDAARHGLH    93
S.coelicolor3       ET--DADLLGTWFRQAADSGSLERYLETFSHTVGVMQ-TRDALVRVAAECAEDLAEDGVV   117

S.antibioticus      WLEIQVDPTSYAPRLGGLIPA------------LEVILDAVDTA---SRDTGLGMRVLVA   145
S.coelicolor2       WLEIQVDPTSYAPRLGGLIPA------------LEIILDAVETT---VRDTGIGMRVLVA   145
S.virginiae         WLEIQVDPTSYAPLLGGMIPA------------VEIILDAVDAA---SRETGLGMRVLIA   141
S.avermitilis       WLEIQVDPTSYAPRLGGLIPA------------MEVILDAVDSA---ARETGLGMRVLVA   141
Homo sapiens        YVEVRYSPHLLANSKVEPIPWNQAEGDLTPDEVVALVGQGLQEG---ERDFGVKARSILC   153
Mus musculus        YVEVRYSPHLLANSKVDPMPWNQTEGDVTPDDVVDLVNQGLQEG---EQAFGIKVRSILC   153
E.coli              YVELRFSPGYMAMAHQLPVAG-----------VVEAVIDGVREG---CRTFGVQAKLIGI   139
S.coelicolor3       YAEVRYAPEQHLEKGLTLEEV------------VEAVNEGFREGERRARDNGHRIRVGAL   165
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Figure 32 Amino acid sequence comparison of adenosine deaminase from *Streptomyces antibioticus* (StADA), *S. coelicolor* A3(2) No.2 (NP628805), *S. virginiae* (BAA09298), *S. avermitilis* MA-4680 (NP826083), Human (P00813), Mouse (MuADA) (NP031424), *Escherichia coli* W3110 (BAA15374), and *S. coelicolor* A3(2) No.3 (NP629054). Residues highlighted in gray are amino acids that present in the active site according to MuADA (His15, His17, Gly184, His214, Glu217, His238, Asp295, and Asp296) and StADA (His16, His18, Asn177, His204, Glu207, His228, Asp285, and Asp286).

Figure 32 (Continued)

Discussion

1. Determination of the Complete Nucleotide Sequence of the Novel *ada* Gene from *S. antibioticus* by Normal PCR, Inverse PCR, and Single Primer-PCR.

The ability of *Streptomyces* to tolerate the endogenous antibiotic is a prerequisite for antibiotic production, because antibiotics are often exhibited inhibitory effect on the cellular metabolism. Previous study had suggested that a novel ADA, namely ADA-II, is probably the key enzyme that confers *S. antibioticus* resistant to 2'-dCF (Pornbanlualap, 1994). The goal of this thesis is to clone, sequence, and express the gene coding for ADA-II, a novel enzyme that is not inhibited by the naturally occurring transition state analog, 2'-dCF. The *ada* gene from *E. coli* had been successfully cloned using the functional complementation approach (Chang *et al*., 1991) but not in *S. antibioticus*. In this approach, *E. coli* mutant lacking the *ada* gene was genetically constructed. This mutant failed to grow on minimal medium supplemented with diaminopurine (DAP). However, when obtained the recombinant plasmid carrying the *ada* gene by transformation, these mutants will grow on minimal medium supplemented with DAP. DAP riboside can be converted to guanosine by ADA and thus fulfilled the host's purine requirement. However, the attempt to clone the *S. antibioticus ada* gene by functional complementation had been unsuccessful (Charopagorn, 2004). This is probably due to the fact that ADA from different organism exhibits different substrate specificity. It is postulated that the ADA from *Streptomcyes* may has high specificity toward its substrate (adenosine) and thus will not recognize DAP riboside as substrate. For this reason, PCR based approaches were used to clone *ada-II* gene from *S. antibioticus*.

Determination of the complete sequence of *ada* gene in *S. antibioticus* by PCR base methodology was divided into three parts: (i) Determination of the central region of the *ada* gene by normal PCR, using two ADA specific primers, (ii) Determination of the 3'-downstream region from the central *ada* gene by IPCR, using two ADA specific primers whose nucleotide sequence are inverse (or reverse) to that of normal

primers, and (iii) Determination of the 5'-upstream region from the central *ada* gene by the SP-PCR, using only one ADA specific primer.

(1). To clone and determine the central region of the *ada* gene, two ADA specific primers were designed. The gene coding for ADA from a variety of organisms, including human (Wiginton *et al*., 1983), murine (Yeung *et al*., 1985), *E. coli* (Chang *et al*., 1991), and *S. coelicolor* (Charopagorn and Pornbanlualap, 2008) has been cloned and sequenced. Based on the two highly conserved regions, PKAH(E)LHL and RIG(D)HGV(L)R, obtained through multiple alignments of ADA proteins, two degenerated primers were synthesized and used to amplify the central region of *ada* gene (Figure 6). When the obtained 657 bp PCR amplified fragment was sequenced, it showed 85% identity to that of the *ada* gene in *Streptomyces sp* (Figure 10). This result suggested that the designed primers are specific toward the *ada* gene from *Streptomyces*, GC rich organism (Wright *et al*., 1992), and that the amplified fragment is probably an *ada* gene.

(2). To determine the 3'-downstream region of *ada* gene by inverse PCR (IPCR), two inverse primers were synthesized and used to amplify the circular DNAs which were used as template. *S. antibioticus* DNA was digested with *Xho*I, *Psh*AI, and *Bam*HI (Figure 13). *Xho*I and *Psh*AI used in this experiment do not cut anywhere within the central region of the *ada* gene, whereas *Bam*HI cleaves once within the gene. All these enzymes are six-nucleotide cutter. The *Xho*I- and *Psh*AI-digested DNA consisted of mostly > 20 kb which is too big for preparation of small circular DNA. The *Bam*HI-digested DNA, on the other hand, generated 1-20 kb which is more suitable for preparing small circular DNA. It has been demonstrated that the circular DNA suitable to be used as template for IPCR should be no greater than 2-3 kb (Ochman *et al*., 1988). Thus, the *Bam*HI digested DNA fragments were gel purified and used for preparing circular DNA. A single band of 1.5-kb was obtained from IPCR approach. The result showed that this 1.5-kb PCR product contained downstream region and right chromosomal DNA region from central region of the *ada* gene. Normally, IPCR method is difficult to apply to the GC rich DNA and stable secondary structure of the gene for *Streptomyces* (Kuno *et al*., 1998). However, the

downstream region of *ada* gene of *S. antibioticus* has been successfully cloned by this approach.

(3). Because IPCR was used unsuccessfully in determined the 5'-upstream region of the *ada* gene (data not shown), thus, single primer polymerase chain reaction (SP-PCR) was carried out. SP-PCR method is convenient because it requires no enzymatic modification of the template DNA but requires specific condition (Parks *et al*., 1991). The advantage of this method is requires only one primer that acts as forward and reverse. SP-PCR requires a high T_m of primer and a high annealing temperature as amplification condition (Aquino and Luiz, 2004). In our experiment, using InF3 as a primer at annealing of 63°C, the 2.8 kb fragment was obtained which correspondent to the upstream region and left chromosomal DNA region from central region of the *ada* gene. This data indicated that the appropriate condition to amplify unknown region was required for each primer used. The larger of PCR fragment was obtained if extension time of PCR process was increased (Aquino and Luiz, 2004).

By combining the nucleotide sequence of the central region of *ada* gene obtained by normal PCR, upstream region obtained by the IPCR, and downstream region obtained by SP-PCR, a complete ORF of the putative *ada* gene from *S. antibioticus* was obtained (Figure 22). It indicated that these approaches can be used for complex genome. Both of them are convenient, low-cost, and less time-consuming to identify the uncharacterized region but they appropriate to a short stretch of known sequence (Ochman *et al*., 1988).

2. Purification and Characterization of the Putative Adenosine Deaminase

Two forms of ADA from *S. antibioticus* had been previously purified and characterized. Both enzymes were demonstrated to be zinc-metalloenzyme with a stoichiometry of 1 zinc per subunit of protein. ADA-I with the molecular weight 40 kDa is tightly inhibited by 2'-dCF whereas ADA-II with a molecular weight of 38 kDa is not or poorly inhibited by 2'-dCF. 2'-dCF has been demonstrated to bind 100,000-fold more tightly to ADA-I than to ADA-II (Pornbanlualap, 1994). To

determine whether the ORF obtained encoded for ADA-I or ADA-II, the *ada* gene was successfully expressed in pET26b as non his-tagged protein, in pET28b as N-terminus his-tagged protein, and in pTYB12 as intein fusion protein.

To purify and characterize the protein, the putative *ada* gene was expressed as non his-tagged protein by addition of 1 mM IPTG to *E. coli* BL21 (DE3) cells carrying pET26-*ada.* Analysis of the protein content of the induced cells on SDS-PAGE revealed that a protein with molecular weight of ~37.8 kDa was expressed upon induction. However, the level of *S. antibioticus* ADA induced was significantly lower than that of *S. coelicolor* ADA expressed using an identical pET/Bl21 expression system (Chalopagorn, 2004). Furthermore, *S. antibioticus* ADA was expressed in the form of insoluble inclusion bodies whereas that of *S. coelicolor* was expressed as soluble protein.

At least three possibilities can be accounted for low level of expression and formation of inclusion bodies. First, the recombinant ADA may be toxic to the *E. coli* host when expressed. It had been demonstrated that with pET system where the target gene is placed under the control of T7 promoter, low level of protein is being expressed because of leaky expression, even in the absence of inducer (Donahue and Bebee, 1999). Second, the *S. antibioticus ada* gene that is enriched with rare codons tends to express poorly and tends to form inclusion bodies in host cells because its codon usage is difference (Jana and Deb, 2005). Finally, when expressed, the recombinant ADA from *S. antibioticus* may not able to fold probably in the *E. coli* cytoplasm. For example, the predicted protein sequence of *ada* gene from *S. antibioticus* contains four cysteine residues. Thus, this protein whose structure is depended on formation of correct disulfide bond might not be produced in the correct conformation and lead to a non-native like structure (Makrides, 1996).

To prevent and reduce the formation of inclusion bodies, cells carrying the recombinant plasmid was grown at lower temperature (16º-25ºC) and induced with lower concentration of IPTG (0.1-0.5 mM) (data not shown). It has been reported that the *in vivo* aggregation of recombinant proteins can be reduced by growing cells at

low temperature. The expression rate of heterologous protein occurs at a much slower rate at 16ºC than at 37ºC (Marx *et al*., 2007). Furthermore, induction with low concentration of IPTG reduced the expression rate and lead to reduction of IBs formation (Austin *et al*., 2004). Because these conditions had failed to prevent the formation of inclusion bodies, therefore, refolding *in vitro* is an alternate approach to solubilize inclusion bodies into the active form.

To unfold and refold the ADA in the inclusion bodies into active form, recombinant his-tagged protein was used in the experiment. Inclusion bodies produced from BL21 (DE3) carrying pET28-*ada* were unfolded by dissolving in 8 M urea or 6 M guanidine hydrochloride. Inclusion bodies have been suggested to form partially folded protein intermediates which have native-like secondary structures. To avoid the loss of secondary structure, a mild solubilization condition was employed by using extraction buffer with alkaline pH in the presence of 2 M urea (Singh and Panda, 2005). With this approach, more than 60% of the his-tagged ADA, 40 kDa, remained in the pellet after solubization. After dialysis of soluble protein and centrifugation, only few his-tagged ADA were detected in the supernatant (Figure 30, lane 5). The refolded his-tagged protein was subsequently purified by Ni^{2+}-NTA affinity column and eluted with elution buffer. This data indicated that his-tagged ADA was successfully refolded using mild condition. During refolding the protein by gradually reducing the concentration of urea from 2 M to 0 M urea, the aggregation of proteins was observed. It has been recently reported that protein aggregation is a selective process. The IBs effect on soluble protein resulting in fast aggregation of soluble protein (Carrio *et al*., 2005). It can be explained that the aggregation of ADA protein is occur through specific intermolecular interactions between aggregating polypeptidic chains during refolding. Unfortunately, refolding of inclusion bodies ADA into bioactive forms had been failed and resulted in poor recovery, from 100% to 6%. The main reasons for the poor recovery of bioactive forms are the loss of secondary structure and the aggregation during refolding by specific interaction (Singh and Panda, 2005). However, the function of the eluted his-tagged protein, refolded protein, was unknown. Although various substrates such as adenosine, 2'-deoxyadenosine, adenine, AMP, ADP, and Ara-A were tried but the activity was not

detected. This result suggested that the eluted protein is a novel protein which specific to another substrate or it is a misfolding protein.

3. Gene Prediction and Proposed Catalytic Mechanism

Determination of the crystal structure of murine ADA complexed with transition state analogue, HDPR, indicated that five catalytic residues, namely Gly184, Glu217, His238, Asp295 and Asp296, interacted with the inhibitor and essential for catalysis. In addition, zinc cofactor which involved in activation of water molecule is coordinated to His15, His17, and His214 at the active site of enzyme (Wilson *et al*., 1991). These eight residues that involved in the deamination of adenosine to inosine are conserved in ADA isolated from various organisms, ranging from *E. coli* to human (Figure 30) (Chang *et al*., 1991).

The catalytic mechanism of MuADA has been proposed to occur by addition-elimination (SN2 mechanism), with formation of a tetrahedral transition state or intermediate at the C-6 position of the purine ring (Kurz and Frieden, 1987). In step 1, the water molecule is activated by coordination with zinc cofactor and is positioned to attack on the C6 of adenosine by hydrogen bonding to Asp295. In step 2, His238 abstracts the proton from water molecule, thereby creating the attacking hydroxyl group. The addition of hydroxyl group on C6 of adenosine changes the hybridization of this carbon from sp^2 to sp^3. Glu217 further facilitates the formation of a tetrahedral C6 by donating a hydrogen bond to N1 of purine ring and reduces the double bond character of the N1-C6 double bond. In step 3, the tetrahedral intermediate collapses with the amino group becoming protonated by His238. The two remaining conserved residues at the active site, namely Gly184 and Asp296, have been suggested to involve in substrate, transition-state and product binding in the ES, ETS and EP complexes, respectively. Gly184 forms a hydrogen bond with N-3 and Asp296 forms a hydrogen bond with the N-7 of purine ring, respectively (Wilson and Quiocho, 1993; Mohamedali *et al*., 1996; Sideraki *et al*., 1996).

As shown in figure 32, seven of catalytically important residues, namely as His16, His18, His204, Glu207, His228, Asp285, and Asp286, were conserved in the protein sequence of *S. antibioticus*. The remaining non-conserved residue, namely Gly184 in MuADA, had been substituted with Asn. This data strongly suggested that the cloned gene of *S. antibioticus* is probably an adenosine deaminase (StADA). Although StADA and MuADA shares 27% protein sequence identity, it shows that StADA contains 7 of the 8 active site residues. Because the conserved active site residues in MuADA are found in StADA, thus, the catalytic mechanism of StADA is proposed to occur by SN2 mechanism also (Figure 33). The four active site residues in StADA, His16, His18, His204 and Asp285, are thought to be important for zinc binding. The three conserved residues, Glu207, His228, and Asp285, in the active site of StADA are proposed to play similar catalytic role as Glu217, His238, and Asp295 of MuADA. The steps of catalysis of StADA were proposed as the following; (i) the water molecule is activated by coordination with zinc cofactor and is positioned to attack on the C6 of purine ring by hydrogen bonding to Asp285 (ii) His228 abstracts the proton from water molecule, thereby creating the attacking hydroxyl group. The addition of hydroxyl group on C6 of adenosine changes the hybridization of this carbon from sp^2 to sp^3. Glu207 further facilitates the formation of a tetrahedral C6 by donating a hydrogen bond to N1 of purine ring to facilitate the unstable formation, sp^3. (iii) the tetrahedral intermediate collapses with the amino group becoming protonated by His228. The Asn177 and Asp286 in the active site involve in substrate binding, transition state and product rather than catalysis and form hydrogen bond at N-3 and N-7 of the purine ring, respectively. Because chemical composition of the R group of Asn is same as Gly, polar uncharged, it is assumed that Asn177 of StADA can also form hydrogen bond to N-3 of purine ring at equivalent position of Gly184 of MuADA. However, three-dimensional structure of MuADA has been determined by X-ray crystallography. This enzyme contains a central $(\beta/\alpha)_8$ barrel and five additional helices (Wilson *et al*., 1991). Although the three-dimensional structure of StADA has not been determined and protein sequence of MuADA and StADA identities only 27%, architecture of this enzyme is probably folded into the $(\beta/\alpha)_8$ barrel, which is found in 1/10 of known enzymes.

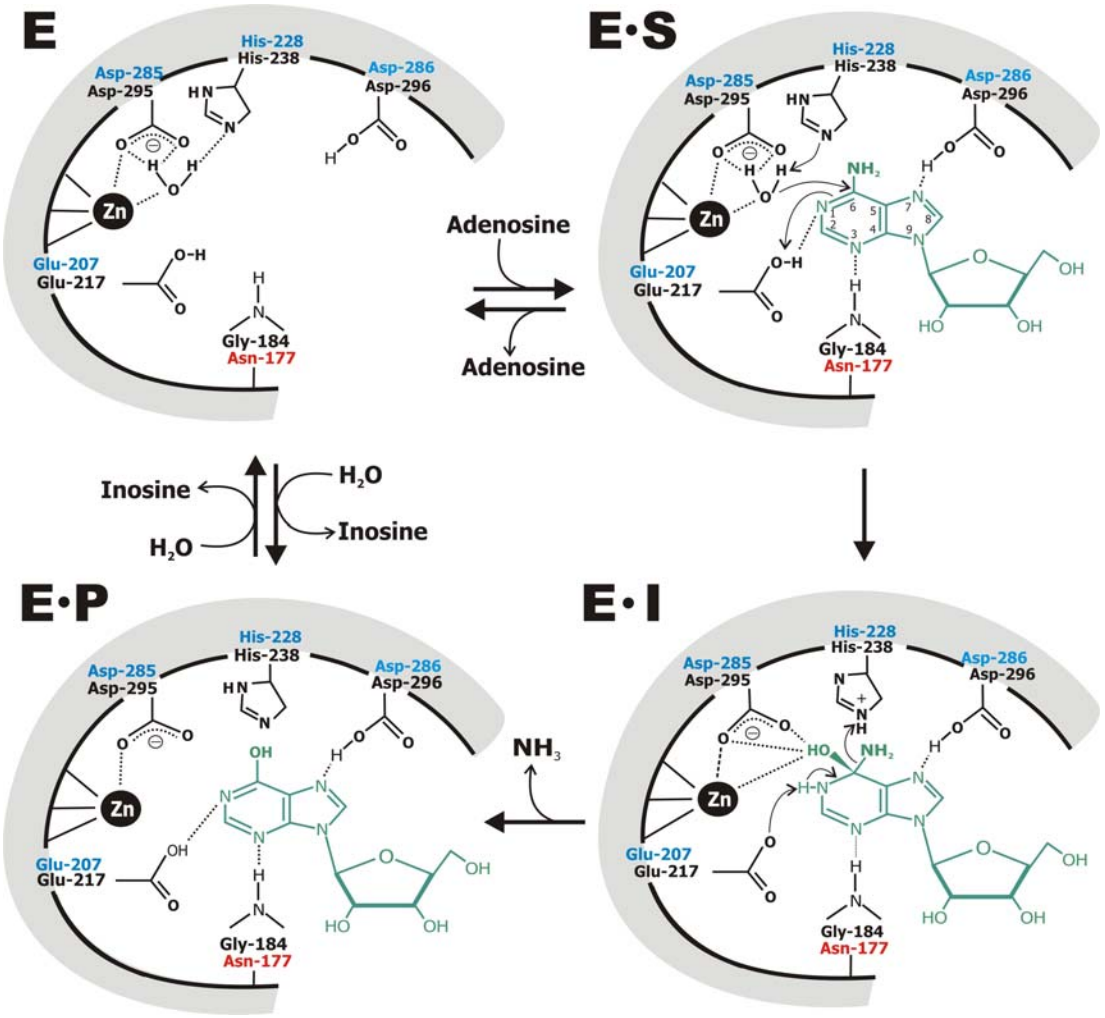

Figure 33 Mechanism of the reaction catalyzed by adenosine deaminase. Active site residues of MuADA which interacts with substrate, intermediate and product are indicated in black. The conserved active site residues of StADA located at the equivalent positions are indicated in blue and the non-conserved residue is indicated in red. Substrate, tetrahedral intermediate and product are shown in green. The addition-elimination mechanism assumes the formation of a tetrahedral intermediate in the EI complex. This diagram is modified from Sideraki *et al*., 1996.

Thus, this thesis reports the complete nucleotide sequence of StADA. It remains to be demonstrated whether this protein is ADA-I or ADA-II. Although both of these enzymes exhibit similar kinetic properties, substrate specificity and zinc requirement, they are markedly differed in inhibition by 2'-dCF (Pornbanlualap, 1994). Similar to ADA from most species, ADA-I is potently inhibited by 2'-dCF, a transition state analog, with K_i of 10^{-9} M. However, ADA-II is weakly inhibited by 2'-dCF with K_i of 10^{-3} M. This data suggested that ADA-II is a novel ADA that resistant to 2'-dCF during the biosynthesis of 2'-dCF in *S. antibioticus*. Amino acid sequencing indicated that the N-terminus of ADA-I was NH_2-TETLRRLPKAVLHD HLDGGLRPATVVELAA (Pornbanlualap, 1994). Comparison protein sequence, the cloned protein (StADA) in our thesis work is not similarly to ADA-I sequence. In addition to, it has been reported that the molecular weight of ADA-II that is not inhibited by 2'dCF is 38 kDa. In contrast, the molecular weight of ADA-I that is tightly inhibited by 2'-dCF is 40 kDa (Pornbanlualap, 1994). However, the calculated molecular weight of cloned protein (StADA) is 37.8 kDa which is closed to that of ADA-II form.

When the right chromosomal DNA region of the *ada* gene obtained by the IPCR was sequenced, a putative *tet*R gene which is similarly to TetR family of *S. griseus* subsp. *griseus* (BAG19708.1) with identity of 74% was obtained (Figure 17). The TetR family proteins had been demonstrated to be regulatory genes whose products are involved in regulating the biosynthesis of antibiotics. For example a protein of TetR family in *S. antibioticus*, SimReg2, located in simocyclinone biosynthetic gene cluster which is regulates the export protein (SimEX). In *S. griseus* subsp. *griseus*, it contains NonG which is probably regulation of antibiotic nonactin biosynthesis (Ramos *et al*., 2005). Furthermore, as the result shown in Figure 20, the left chromosomal DNA region of the putative *ada* gene is contains *nusG* and *secE* genes which are similar to *S. griseus* subsp. *griseus* (BAG19704.1) and *S. galbus* (CAA65164.1) with identities 88% and 95%, respectively. The NusG protein is required for N-antitermination in the transcription system of Rho-dependent terminators (Weisberg and Gottesman, 1999). For example, NusG protein of *S. virginiae* has been reported that it is essential for virginiamycin antibiotic production

(Miyake *et al*., 1994). In addition, SecE protein is essential required for protein export to extracellular environment in gram-positive bacteria such as *Streptomyces*, *Mycobacterium*, and *Bacillus* etc. Thus, both of proteins are importance for cellular function and antibiotic production (Miyake *et al*., 1994).

As we known, *S. antibioticus* can produce natural 2'-dCF antibiotic, which is strong inhibitor to ADA. Thus, the ADA-II confers *S. antibioticus* to resistant the 2'-dCF during the antibiotic production. Because the cluster genes of 2'-dCF biosynthesis in *S. antibioticus* have been not well known, here, we propose the four genes (*tet*R, novel *ada*, *nus*G, and *sec*E) involved in the 2'-dCF biosynthetic genes (Figure 34). In the rich environments, TetR repressor binds to the *tet* promoter and suppresses transcription of the 2'-dCF cluster genes. When the environments change to starvation, *S. antibioticus* must responds quickly to survive. Because signal molecules bind to a TetR repressor, RNA polymerase can binds to *tet* promoter, thus, the genes encoding for 2'-dCF production can be transcribed. During transcription, the NusG protein (antiterminator) binds to Rho protein (termination factor). Thus, the RNA polymerase can read through the transcription termination and transcribed to the next genes. For cellular function during 2'-dCF biosynthesis, ADAII confers *S. antibioticus* to resistant the 2'-dCF because the ADAI was inhibited by 2'-dCF. The accomplished 2'-dCF antibiotic is exported to extracellular environment by SecE protein.

In summary, a complete ORF of the gene encoding for a putative *ada* gene had been determined. The ADA protein as non his-tagged protein, his-tagged protein, and intein fusion protein was successfully expressed. However, molecular weight and sequence of StADA is not similar to ADA-I. Because the molecular weight of the expressed non his-tagged protein is ~37.8 kDa, it is similar to that of ADA-II (38 kDa) purified from *S. antibioticus* cells. By determination of left and right chromosomal DNA region of the *ada* gene, the *ada* gene locates in the cluster of antibiotic biosynthetic genes. Thus, the putative *ada* gene is a probably ADA-II which confers *S. antibioticus* resistant to 2'-dCF during the 2'-dCF biosynthesis. The entire 2'-dCF antibiotic genes cluster can be obtained (Figure 34).

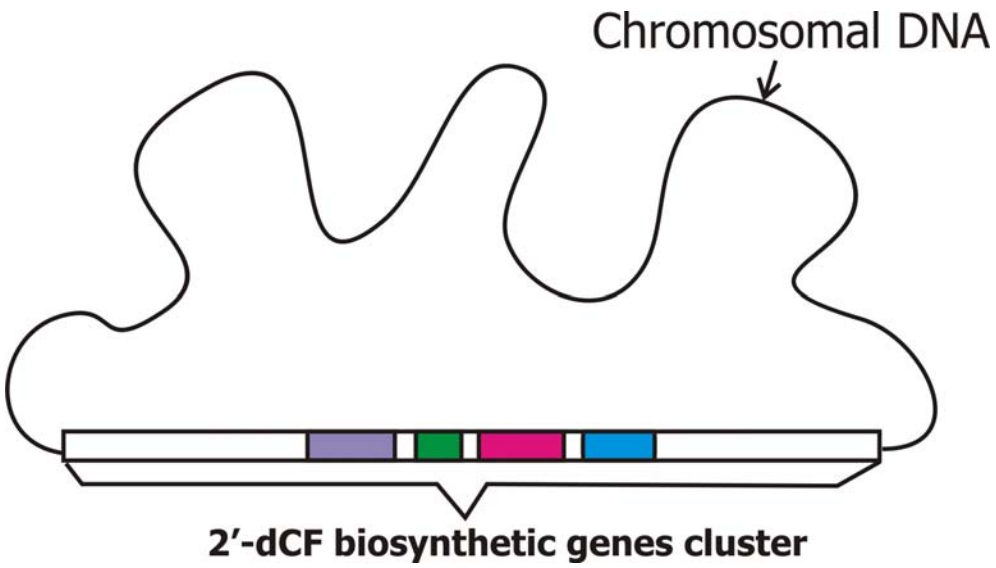

Figure 34 The proposed putative 2'-dCF biosynthetic genes cluster. The assemble nucleotide sequence, approximately 4.5 kb, reveals the cluster of putative 2'-dCF antibiotic production genes. The cluster of 2'-dCF biosynthesis contains *nus*G (purple), *sec*E (green), *ada*II (pink) and *tet*R (blue). It can be proposed the pathway of 2'-dCF biosynthesis from the following. The TetR protein binds to *tet* promoter and controls genes whose products are involved in biosynthesis of 2'-dCF. When *S. antibioticus* grown in the starvation environment, small signal molecules bind to TetR repressor, thus, the genes encoding for 2'-dCF production can be transcribed by RNA polymerase. Because NusG protein suppresses the transcription termination, then, the RNA polymerase can read through the termination to the next genes. During the 2'-dCF biosynthesis, the ADAII protein which is important for cell growth is resistant to the 2'-dCF. The accomplished 2'-dCF is exported to extracellular environment by SecE protein. The black line indicated as chromosomal DNA of *S. antibioticus*.

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusion can be drawn as follow:

1. The complete open reading frame (ORF) of putative *ada* gene sequence from *S. antibioticus* was obtained by combined nucleotide sequence, 4.5 kb in length, from normal PCR, IPCR, and SP-PCR.

2. The putative *ada* gene of *S. antibioticus* is 1041 bp that that contains a high GC content of 74% and encodes for protein with 346 amino acid residues (37.8 kDa).

3. The deduced amino acid sequence is similar to ADA from *S. coelicolor* A3(2), *S. avermitilis* MA-4680, and *S. verginiae* with homology of 88%, 87%, and 84%, respectively.

4. The ADA protein as non his-tagged protein, his-tagged protein, and intein fusion protein was successfully expressed. All of proteins formed inclusion bodies and can not assay for the activity.

5. The expressed his-tagged ADA, 40 kDa, was successfully refolded using mild condition and was purified by Ni^{2+}-NTA affinity column. It can be eluted by buffer B containing 100 mM imidazole. However, the function of refolded protein was not detected.

6. The molecular weight and sequence of the putative *ada* is not similar to ADA-I. Because the molecular weight of the expressed non his-tagged protein is ~37.8 kDa, it is similar to that of ADA-II (38 kDa), resistant form to 2'-dCF.

7. By determination of DNA region of the *ada* gene, the *ada* gene locates in the cluster of antibiotic biosynthetic genes. Thus, the putative *ada* gene is a probably ADA-II which confers *S. antibioticus* resistant to 2'-dCF during the 2'-dCF biosynthesis. Moreover, the entire 2'-dCF antibiotic genes cluster can be obtained

8. A concerted addition and elimination type (SN2) mechanism is proposed for ADA of *S. antibioticus* based on multiple alignment of ADA from various organisms.

Recommendation

1. The upstream and downstream regions of the putative 2'-dCF biosynthetic genes should be cloned and sequenced in underway.

2. To avoid the inclusion bodies problem, the putative *ada* gene of *S. antibioticus* should be expressed in other vector such as pGEX or in other expression systems such as *Streptomyces*, yeast, insect cells *etc*.

3. However, the IPCR and SP-PCR approaches can be used for complex genome. Both of them are convenient, low-cost, and less time-consuming to identify the uncharacterized region but they appropriate to a short stretch of known sequence.

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