PCR Amplification of the ada Gene from S. Coelicolor using Designed Primers – The attempt to clone novel adenosine deaminase from *S. antibioticus* by functional complementation resulted in clones containing *gua*A gene rather than *ada* gene. To determine whether functional complementation is suitable for cloning of *ada* gene, the gene coding for putative adenosine /AMP deaminase gene from *S. coelicolor* had been amplified by PCR reaction and used as positive control for the experiment. Using chromosomal DNA from *S. coelicolor* as template and the designed forward and reverse primer (Table 1), a 1.1 kb PCR product that corresponded to the expected full length adenosine/AMP deaminase gene was obtained (Figure 6). This PCR product was ligated into pGEM-T vector, transformed into *E. coli* JM109, and streaked on LB contain IPTG and X-Gal. Ten white colonies were obtained. One white colony was found to contain pGEM with 1.1 kb insert, pGEM-a*da*. This colony was inoculated into 100 ml LB contain ampicillin, grown overnight and the plasmid was by alkali lysis method. The obtained pGEM-*ada* was double digested with *Nde*I and *Bam*HI and the 1.1 kb fragment was gel purified. This gel purified 1.1 kb fragment was ligated into pET-15 that had been previously digested with *Nde*I and *Bgl*II. The ligation mixture was transformed into the expression host, BL21 (DE3). One colony was found to contain plasmid, designated as pET-*ada*, was found to contain 1.1 kb insert. The presence of the 1.1 kb insert was determined by digesting pET-*ada* with *Nde*I. Digesting of the pET-ada with *Nde*I resulted in two bands, one migrating at size corresponding to a 6.8 kb fragment (Figure 7, lane 5).

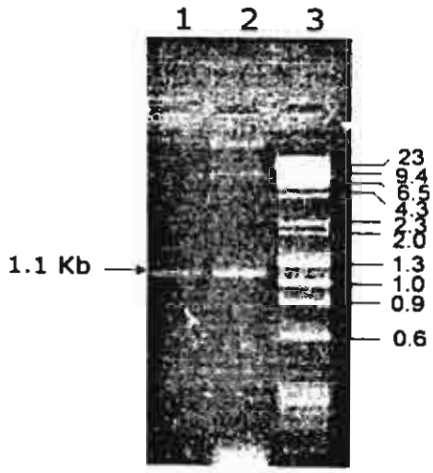

Figure 7 PCR amplification of the *S. coelicolor* chromosomal DNA with the designed primers. *Lane* 1, PCR product obtained using 50 ng of chromosomal DNA as template; *lane* 2, PCR product obtained using 100 ng of chromosomal DNA as template; *lane* 3, mixture of λ DNA digested with *Hind*III and ϕX 174 DNA digested with *Hae*III.

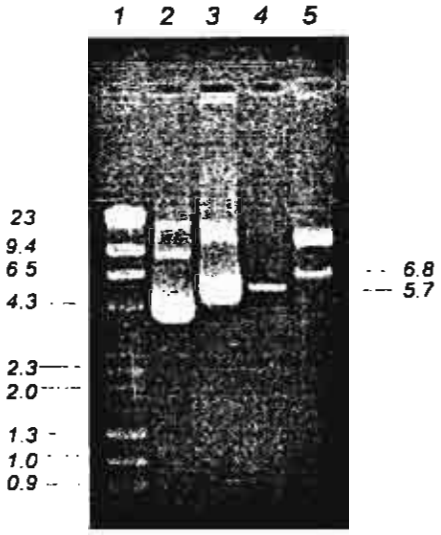

Figure 8. Determination of the presence of the *S. coelicolor ada* gene in pET-*ada*. *Lane* 1, I DNA digested with *Hind*III and ϕX 174 DNA digested with *Hae*III; *lane* 2, super-coiled pET-15b; *lane* 3, supercoiled pET-*ada*; *lane* 4, linear pET-15b by digestion with *Eco*RI (5.7 kb); *lane* 5, pET-*ada* linearizd by digestion with *Nde*I

Purification of the Adenosine Deaminase from E.coli BL21(DE3) Carrying the Recombinant pET-ada – Adenosine/AMP deaminase from *S. coelicolor* had been purified from 1 L of culture of *E. coli* BL21(DE3) carrying the pET-*ada*. This purification scheme resulted in 16.3 mg of approximately 90% pure protein, with 70% recovery. The specific activity of the purified protein is 27 µmole/min/mg. The recombinant enzyme contained polyhistidine-tagged at its N-terminus can be easily purified with Ni^{2+}-NTA affinity column. The purification scheme of the enzyme is summarized in table 2. The purity of enzyme at each step of purification was also monitored on SDS-PAGE gel electrophoresis (Figure 9). Upon induction with lactose, approximately 15-20% of the total protein was consisted of the enzyme (Figure 9, *lane* 2). When loaded onto the Ni^{2+}-NTA column, the enzyme bound tightly to the column. After washing the column with buffer B containing 500 M NaCl, the enzyme was eluted with buffer B containing 100 mM imidazole. The eluted adenosine deaminase that contained polyhistidine-tagged at its N-terminus migrated on SDS-PAGE gel with molecular weight of approximately 46 KDa (Figure 9, *lane* 7).

Table 2. Summary of the Purification of adenosine deaminase from *S. coelicolor*.

Step	Total Protein (mg)	Total Unit (μmole/min)	Specific activity (μmole/min/mg)	Fold purification	Recovery (%)
Cell-free extract	240	72.0	0.3	1.0	100
$(NH_4)_2SO_4$	19.2	65.3	3.4	11.3	91
Ni^{2+}-NTA affinity	16.3	55.5	8.1	27.0	70

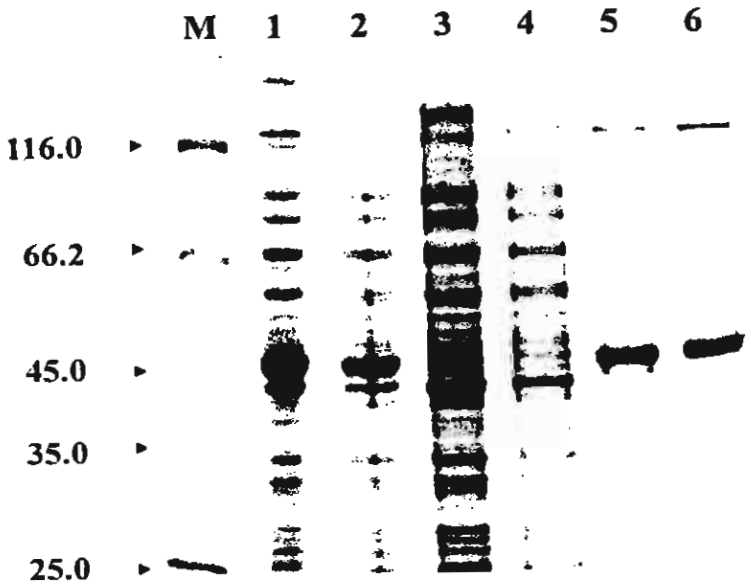

Figure 9. Analysis of the purification of adenosine deaminase in each step of purification. *Lane 1*, molecular marker; *lane 2*, cell-free extract after induced with lactose for 4 hours; *lane 3*, ammonium sulfate fractionation; *lane* 4, flow through fraction; *lane* 5, protein eluted with buffer contain 20 mM imidazole; *lane* 7, protein eluted with 100 mM imidazole; and *lane* 8, protein eluted at 250 mM imidazole.

Substrate Specificity of Adenosine/AMP deaminase from *S. coelicolor*

The K_m, K_i, and V_{max} values of purified putative adenosine/AMP deaminase with substrates/inhibitors are shown in Table 3. Substrate specificity studies showed that adenosine is the best substrate with relative V_{max} of 100%. Removal of the ribosyl moiety from adenosine (i.e., adenine) resulted in completely loss of activity as substrate (V_{max} <0.01%). Substitution of the hydroxyl group at 2'-position with hydrogen (i.e., 2'-deoxyadenosine) and inversion of the hydroxyl group at the 2'-position (i.e., ara-A) resulted in loss of 84% and 99% activity. Additions of the phosphate group(s) to the 5'-

postion of the ribosyl moiety (i.e., 5'-AMP, 5'-ADP, and 5'-ATP) resulted in completely loss of activity as substrate. This data clearly showed that the purified enzyme is indeed adenosine deaminase and not AMP deaminase. Coformycin and 2'-deoxycoformcyin with the sp^3 hybridization at the C-6 position of the purine ring had been proposed to mimic the transition state formed in the deamination reaction. Studies of the inhibition of *S. coelicolor* adenosine deaminase by these hypothetical transition state analogs showed that these analcgs are potent inhibitor of the enzyme. Thus, this enzyme is competitively inhibited by coformycin and 2'-deoxycoformycin with K_i of 2 x 10^{-12}M and 2 x 10^{-13} M, respectively (data not shown).

Table 3. Comparison of K_m, K_i, and V_{max} values for adenosine analogs interaction with adenosine deaminase from *S. coelicolor.*

Substrate or inhibitor	K_m (μM)	K_i (μM)	V_{max} (μmole/min/mg)	Relative V_{max} (%)
Substrates				
Adenosine	100		23.0	100
2'-Deoxyadenosine	500		3.7	16
Ara-A	ND			< 0.1
Adenine	ND			< 0.01
5'-AMP	ND			< 0.1
5'-ADP	ND			< 0.1
5'-ATP	ND			< 0.1
Inhibitors				
Coformycin		0.00025		
2'-Deoxycoformcyin		0.0025		

ND -Not determined

Finally, substrate specificity studies had shown that putative gene isolated from *S. coelicolor* is adenosine deaminase rather than AMP deaminase. To determine whether functional complementation can be used to clone adenosine deaminase from *S. antibioticus*, *ada* gene from *S. coelicolor* was cloned into pBluescript and then transformed

into Sϕ3834. The transformants was platted onto minimal plate supplemented with DAP. This experiment should serve as a positive control and provide us with information of whether functional complementation can be used to clone ada gene from *S. antibioticus*. When Sϕ3834 carried pBluescript-ada was platted onto minimal medium supplemented with DAP, no growth was observed on the plate even after two days (Result not shown). This result indicated to us that functional complementation could not be used to clone ada gene from S. antibioticus. One possible explanation for this observation is that, unlike other deaminases, adenosine deaminase from *S. antibioticus* is extremely specific for its substrate adenosine and could deaminate diaminopurine riboside to guanosine. This resulted in the ability of the clone to utilize the supplemented DAP and could not growth on minimal medium supplemented with DAP.

DISCUSSION

E. coli Sϕ3834, a mutant that had a deletion of *ada* gene and mutation in *gua*A gene, had been constructed by Chang *et al* for the purpose of cloning *ada* genes from other source by the functional complementation (9). This *E. coli* strain had been successfully used in cloning of the *E. coli* adenosine deaminase (9). The rational of this approach is as the following. AMP and GMP which are precursors for biosynthesis of nucleic acids can be either supplied by the *de novo* or salvage pathway (Figure 10). Mutation in *gua*A gene in *E. coli* Sϕ3834 prevents the conversion of XMP to GMP and therefore results in a blockage of *de novo* biosynthesis of GMP (Figure 10A). Thus, this mutant has to depend on its salvage pathway to utilize exogenous purines (i.e., guanine, hypoxanthine, guanosine, or inosine) as precursors for synthesis of nucleotide. Deletion of *ada* gene from Sϕ3834 results in the inability to utilize exogenous adenine or adenosine for synthesis of inosine and IMP. However, when minimal medium was supplemented with guanine or hypoxanthine, mutant will grow because they can convert these bases to their corresponding nucleosides (e.g., inosine or guanosine), which are subsequently phosphorylated to corresponding nucleotides (e.g., IMP or GMP).

With the except of the presence of amino group at the C-2 position, 2,6-diaminopurine riboside (DAP-R) is structurally similar to adenosine and is a moderate substrate for adenosine deaminase. Thus 2,6-diamopurine riboside will be deaminated to guanosine by the action of adenosine deaminase. When mutant *E. coli* Sϕ3834 received the *ada* gene though transformation, it will able to utilize 2,6-diamopurine

supplemented and grow on minimal medium, because 2,6-diamopurine and *D*-ribose 1-phosphate can be converted to 2,6-diaminopurine riboside (DAP-R) by action of purine nucleoside phosphorylase (PNPase) (Figure 10B). Deamination of DAP-R by adenosine deaminase would result in formation of guanosine, which is subsequently phosphorylated GMP and provided nucleotide precursor that is essential for growth. As a result, *E. coli* Sϕ3834 carrying plasmid containing *ada* gene will grow on medium minimum medium supplement with DAP.

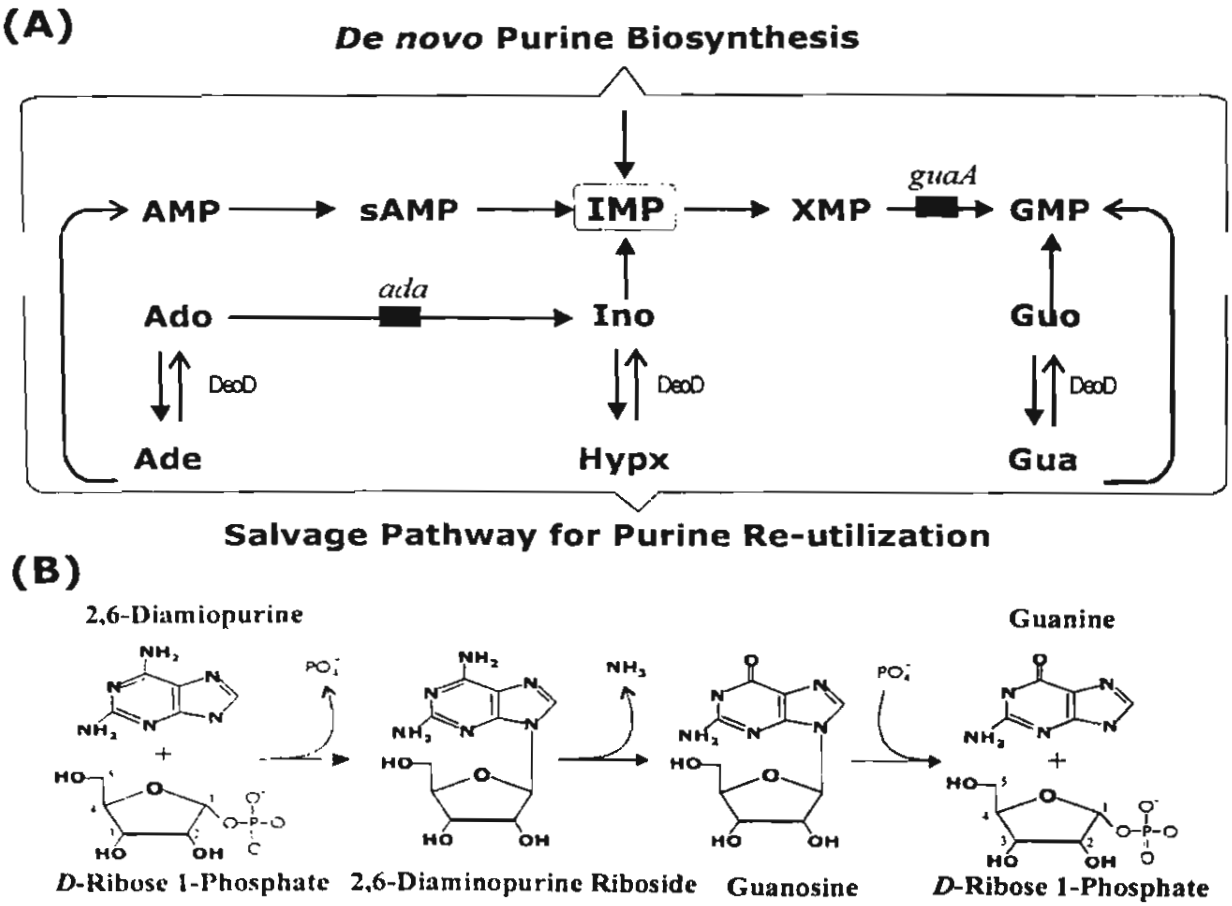

Figure (10). Purine metabolic pathways in the *E. coli* mutant Sϕ3834. Panel A shows purine metabolism in *E. coli*. Sϕ3834 (*rps*L, *ada-uid-man*, *met*B, *gua*A, *ura*A::Tn10) is a purine requiring nutritional auxotroph, which has a deletion of *ada* gene and mutation in *gua*A gene. The mutation in the *gua*A gene results in the loss of the guanylate synthetase activity and provide the basis for the guanine (G) or guanosine (GR) requirement. Addition abbreviation; Ade, adenine; Ado, adenosine; Hyx, hypoxanthine, Ino, inosine. Panel B shows the pathway leading to the synthesis of guanine and guosine from 2,6-diaminopurine (DAP) and *D*-ribose 1-phosphate by adenosine deaminase (*ada*) and purine nucleoside phosphorylase (*deo*D). Sϕ3834 can grow on DAP if adenosine deaminase is provided because GMP can be synthesized from guanosine or guanine as indicated.

Cloning and Sequencing of the *gua*A gene from *S. antibioticus*

In the attempt to clone the *ada* gene from *S. antibioticus* by functional complementation, clones that carried *gua*A gene rather than *ada* gene had been obtained unexpectedly. To confirm that the recombinant plasmid isolated from these clones contain *gua*A gene, a set of primers that contained sequence complementary to the highly conserved region in the *gua*A gene was used to amplify the *gua*A gene by PCR reaction (Figure 6). PCR amplification resulted in a single band with size approximately 800 bp, indicating that recombinant plasmid indeed contain *gua*A gene. However, since our original goal is not to clone the *gua*A gene, the nucleotide sequence of *gua*A gene carried on the recombinant plasmid isolated will not be further characterized or sequenced.

Cloning of Adenosine Deaminase gene from *S. antibioticus*

In attempt to clone *ada* gene from *S. antibioticus* by functional complementation, it was surprising that all of the clones obtained contained *gua*A gene rather than *ada* gene. In principle, with functional complementational approach, there will be equal chance of obtaining *gua*A gene and *ada* gene. Thus, an alternate approach was used to clone the *ada* gene from *S. antibioticus*. Because alignment of the amino acid sequences of *E. coli*, human, and murine adenosine deaminase revealed several highly conserved regions, namely HLDG, DLAGDE, TVHAGE and NTDDP, thus a set of degenerated forward primers that contained the highly conserved HLDG and reverse primers that contained the highly conserved TVHAGE of the *ada* gene were synthesized and used to amplify the *S. antibioticus ada* gene by PCR amplification. The single PCR product with approximately 500 bp was obtained from using ADA primers. However, when this 500 bp fragment was sequenced, its nucleotide sequence did not show any similarity to adenosine deaminase. This data suggested that highly conserved region found in *ada* gene found in several organisms may be slightly different in *S. antibioticus*. In summary, these highly conserved HLDG and TVHAGE that existed among *E. coli*, human, murine adenosine deaminase may not be conserved or slightly altered in *S. antibioticus* adenosine deaminase.

Because cloning of *ada* gene from *S. antibioticus* by functional complement had failed to obtain clone that contained the *ada* gene, there are at least three possible explanations for this result. First, the structural gene of the *ada* gene from *S. antibioticus* may contain an internal *Bam*HI site. Thus, when *S. antibioticus*

chromosomal DNA was digested with *Bam*HI into 2-8 kb fragments, the *ada* gene might be also being digested. Second, the *ada* gene from *S. antibioticus* might not express well in *E. coli*, since the codon usage between two microorganisms are differed markedly. For example, the codon AAA that specified for lysine occurred at 36% in *E. coli* but only at 1.3% in *S. antibiotiucs* (Table 4). Finally, the substrate specificity of *E. coli* adenosine deaminase may be significantly differed from that of *S. antibioticus*. For example, 2,6-diaminopurine riboside which is substrate for *E. coli* adenosine deaminase can be converted to guanosine. However, 2,6-diamopurine riboside may or may not be the substrate for *S. antibioticus* adenosine deaminase, since this nucleoside analog had not been tested as substrate for *S. antibioticus* adenosine deaminase. Therefore, 2,6-diaminopurine that was supplemented into the minimal medium may be unable to be utilized by clone that contained the recombinant plasmid that carried the *S. antibioticus ada* gene as precursor for synthesis for guanosine.

Because the nucleotide sequence of putative adenosine/AMP deaminase from *S. coelicolor* is known, thus, to determine whether functional complementation can be used to clone the *ada* gene from *S. antibioticus*, and the *ada* gene from *S. coelicolor* was amplified by PCR and inserted into pBluescript. The obtained recombinant pBluescript-ADA was transformed into SΦ3834 and clones that carried pBluescript-ADA were selected on LB supplemented with ampicillin. If functional complementation can be used to clone *ada* gene from *S. antibioticus*, SΦ3834 transformed with pBluescript-ADA should be able to grow in on minimal medium plate supplemented with 2,6-diaminopurine. However, our result showed that SΦ3834 carrying pBluescript-ADA failed to grow even after 2-3 days. This result suggested that adenosine deaminase from *S. coelicolor* is likely cannot deaminated 2,6-diaminopurine riboside to guanosine. The substrate specificity of adenosine deaminase from *S. antibioticus*, had been characterized. Adenosine, 2'-deoxyadenosine, ara-A were found to be substrates for the enzyme (S. Pornbanlualap, 1995). However, 2,6-diaminopurine riboside had not been tested as the substrate for this enzyme. Thus, to determined whether the functional complementation can be used to clone *ada* gene from *S. antibioticus*, 2,6-diamopurine riboside must be tested first to see if this analog is a substrate for *S. antibioticus* adenosine deaminase in the future.

Over-expression, Purification and Characterization of Putative *S. coelicolor* Adenosine/ AMP deaminase.

To characterize the putative adenosine/AMP deaminase from *S. coelicolor*, the gene was cloned into an expression vector, pET-15b. Substrate specificity of the enzyme purified was characterized. Adenosine, 2'-deoxyadenosine, and ara-A were substrates for the enzyme. Removal of the ribosyl moiety of adenosine (i..e, adenine) resulted in complete loss of activity. Addition of phosphate group to the 5'-position of ribosyl moiety of adenosine (i.e., 5'-AMP, 5'-ADP, 5'-ATP) resulted in completely loss of activity as substrate. This data clearly suggested that the enzyme is adenosine deaminase rather than AMP deaminase, because AMP is not substrate for the enzyme. This result further supports the hypothesis proposed by Schramm and coworkers that prokaryotes lack specific AMP deaminase whereas eukaryotes lack AMP nucleosidase. Both AMP deaminase and AMP nucleosidase has been proposed to play essential function in regulation and maintenance of the intracellular concentration of AMP. (Chapman and Atkinson, 1973). The adenine nucleotide pool, also referred to as the adenylate energy charge, is highly regulated in prokaryotes and eukaryotes (Chapman, 1973). The adenylate energy charge is defined by the ratio of ATT:ADP:AMP and is expressed as:

$$\text{Adenylate energy charge} = \frac{\text{High energy phosphate bond}}{\text{Total phosphate bond}} = \frac{\text{ATP} + \tfrac{1}{2}\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

Adenylate energy charge of 0.7-0.9 is essential for normal cellular function. Maintenance of the adenylate energy charge can be accomplished by removal of AMP from the adenine nucleotide pool. Two enzymes have been demonstrated to be involved in regulation of the adenine nucleotide pool. In eukaryotes, regulation of adenine nucleotide pool occurs by deamination of AMP to IMP and NH_3 by AMP deaminase. In eukaryotes, regulation of adenine nucleotide pool occurred by hydrolysis of AMP to adenine and ribose 5-phosphate by AMP nucleosidase (Schramm and Leung, 1973). Although both enzymes catalyzed the degradation of AMP and are regulated allosterically by ATP activation and inorganic phosphate inhibition, the nucleotide sequences from *E. coli* AMP nucleosidase and *Saccharomyces cerevisiae* show no significant homology, suggesting divergent evolution (Leung *et al.*, 1989).

Three-Dimensional Structure, Kinetic and Catalytic Mechanism of *S. coelicolor* Adenosine Deaminase

The three-dimensional structure of recombinant murine ADA produced in *E. coli* was reported (Wilson et al, 1991). The overall architecture enzyme is $(\alpha/\beta)8$ central barrel that found in several enzymes. The enzyme a contains a parallel α/β-barrel motif with eight central β strands and eight peripheral α helices, which is common structure found in 1/10 of known enzymes; it also contains five additional helices which located in long peptide segment between $\alpha 1$ and $\beta 1$. Following $\alpha 8$, the polypeptide chain terminated into two antiparallel COOH-terminal helices that lies across the NH2-terminal of the β barrel. The oblong-shaped deep active site is lined by the COOH-terminal segments and connecting loops of the β-barrel strands. The active site also contains a zinc atom, which lies in the deepest part of the active site pocket and is coordinated by five atoms –three Nε2 atoms of His^{15}, His^{17}, His^{214}, and the Oδ2 of Asp^{295} and participates directly in the deamination mechanism. There are several hydrogen bonds between the substrate and the enzyme that stabilize the binding of substrate and the transition state. There is one zinc atom bound per enzyme molecule.

Although the three dimensional structure of *S. coelicolor* adenosine deaminase has not been determined, the three dimensional structure of this enzyme can be prediction by alignment of its amino acid sequence to that of the murine adenosine sequence. The alignment of amino acid sequence of murine and *S. coelicolor* is shown in figure (X). The result of the amino acid alignment of adenosine deaminase from several sources showed that most amino acid residues that had been proposed to be involved in substrate binding and catalysis are highly conserved among adenosine deaminase as well as AMP deaminase. These highly conserved sequences are summary in table 9.

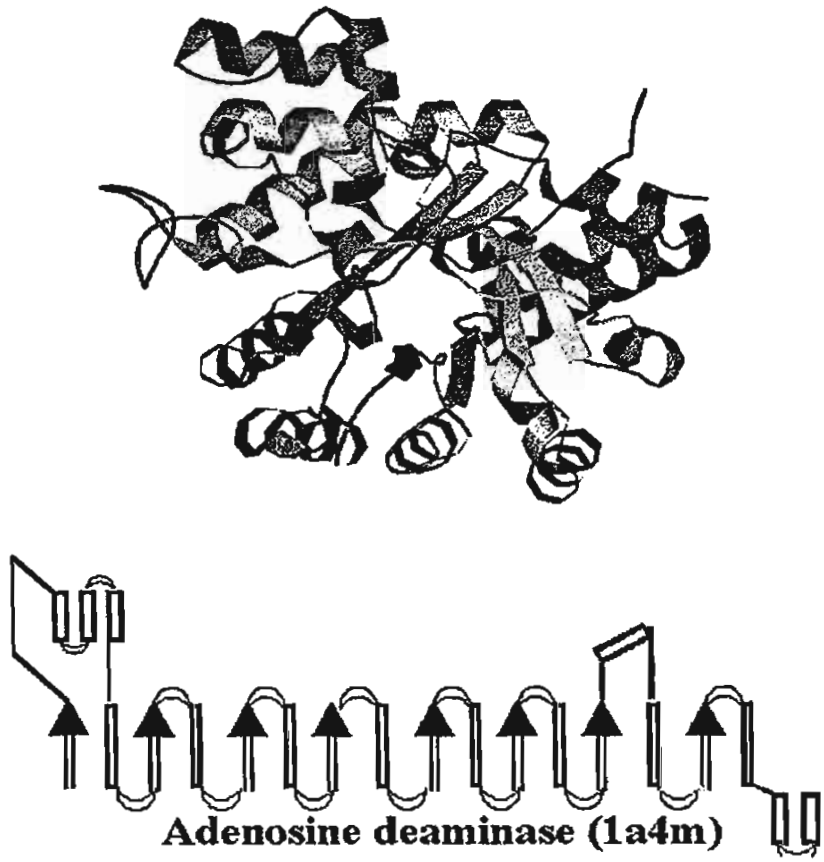

Figure (11). Ribbon diagram representation of the three dimensional structure of murine adenosine deaminase. In this diagram, the coiled structure represents alpha helices, arrows represent beta strands, and β represents residue in isolated beta bridge. Residues involves in catalytic site are indicated by the triangle above it.

Table (3). Sequence alignment of adenosine deaminase from various organisms

Enzyme	Conserved sequences	Reference
Adenosine Deaminase		
E. coli	----^{194}TVHAGE-----(44)----C^{244}---(31)---SINTDDP^{279}-----(1)	
Mouse	----^{212}TVHAGE-----(44)----C^{262}---(31)---SLNTDDP^{297}-----(2)	
Human	----^{212}TVHAGE-----(44)----C^{262}---(31)---SLNTDDP^{297}-----(3)	
S. coel	----^{222}TIHAGE-----(44)----C^{283}---(31)---TVNTDNR^{318}-----(4)	
AMP Deaminase		
Yeast	-----^{421}MHAH------(206)---C^{631}---(74)---SLSTDDP^{709}-----(5)	
Rate	-----^{362}VHAG------(207)---C^{573}---(74)---SLSTDDP^{651}-----(6)	
Human (M)	-----^{362}VHAG------(207)---C^{573}---(74)---SLSTDDP^{651}-----(6)	
Human (L)	-----^{362}VHAG------(207)---C^{573}---(74)---SLSTDDP^{651}-----(6)	

Thus, based on amino acid sequence alignment of adenosine deaminase, it is proposed that amino acid residues in the active site of enzyme that involved in substrate binding and catalysis are similar to that of murine adenosine deaminase reported. That catalytic mechanism of deamination of adenosine to ionsine by the enzyme is proposed to occur as the following. The zinc cofactor activates a liganded water molecule from which the nearby His248 abstrats a proton, thus creating the attacking hydroxyl group. The incipient hydroxyl is oriented for attack on the C6 of the substrate through its interaction with Asp316, His328, and the zinc. Asp316 is thought to hydrogen bound to the catalytic water and share a zinc ligand site with it. The protonated Glu227 facilitate the reaction by donating a hydrogen bond to N1 of the purine, thus enabling the formation of a tetrahedral C6. The source of the proton added to the amino leaving group is still in question, with His248 being a possible candidate. Residues Asp316 and Gly197 participate in hydrogen bond with N7 and N9 of adenosine, respectively, thus reducing the aromaticity of the purine ring and facilitating nucleophilic attack at C6 (Figure 12)

In addition to its important role in maintenance of immune system, adenosine deaminase is a favorite enzyme for mechanistic studies because it is a small monomeric enzyme that catalyzes a relatively simple chemical reaction with remarkable efficiency. The rate enhancement afforded to the reaction by the enzyme is on the order of 2×10^{12} (12). The reaction catalyzed by adenosine deaminase appears to be diffusion control limited reaction, with a k_{cat} of 375 s^{-1}. Several binding studies, using ground state and transition state analogs, suggested that the reaction involved a tetrahedral intermediate at the C6 of the purine ring. Thus, molecules such as coformycin, 2'-deoxycoformycin and 6-hydroxyl-1,6-dihydropurine riboside that resemble the structure of the transition state formed in the reaction are powerful inhibitor of the enzyme, with K_i between 10^{-9} to 10^{-13} M (Kati and Wofenden, 1987). The catalytic mechanism of murine adenosine deaminase had been proposed to occur by a stereospecific addition-elimination (with a tetrahedral intermediate) or SN2 type mechanism. In SN2 type mechanism, the zinc cofactor activates a liganded water molecule from which the nearby His238 abstracts a proton, thus creating the attacking hydroxyl group. The incipient hydroxyl is oriented for attack on the C6 of th3e substrate through its interaction with Asp295, His238, and the zinc. Asp295 is thought to hydrogen bond to the catalytic water and share a zinc ligand site with it. The protonated Glu217 facilitates the reaction by donating a hydrogen bond to N1 of the purine, thus

enabling the formation of a tetrahedral C6. The source of the proton added to the amino leaving group is still in question, with His238 being a possible candidate. Residue Asp296 and Gly184 participate in hydrogen bonds with N7 and N9 of adenosine, respectively, thus reducing the aromaticity of the purine ring and facilitating nucleophilic attack at C6 (Figure 12).

Although the three dimensional structure of *S. coelicolor* adenosine deaminase has not been determined, architecture enzyme is likely to be similar to $(\alpha/\beta)8$ central barrel that had been reported for murine adenosine deaminase. Several evidences suggested this hypothesis. First, alignment of the amino acid sequence of adenosine deaminase from S. coelicolor to other organism showed that amino acid residues that involved in substrate binding and catalysis are the highly conserved among the this enzyme (Figure 12). Asp-295 that had been reported to be essential in stabilization of the unstable tetrahedral transition state in murine enzyme is in placed by Asp-316 in *S. coelicolor* (Figure 12). Similarly, His-238 that had been proposed to activate the water molecule and made it more nucleophilic in murine enzyme had been in placed by His-248 in *S. coelicolor*. Similarly, Glu-217 and Gly-184 that are involved in binding to the N1 and N3 of adenosine in murine enzyme had been in place by Glu-227 and Gly-197 respectively in *S. coelicolor*. Second, similar to murine enzyme, S. coelicolor adenosine deaminase also required Zn as cofactor. Incubation of *S. coelicolor* adenosine deaminase resulted in loss of catalytic activity (Figure 12). Finally, similar to the murine enzyme, coformycin and 2'-deoxycoformycin who structures mimic the tetrahedral transition state formed in the deamination reaction inhibit *S. coelicolor* potently, with Ki between 10^{-11} to 10^{-12} M (Figure 12).

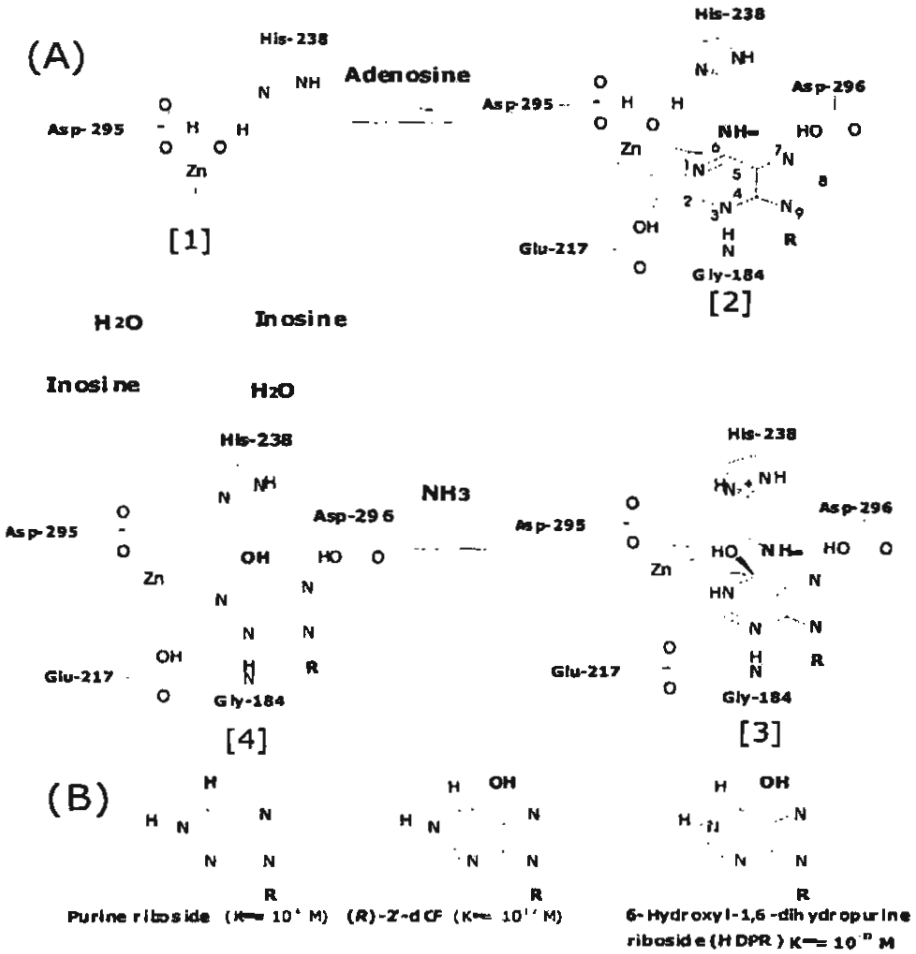

Figure (12). Schematic diagram of the mechanism for the reaction catalyzed by adenosine deaminase. Dashed lines indicate noncovalent interaction between neighboring atoms. (Step 1) The zinc cofactor is coordinated to three His residues (not shown), Asp295, and the catalytic water. The water is oriented for attack by hydrogen bonds to His 238 and Asp295. (Step 2) His 238 abstracts the proton from the catalytic water, and the hydroxylate attacks the substrate's at C6; the N1=C6 double bond is lost, and N1 becomes protonated by Glu217. Also shown are Asp 296 and Gly 184, donating hydrogen bonds to N7 and N3, respectively. (Step 3) The tetrahedral intermediate collapses with the amino group becoming protonated, possibly by His 238, and leaving in the form of ammonia. (Step 4) The enol form of the product inosine is shown to bound to the active site.

Table 4. A comparison of codon usage between average values of *E. coli*, *B. subtilis* and *Streptomyces*

	Codon	*E. coli*	*Streptomyces*
Arg	CGA	3.1	3.6
	CGC	22.0	37.1
	CGG	4.6	30.9
	CGU	24.7	6.2
	AGA	2.0	1.2
	AGG	1.3	4.9
Leu	CUA	3.0	0.3
	CUC	9.8	34.5
	CUG	54.8	56.1
	CUU	9.9	1.9
	UUA	10.3	0.3
	UUG	11.2	2.6
Ser	UCA	6.3	1.4
	UCC	9.6	21.3
	UCG	7.9	16.1
	UCU	10.4	0.8
	AGC	15.0	15.1
	AGU	7.1	1.4
Thr	ACA	6.4	1.7
	ACC	24.6	42.9
	ACG	12.5	17.7
	ACU	10.5	1.1
Pro	CCA	8.1	1.2
	CCC	4.2	25.3
	CCG	24.2	29.7
	CCU	6.5	2.2
Ala	GCA	20.6	5.1
	GCC	23.7	73.5
	GCG	33.3	45.4
	GCU	17.8	3.3
Gly	GGA	6.7	7.6
	GGC	30.7	60.1
	GGG	9.6	17.2
	GGU	28.0	7.9
Val	GUA	11.8	1.9
	GUC	14.3	43.2
	GUG	25.3	30.4
	GUU	20.4	1.7
Lys	AAA	36.9	1.3
	AAG	11.9	21.5
Asn	AAC	24.2	23.2
	AAU	15.9	1.1
Gln	CAA	13.0	1.9
	CAG	30.1	23.5
His	CAC	11.0	21.0
	CAU	11.5	2.5
Glu	GAA	43.7	9.2
	GAG	19.3	48.9
Asp	GAC	22.3	60.2
	GAU	32.0	3.2
Tyr	UAC	13.4	23.1
	UAU	14.9	1.3
Cys	UGC	6.2	7.9
	UGU	4.7	0.9
Phe	UUC	18.2	28.6
	UUU	18.5	0.5
Ile	AUA	3.8	1.1
	AUC	27.1	28.3
	AUU	27.0	0.6
Met	AUG	26.5	15.9
Trp	UGG	12.8	16.2
Ter	UAA	2.0	0.2
	UAG	0.2	0.7
	UGA	0.8	2.3

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3. ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ (Publication)

None

4. กิจกรรมอื่นๆ

None

5. ปัญหาและอุปสรรค (Problem encountered)

None

6. **ความเห็นและข้อเสนอแนะ** (Suggestion and comment)

The result from this study suggests that cloning of adenosine deaminase from *Streptomyces antibioticus* by functional complementation may not work. This approach will work only if DAP riboside is a substrate for the enzyme. Thus, new protocol for cloning of this gene is currently underway. This approach is based on the use of primers that contained the highly conserved region of adenosine deaminase along with the universal primer which existed on pBluescript. These primers will be used to amplify the ada gene from *S. antibioticus* chromosome. In the mean time, a manuscript on "Cloning, purification and characterization of the putative AMP/adenosine deaminase from Streptomyces coelicolor" is being prepared. It will be submitted to international journal in a few months.

7. **งานที่จะทำในปีต่อไป** (Future work).

New approach for cloning of adenosine deaminase gene from *S. antibioticus* is currently underway. This approach is based on the use of primers that contained nucleotide sequence that are identical to the highly conserved region among adenosine deaminases isolated from various sources. These primers will be used to amplify the *ada* gene from *S. antibioticus* chromosome.