รายงานวิจัยฉบับสมบูรณ์

โครงการ Cloning of 2'-Deoxycoformycin Gene Cluster from *Streptomyces antibioticus*

โดย ผศ.ดร. สมชัย พรบันลือลาภ และ รศ.ดร. วิไล สันติโสภาศรี

1 กรกฎาคม 2548

สัญญาเลขที่ MRG4680105

รายงานวิจัยฉบับสมบูรณ์

โครงการ Cloning of 2'-Deoxycoformycin Gene Cluster from *Streptomyces antibioticus*

คณะผู้วิจัย	สังกัด
1. ผศ.ดร. สมชัย พรบันลือลาภ	มหาวิทยาลัยเกษตรศาสตร์
2. รศ.ดร. วิไล สันติโสภาศรี	มหาวิทยาลัยเกษตรศาสตร์

สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

รายงานโครงการวิจัยประจำปีที่ 2

(1 กรกฎาคม 2546-1 กรกฎาคม 2548)

โครงการ Cloning of 2'-deoxycoformycin gene cluster from *Streptomyces antibioticus*

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน

สมชัย พรบันลือลาภ

ชื่อสถาบันของหัวหน้าโครงการวิจัยผู้รับทุน

Department of Biochemistry

Kasetsart University

ชื่อนักวิจัยที่ปรึกษา

วิไล สันติโสภาศรี

ชื่อสถาบันของนักวิจัยที่ปรึกษา

Department of Biochemistry

Kasetsart University

สนับสนุนโดยทบวงมหาวิทยาลัย และ สำนักงานกองทุนสนับสนุนการวิจัย

สัญญาเลขที่ MGR4680105

โครงการ: Cloning of 2'-deoxycoformycin gene cluster from Streptomyces antibioticus

1.2 รายละเอียดผลการดำเนินงานของโครงการ

1. สรุปย่อ (Summary)ประกอบด้วยวัตถุประสงค์และการดำเนินงานวิจัยรวมทั้งผลงานวิจัย

Functional complementation approach had been used to clone a novel adenosine deaminase gene (*ada*), a gene that presumably confers *Streptomyces antibioticus* resistant to 2'-deoxycoformycin (2'-dCF). Plasmid library containing genomic DNA of *S. antibioticus* were prepared and transformed into *E. coli* Sϕ3834, a strain that had deletion of *ada* gene and mutation in *gua*A gene. The transformed colonies were screened for the presence of *ada* gene by their resistance to ampicillin and their ability to utilize diamino purine (DAP) for growth in minimal medium. Unexpectedly, all of the three positive clones obtained were found to contain the *gua*A gene rather than *ada* gene. In principle, there is equal chance of obtaining either *ada* or *gua*A gene with this approach. To test whether functional complementation could be used to clone *S. antibioticus ada* gene, putative adenosine/AMP deaminase gene from *S. coelicolor* were amplified by PCR, cloned into pBluescript, and transformed into Sϕ3834. The transformed cell carrying pBluescript-ADA failed to grow on minimal medium supplemented with DAP. This result suggests that functional complementation may not suitable for cloning of ADA from *Streptomyces* because substrate specificity of *Streptomyces* enzyme may be differed significantly from that of *E. coli*.

To over-express and characterize the putative ADA from *S. coelicolor*, the *ada* gene was inserted into pET-15b and transformed into BL21(DE3). Protein was purified and characterized. The result indicates the enzyme is a zinc metalloenzyme and could deaminate adenosine and 2'-deoxyadenosine but not AMP. Coformycin and 2'-dCF whose structures mimic the tetrahedral transition state of the reaction were found to be a potent inhibitor, with K_i of 2.5×10^{-10} and 2.5×10^{-9} M, respectively. Thus, it is proposed that the catalytic mechanism of ADA from *S. coelicolor* is probably similar to that of the murine enzyme.

2. ผลงานวิจัยที่ทำในรอบปี (Full year progress report)

(1). วัตถุประสงค์ของโครงการ (Specific aims)

2'-Deoxycoformycin (2'-dCF) and 9-β-*D*-arabinofuranosyladenine (ara-A) are two naturally occurring nucleoside antibiotics that have been isolated from *Streptomyces antibioticus* (1). 2'-dCF is a tight binding inhibitor of adenosine deaminase (ADA) and is successfully used in the treatment of T-cell acute lymphoblastic leukemia, chronic lymphoblastic leukemia, and hairy cell leukemia. When phosphorylated to ara-ATP, ara-A inhibits the replication of DNA virus. Both ara-A and 2'-dCF have been approved by the Federal Food and Drugs Administration (FDA) in the U.S.A to be used for treatments of various forms of leukemia.

Because antibiotics (i.e., 2'-dCF) produced by *Streptomyces* are often toxic to the organisms, therefore, the ability of *Streptomyces* to tolerate the inhibitory effect of antibiotics is an essential prerequisite for antibiotic biosynthesis (2). Recently, two distinct forms of adenosine deaminase, designated as ADA-I and ADA-II, had been purified from *S. antibioticus* (3). The molecular weight of these two enzymes is approximately the same. The molecular weight of ADA-I is 40 KDa and that of ADA-II is approximately 37 KDa. These two enzymes, however, are markedly differed in the inhibition by 2'-dCF. Inhibition studies indicated that ADA-I is inhibited by 2'-dCF but not ADA-II. This data suggested that ADA-II is the key enzyme that conferred *S. antibioticus* selectively resistant to 2'-dCF during the biosynthesis of 2'-dCF.

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. By cloning of the antibiotic resistant gene, namely *ada*-II, the entire genes encoding for 2'-dCF biosynthetic enzymes can be obtained. Thus, the aim of this project is to clone the novel adenosine deaminase, namely ADA-II, from *S. antibioticus* by functional complementation.

(2). การดำเนินงานวิจัยตามตามวัตถุประสงค์ (Materials and methods)

Bacterial strains, plasmids, and media – *E. coli* strain sϕ3834 (rpsL, Δadd-uid-man, metB, guaA, uraA::Tn10) and *Streptomyces antibioticus* was from Dr. Robert Suhadolnik (Temple University School of Medicine, USA). *E. coli* strain DH5α and pBluescript were from Stratagen. The pET-15b was from Novogen. The plasmid map is shown in Figure 1. *E. coli* culture was grown either in LB broth or minimal medium as described by Sambrook et al (4). *S. antibioticus* was grown on YEME medium

according to Hopwood et al (5). Where applicable, medium was supplemented with 100 µg/ml ampicillin, 33 µg/ml tetracycline, or 50 µg/ml streptomycin sulfate. Restriction enzymes and DNA size marker were from New England BioLabs, Inc. QIAEX-II gel extraction kit was from Qiagen. Diaminopurine (DAP), isopropylthio-β-galactoside (IPTG) and thiamine were purchased from Sigma. All other chemicals were of the highest purity commercially available.

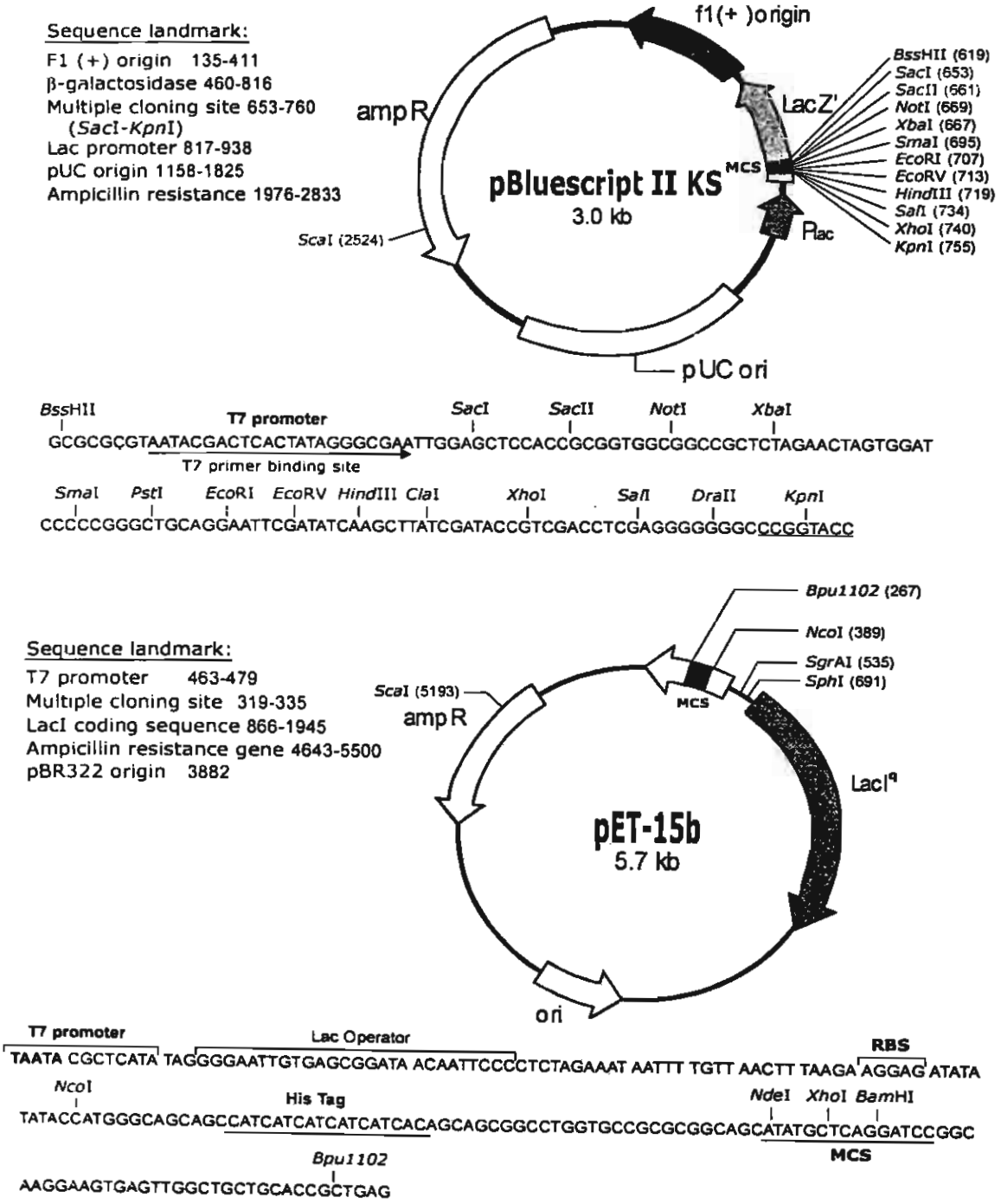

Figure 1. Diagram of pBluescript and pET-15b. Multiple cloning sites (MCS) are in black boxes. Note that pBluescript also contain site for the universal T_7 primer and T_3 primer.

Gene manipulation – Chromosomal DNA from *Streptomyces* was isolated as described by Hopwood et al (5). The plasmid was propagated on *E. coli* DH5α, isolated and purified by the standard alkali lysis procedure as described (1). Competent cells were prepared either by simple or complex multivalent cations procedure as described by Sambrook et al (4). Ligation of 8-10 kb *S. antibioticus* DNA to the *Bgl*II digested/alkali phosphatase dephosphorylated pBluescript was performed as described by the manufacturer, using T4 DNA ligase. *S. antibioticus* DNA and plasmid were ligated at the DNA ratio of 3:1 with T4 DNA ligase for 6 hours at 10°C. The ligated DNA was transformed into competent *E. coli* Sϕ3834 as described (4). After allowing cells to recover by growing in SOC medium for 45 hours, cells were platted on LB plates supplemented with ampicillin.

Screening of Clone Containing ada gene by Functional complementation - A two-step method was used to screen *E. coli* Sϕ3834 for clone that contains either *ada* or *gua*A gene (figure 2). The transformed colonies were first selected for the presence of plasmid on LB medium plus ampicillin. These colonies were subsequently screened for the presence of *ada* gene by the ability to grow on minimal medium plates containing 2,6-diaminopurine (DAP) with tooth-pick (figure 2). Positive clones obtained were screened for the presence of DNA insert on LB containing X-Gal.

Detection of DNA Insert in Recombinant Plasmid by Polymerase Chain Reaction - The presence of *gua*A gene in the recombinant plasmid isolated from positive clones was detected by polymerase chain reaction (PCR), using primers contain the sequence complementary to the conserved region of *gua*A gene (Table 1). Reaction mixtures of 15 μl contained 300 ng of recombinant plasmid, 1x PCR buffer, 0.15mM dNTP, 1.2mM $MgCl_2$, 0.5 unit Tag DNA polymerase, 0.13 μM Gua1 (5'-CAGTTCCA CCCGGAAGT-3') and Gua2 (5'-CCCGGTCCCGGGAACGG-3') primers. Amplification of *gua*A gene was performed on Perkin Elmer Thermal Cycle. Template DNA was denatured at 93°C for 1 min, annealed at 53°C for 40 sec, and extended at 72°C for 1 min 40 sec for 35 cycles. PCR product was analyzed on 1.2% agarose gel electrophoresis.

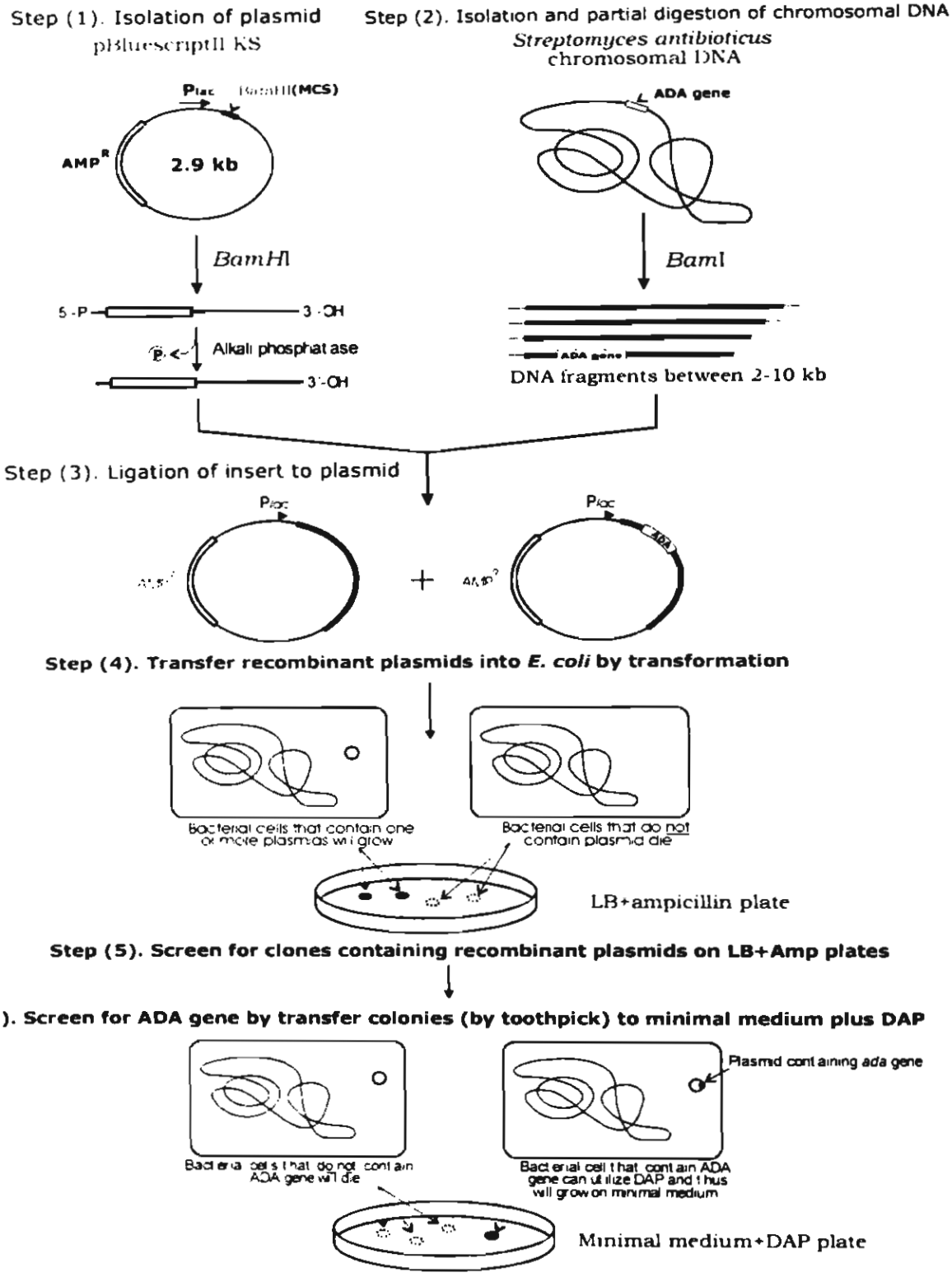

Figure 2 Outline of Strategy of Cloning of *Streptomyces antibioticus* Adenosine Deaminase Gene by Functional Complementation Approach.

Table 1. List of oligonucleotide primers

Primers	Sequence(5'→3')	Length (nt)	%GC	Tm (°C)*
Universal primers of pBluescriptII KS*				
T_3	5' AATTAACCCTCACTAAAGGG 3'	20	40	56
T_7	5' GCCCTATAGTGAGTCGTATTAC 3'	22	45	64
Primers of adenosine deaminase gene for *S. coelicolor*				
Nok1	5' TTTAAGCTTCATATGACGAGCCGAAGCACCGAG 3'	33	48	69
Nok2	5' TTTAGATCTTCAGCCGTCGGATTCCGAAGA3'	30	46	67
Primers for conserved region of GMP synthetase gene				
Gua1	5' CAGTTCCACCCGGAAGT 3'	17	59	54
Gua2	5' CCCGGTCCCGGGAACGG 3'	17	82	62

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

Amplification and Cloning of the ada Gene from Streptomyces coelicolor – To clone the *ada* gene from *S. coelicolor*, chromosomal DNA was isolated and used as template for PCR amplification. Based on the nucleotide sequence of the putative adenosine/AMP deaminase gene obtained from Genebank (Accession No. SCO4901, figure 3), forward primer (Nok-1: 5'-TTTAAGCTTCATATGACG AGCCGAAGCACCGAG-3', where the underline indicated the added *Hind*III and *Nde*I linker) and reverse primer (Nok-2: 5'-TTTAGATCTTCAGCCGTCGGATTCCGAAGA-3', where the underline indicated the added on *Bgl*II linker) were synthesized by KU-vector at Kasetsart University. The optimum PCR conditions were first established for efficient amplification of the *ada* gene and then subsequently used as condition for amplification. Reaction mixture contained 100 ng of *S. coelicolor* chromosomal DNA, 0.3 μM of forward and reverse primers, 200 μM of dNTP, 0.06 unit of Tag DNA polymerase in 50 mM Tris-HCl pH 8 9, 2.5 mM $MgCl_2$, and 1% Triton 100X. The reaction was initiated by denaturation at 93°C for 5 min and subjected to 35 cycles of 1 min denaturation at 93°C, 1 min of annealing at 68°C, and 1.3 min of extension at 72°C. After 35 cycles, the reaction was incubated at 72°C for 10 min and then cooled to 4°C. After electrophoresis, the amplified ~1.1 kb PCR product was gel purified and ligated into pGEM-T vector (25 ng), using 1.5 units of T_4 DNA ligase. The ligated DNA was transformed into competent *E. coli* JM109 and platted on LB supplement with ampicillin, IPTG and X-Gal. One of the

white colonies were found to contain the *ada* gene after digestion with *Nde*I and *Bgl*II. Colony that contained the plasmid with *ada* gene was grown overnight in 100 ml LB and plasmid was purified by alkali method. The purified plasmid (20 µg) was double digested with *Nde*I and *Bgl*II and separated on 1.5% agarose gel. The 1.1 kb fragment was gel purified with QIAEXII gel extraction kit (Qiagen) and ligated to pET-15b which had been previously double digested with *Nde*I and *Bam*HI. The ligation mixture was transformed into *E. coli* BL21 (DE3) and platted on LB containing 60 µg/ml ampicillin. 100 colonies were obtained. After screened 20 colonies for recombinant plasmid by digestion with *Nde*I restriction endonucleases, one colony was found to contain the *ada* gene. This recombinant plasmid was designated as pET-*ada*.

Assay for Adenosine Deaminase Activity - The activity of adenosine deaminase was assayed spectrophotometrically by the decrease in absorbance at 265 nm on conversion of adenosine to inosine with Shimazu spectrophotometer. One milliliter reaction containing 0.04-0.08 µmole of adenosine in 50 mM potassium phosphate was incubated for 1 min a cuvette prior to the addition of enzyme. The amount of production formed was calculated using molar extinction coefficient of 8.3 mM^{-1}. For determination of the K_m and V_{max} for ara-A, 2'-deoxyadenosine, Adenine, AMP, ADP, ATP, the same wavelength and molar extinction coefficient (8.3 mM) was used. One unit of enzyme activity is defined as the amount of enzyme needed to convert 1 µmole of substrate into product at 37°C in minute. Protein concentration was determined by the Bradford method, using bovine serum albumin as standard.

Purification of the Recombinant Adenosine Deaminase from S. coelicolor - For large scale purification of the adenosine deaminase from *S. coelicolor*, 50 ml of *E. coli* BL21 (DE3) carrying pET-*ada* grown overnight culture in 50 ml of LB supplemented with 60 µg/ml ampicillin was transfer to 1 L of LB plus ampicillin. The culture was allowed to grown to reach an OD_{600} nm of 0.5, the expression adenosine deaminase was induced by addition of 1 mM lactose. After allowed cells to grow for 5 additional hours, cell were harvested by centrifugation at 10,000 rpm at 4°C and suspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 0.5% Tween 20 and 1 mM PMSF]. After breaking cell by sonication, cell-free extract was obtained by centrifugation at 10,000 rpm. The enzyme in supernatant was precipitated by addition of ammonium sulfate at 80% saturation. After centrifugation at 10,000 rpm, the pellet was dissolved in 4 ml of lysis buffer and dialyzed against 3 liters of 50 mM potassium phosphate (pH 7.5)

```
                10        20        30        40        50
  1 ATGACGAGCCGAAGCACCGAGAAGAGCGCCGCGGCGAACCCCGCGGCGGTCTCGAAGACA
    M  T  S  R  S  T  E  K  S  A  A  A  N  P  A  A  V  S  K  T
 61 CCGTCGCCGGACCGGATCCGCCGGGCACCGAAGGTATTGCTGCACGACCACCTCGACGGC
    P  S  P  D  R  I  R  R  A  P  K  V  L  L  H  D  H  L  D  G
121 GGTCTGCGGCCGGGCACGATCGTGGAACTCGCCCGCGAGACGGGCTACGGGGACCTTCCC
    G  L  R  P  G  T  I  V  E  L  A  R  E  T  G  Y  G  D  L  P
181 GAAACCGACGCCGACCTGCTCGGCACCTGGTTCCGGCAGGCCGCCGACTCCGGGTCCCTG
    E  T  D  A  D  L  L  G  T  W  F  R  Q  A  A  D  S  G  S  L
241 GAGCGGTACCTGGAGACCTTCTCCCACACCGTCGGCGTCATGCAGACCCGCGACGCCCTG
    E  R  Y  L  E  T  F  S  H  T  V  G  V  M  Q  T  R  D  A  L
301 GTCCGGGTCGCCGCCGAGTGCGCCGAGGACCTCGCCGAGGACGGCGTCGTCTACGCCGAG
    V  R  V  A  A  E  C  A  E  D  L  A  E  D  G  V  V  Y  A  E
361 GTGCGGTACGCGCCCGAGCAGCACCTGGAGAAGGGGCTGACCCTCGAAGAGGTCGTCGAG
    V  R  Y  A  P  E  Q  H  L  E  K  G  L  T  L  E  E  V  V  E
421 GCCGTCAACGAGGGATTCCGGGAGGGCGAACGTCGCGCCCGGGACAACGGCCACCGCATC
    A  V  N  E  G  F  R  E  G  E  R  R  A  R  D  N  G  H  R  I
481 CGGGTCGGCGCCCTGCTCACCGCCATGCGGCACGCCGCCCGCTCCCTGGAGATCGCCGAA
    R  V  G  A  L  L  T  A  M  R  H  A  A  R  S  L  E  I  A  E
541 CTCGCCAACCGGTACCGCGATCTCGGCGTGGTCGGCTTCGACATCGCGGGCGCCGAGGCC
    L  A  N  R  Y  R  D  L  G  V  V  G  F  D  I  A  G  A  E  A
601 GGCTACCCGCCCACCCGGCACCTGGACGCCTTCGAGTACCTGAAGCGGGAGAACAACCAC
    G  Y  P  P  T  R  H  L  D  A  F  E  Y  L  K  R  E  N  N  H
661 TTCACCATCCACGCGGGCGAGGCCTTCGGGCTGCCGTCCATCTGGCAGGCGCTGCAATGG
    F  T  I  H  A  G  E  A  F  G  L  P  S  I  W  Q  A  L  Q  W
721 TGCGGCGCCGACCGGCTCGGGCACGGGGTGCGCATCATCGACGACATCCAGGTCCACGAG
    C  G  A  D  R  L  G  H  G  V  R  I  I  D  D  I  Q  V  H  E
781 GACGGCTCGGTCAAGCTCGGGCGGCTCGCCTCCTACGTCCGCGACAAGCGGATCCCGCTG
    D  G  S  V  K  L  G  R  L  A  S  Y  V  R  D  K  R  I  P  L
841 GAGCTGTGCCCCAGCTCCAACCTCCAGACCGGCGCCGCCGACTCCTACGCCGAGCACCCC
    E  L  C  P  S  S  N  L  Q  T  G  A  A  D  S  Y  A  E  H  P
901 ATCGGGCTGCTGCGCCGGCTGCACTTCCGGGCCACCGTCAACACCGACAACCGGCTGATG
    I  G  L  L  R  R  L  H  F  R  A  T  V  N  T  D  N  R  L  M
961 TCCCACACCAGCATGAGCCGGGAATTCGAGCACCTGGTCGAGGCATTCGGCTACACGCTC
    S  H  T  S  M  S  R  E  F  E  H  L  V  E  A  F  G  Y  T  L
1021 GACGACATGCAGTGGTTCTCCGTCAATGCGATGAAATCAGCGTTCATTCCTTTCGATGAA
    D  D  M  Q  W  F  S  V  N  A  M  K  S  A  F  I  P  F  D  E
1081 CGACTGGCCATGATCAATGACGTCATCAAGCCCGGATATGCCGAGCTGAAATCCGAATGG
    R  L  A  M  I  N  D  V  I  K  P  G  Y  A  E  L  K  S  E  W
1141 CTGTTCCAGCAGACGGCTTCGACCAGCGGTTCTTCGGAATCCGACGGCTGA
    L  F  Q  Q  T  A  S  T  S  G  S  S  E  S  D  G  *
```

Figure 3. Nucleotide sequence and amino acid sequence of putative adenosine deaminase/AMP deaminase obtained from Genebank (Accession No. SCO4901) from *Streptomyces coelicolor*. The stop codon is represented by asterisk.

containing 1 mM EDTA for four times. The dialyzed protein was added onto Ni^{2+}-NTA resin (1 ml bed volume), which had been previously equilibrated with buffer B [20 mM Tris-HCl (pH 8), 500 mM KCl, and 0.1% Triton X-100). The column was washed with 4 ml of buffer B. The polyhistidine-tagged enzyme was eluted by washing column with buffer B containing 20, 100, and 250 mM imidazole, respectively. The enzyme eluted from column was analyzed on 10% SDS-PAGE.

Analysis of Kinetic Data - Kinetic data were fitted to the appropriate equations using Sigma Plot program. The initial velocity (v) obtained by varying the concentration of substrate was fitted into equation 1, where V_{max} is the maximum velocity, S is substrate concentration, and K_m is the Michaelis constant. Kinetic data obtained from competitive inhibition was fitted into equation 2, where I is the concentration of inhibitor and K_i is the inhibition constant.

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \text{Equation 1}$$

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \qquad \text{Equation 2}$$

(3). ผลงานวิ (Results)

Cloning of Novel adenosine deaminase from S. antibioticus – To clone novel adenosine deaminase from *S. antibioticus,* chromosomal DNA was partially digested with *Bgl*II at DNA/enzyme ratio of 0.6 µg/unit. Under this condition, DNA fragment ranging mostly between 8-10 kb were obtained. These fragments were gel purified, ligated into pBluescript, and transformed into competent *E. coli* Sϕ3834. A two-step method was used to screen the transformed Sϕ3834 clones that contain *ada* gene. This involved screening transformants for (*i*) its ability to grow on LB plates plus ampicillin and (*ii*) its ability to grow on minimal plates plus diaminopurine (DAP). When the transformants were screened for their ability to grow on LB plus ampicillin plates, 10,250 clones were obtained. These colonies were subsequently screened for the presence of *ada* gene by their ability to grow on minimal plates plus diaminopurine (DAP). Three positive clones were obtained.

These 3 positive clones were further characterized by their ability to grow either in LB or minimal plates containing various antibiotics. Because all three clones grew in media containing tetracycline and streptomycin, this result suggested that they are Sϕ3834 and not other bacterial contaminants (Figure 4A and 4B). To confirm that they carried pBluescript, the three clones were streaked on LB supplemented with ampicillin. All three clones but not the untransformed Sϕ3834 were able to grow, indicating that they carried pBluescript (Figure 4C). Because pBluescript contain a multiple cloning site within the α-coding region of the β-galactosidase, insertional inactivation of the α-

peptide by the inserted DNA allows recombinant clones to be directly identified by blue-white screening on LB plus X-gal. The result showed that all 3 positive clones turned white, indicating that they all contained DNA inserts (Figure 5A). To differentiate whether they contained *ada* or *gua*A gene, the 3 positive clones were plated on minimal medium plate supplemented with or without DAP. In minima medium. supplemented with DAP, clone which contained *ada* gene will able to utilize DAP for synthesis of guanine and grow. If the *gua*A gene had been cloned instead, the 3 positive clones would grow on minimal medium without supplemented with DAP. The result showed that all 3 clones could grow on minimal medium either with or without supplemented with DAP (Figure 5B and 5C). This data suggested that they contained *gua*A gene rather than *ada* gene.

To determine the size of DNA inserted in the recombinant plasmid, the three clones obtained were individually inoculated into 200 ml LB plus ampicillin and plasmids were purified. However, these three clones grew extremely slow in LB plus ampicillin and it took two days to obtain sufficient quantity of cells. Furthermore, these clones can be replatted only a few generations, indicating that they were unstable. When plasmids were isolated from these cell cultures, only 5-10 μg of plasmids DNA per 200 ml of cell culture was obtained. This result suggested that the plasmid carried *gua*A gene may be unstable or toxic to the host. It had been reported by other laboratories that expression of *gua*A gene in high copy number plasmid was toxic to *E. coli* host. Determination of the size of plasmid by digestion with *Eco*RI indicated that the size of insert in clone 1 was approximately 10 kb (data not shown).

To confirm that the recombinant plasmid contained the *gua*A gene, specific primer Gua2 that contain sequence complementary to the highly conserved region of *gua*A gene was synthesized and used to amplify the *gua*A gene by polymerase chain reaction. When T3 which is universal primer for pBluescript and Gua2 were used as the forward and reverse primers, amplification of recombinant plasmid isolated from clone 3 resulted in single band with size approximately 2.5 kb (figure 6, lane 2). This result confirmed that clone 3 carried pBluescript with *gua*A gene rather than *ada* gene. However, because the objective of this project is to clone *ada* gene rather than *gua*A gene, the nucleotide sequence of the DNA insert had not been sequenced and will not be analyzed further.

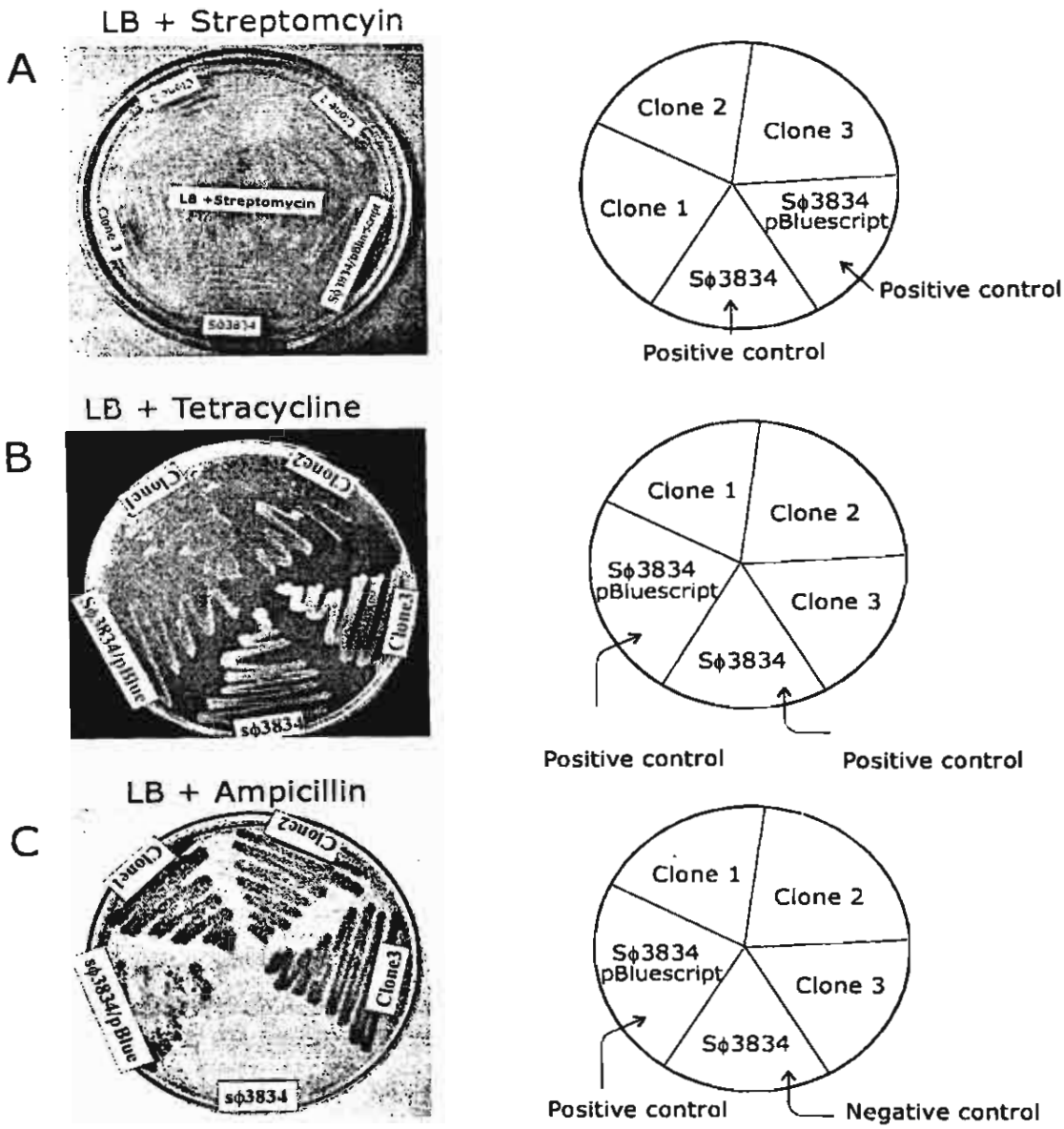

Figure 4. Growth of the three positive clones on L-broth medium supplemented with various antibiotics. A- LB plus tetracycline plate. B- L-broth plus streptomycin plate. C- LB plus ampicillin plate.

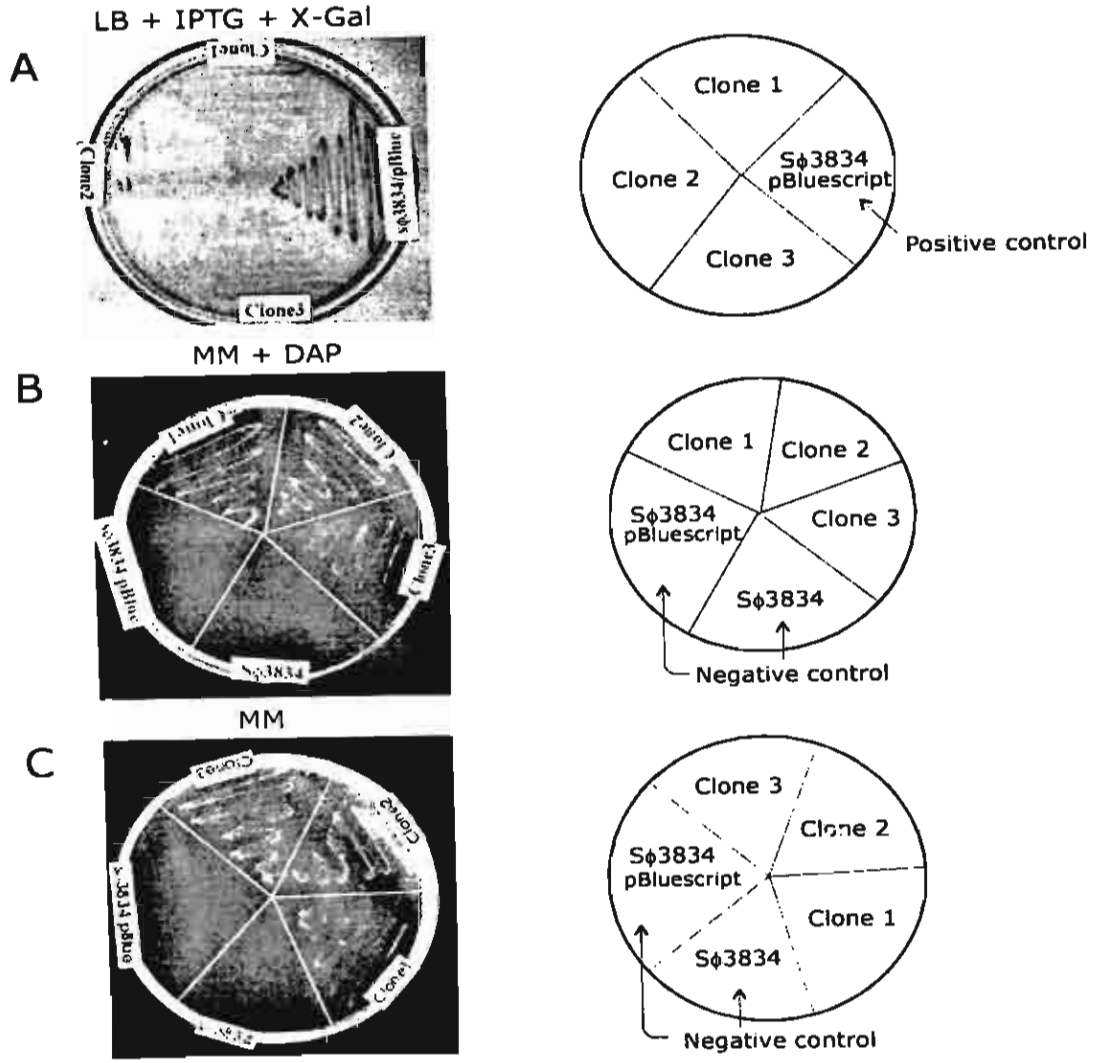

Figure 5. Growth of the three positive clones on Minimal medium supplemented with or without diaminopurine: (A) Minimal medium plus 2, 6-diaminopurine plate, (B) Minimal medium without 2,6-diaminopurine.

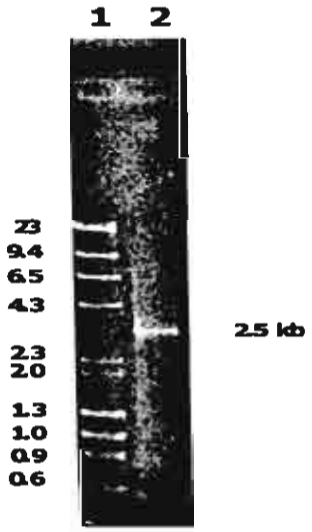

Figure 6. Analysis of the PCR product on 1.2 % gel. *Lane* 1, markers; *lane* 2, PCR amplification of the guaA gene from plasmid isolated from clone 3 using T3 and Gua2 as primers.