CHAPTER II

Literatures Review

1. Life cycle and biology of the *Plasmodium* parasite and *Anopheles* Mosquitos

The malarial parasite have a life cycle which is split between a vertebrate host and an insect vector (Figure 2-1). The plasmodium species, with the exception of *P. malariae* are exclusively parasites of man. The mosquito is always an *Anopheles* mosquito, although, out of the 380 species of *Anopheles* mosquito, only 60 can transmit malaria. Only female mosquito are feed on blood.

During a blood meal the sporozoites from the malaria-infected mosquito salivary gland are injected into the human as the mosquito must inject anticoagulant saliva to ensure an even meal (Figure 2-1,1). Once in the human bloodstream, the sporozoites arrive in the liver and penetrate hepatocytes (Figure 2-1,2), where they remain for 9-16 days, multiplying within the cells and become schizont stage (Figure 2-1,3). This stage is called exo-erythrocytic schizogony (Figure 2-1,A). The schizonts rupture and release thousands of merozoites infect red blood cells and undergo asexual multiplication in the erythrocytes (erythrocytic schizogony) (Figure 2-1,B). The ring stages mature into trophozoites and then into schizonts, which rupture releasing merozoites (Figure 2-1,6). Some parasites differentiate into sexual erythrocytic stage (micro and macro-gametocytes)

(Figure 2-1,7). Blood stage parasites are responsible for the clinical manifestations of the disease.

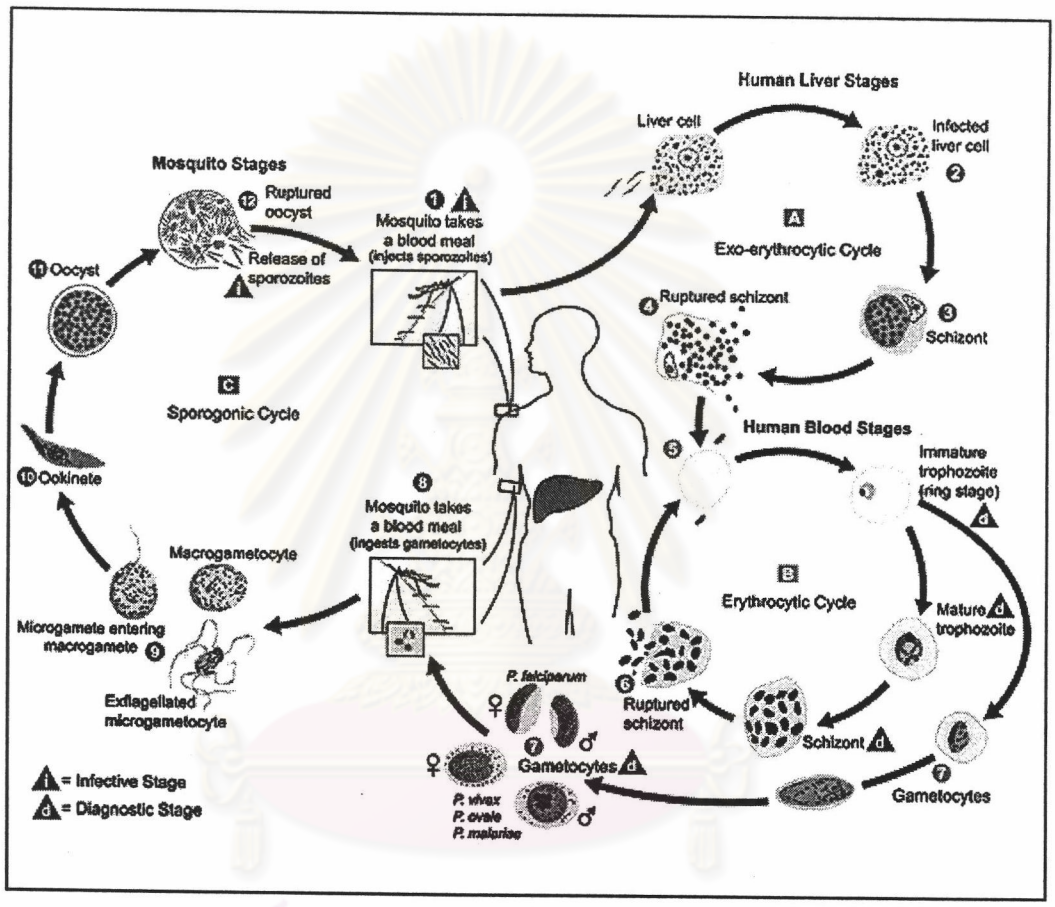

Figure 2-1 Life cycle of the malarial parasite.

(From *http://www.malariatest.com/cycle.html*)

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal (Figure 2-1,8). The parasites' multiplication in the mosquito is known as the sporogonic cycle (Figure 2-1,C). While in the mosquito's stomach, the fertilization occurs after the microgametes exflagellate and penetrate the macrogametes generating zygotes (Figure 2-1,9). The zygotes in turn become motile and elongated (ookinetes) (Figure 2-1,10) which invade the midgut wall of the mosquito where they develop into oocysts (Figure 2-1,11). The oocysts grow, rupture, and release sporozoites (Figure 2-1,12), which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpectuates the malaria life cycle (Figure 2-1,1).

Once injected into the human host, all Plasmodium species will penetrat hepatocytes. However, *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony whereas *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or have a delayed trigger, resulting in dormant hypnozoites. The dormant hypnozoits can persist in the liver and cause relaps by invading thr blood stream weeks, or even years later. Some strains, such as the North Korean strain, seem to consist of sporozoites with universally delayed triggers, so they all form long lasting hypnozoites. *P. vivax* may have an incubation period of up to 10 months. Gametocytes produce in the primary attack seem to contain all the genetic information required to create sporozoites of several different activation times. The same seems true for gametocytes produced in relapses where the hypnozoites become activated.

Sexual development of Plasmodium begins as the merozoites invade the erythrocytes after their release from the liver. Within the erythrocyte, shizogony occurs to produce either more merozoites, or the sexual micro and macrogametocytes (taking 26 hours) (10). In *P. falciparum*, erythrocytic schizogony takes 48 hours and gametocytosis takes 10-12 days. Normally a variable number of cycles of asexual erythrocytic schizogony occurs before any gametocytes are produced (11). The immune system may produce antibodies to the gametocytes at this stage.

Once drawn into the mosquito, the gametocytes increase in volume and escape the erythrocyte. Microgametes are formed by 3 mitotic divisions within the microgametocyte, and are expelled explosively. No further changes affect the female macrogametocyte until fertilization where the plasmalemmas of male and female gametes fuse and the nucleus of the microgamete enters the female cytoplasm (12). After fertilization, the zygote is a motionless globular cell, but after 18 to 24 hours it becomes elongated and motile, containing micronemes and a pellicle. The cell invades the microvillus border, pass through the midgut cells, and lies beneath the basement membrane (12). The ookinete then becomes a static oocyts, between the basal lamina and the basement cell membrane, and bounded by a thick plasmalemma. The chief source of nutrients is the haemolymh in which the oocyst develops. Sporoblasts form, and sporozoites bud off. After the cyst ruptures, the sporozoites escape into the haemocoele and migrate to and penetrate salivary gland cells where they lie in vacuoles for up to 59 days. These sporozoites develop and become up to 1000 times more infective than when in the oocyst (12). They are more antigenic, and bear circumsporozoite

polypeptide on their plasmalemma. Sporozoite motility is involved in their invasion of cells and escape from the salivary gland. The sporozoites are about 12 μM long and 1 μM across, with a single nucleus, anterior to which lie micronemes, and posterior to which lies ER and mitochondria (12). They posses a complex pellicle, which is responsible for motility, and contains circumsporozoite protein. The apical penetrating region contains extensions of the microneme ducts which release an agent which interacts with host cell plasma membrane during penetration.

A bitting mosquito transfers about 10 % of its sporozoite load into the capillaries or perivascular tissue. Now the sporozoites must begin their evasion of the host defences, possibly by binding serum proteins for camouflage (13). Some are destroyed by macrophages, or by antigen specific antibodies in immune individuals, but in non immune individuals, they reach the hepatocytes and initiated schizogony or become hypnozoites depending on their delay trigger. All sporozoites have left peripheral circulation within 45 minutes.

2. Biochemistry of the *Plasmodium* parasite

The malarial parasite exhibits a rapid growth and multiplication rate during many stages of its life cycle. This necessitates that the parasite, like all other organisms, acquire nutrients and metabolize these various biological molecules in order to survive and reproduce. Obviously, the parasite's metabolism will be intertwined with that of the host's because of the intimate relationship between the host and parasite. These host-parasite interactions are further complicated by the complex life cycle of the parasite

involving vertebrate and invertebrate hosts as well as different locations within each of these hosts. A better understanding of the parasite's metabolism may lead to the development of novel therapeutic strategies which exploit the uniqueness of the parasite.

2.1 Carbohydrates and energy production

The blood-stage parasite activity ferments glucose as a primary source of energy. The metabolic steps involved in the conversion of glucose to lactate are essentially the same as that found in other organisms. All of the enzyme activities have been identified in *Plasmodium* and some of the genes were cloned. The parasite exhibits a high rate of glycolysis and utilizes up to 75 times more glucose than uninfected erythrocytes. Most of the glucose is converted to lactate and the high lactate dehydrogenase (LDH) activity is believed to function in the regeneration of NAD^+ from NADH which is produced earlier in the glycolytic pathway by glyceraldehyde-3-phosphate dehydrogenase. The net result of glycolysis is to produce ATP which is the energy currency of the cell. In other words, ATP is needed for anabolic and homeostatic processes.

Most (approximately 85%) of the glucose utilized by the parasite is converted to lactate. However, some of the glycolytic intermediates may be diverted for synthetic purposes. For example, enzymes of the pentose phosphate pathway have been identified. This pathway probably provides some of the ribose sugars needed for nucleotide matabolism and provides for the regeneration of reduced NADPH to be used in biosynthesis or defense

against reactive oxygen intermediates. Similarly, the further metabolism of pyruvate may provide intermediates in several biosynthetic pathway.

Aerobic metabolism involves the further catabolism of pyruvate (glycolysis intermediate preceeding lactate) to carbon dioxide and hydrogen atoms via the tricarboxylic acid (TCA) cycle. The hydrogen atoms are captured by the reduction of NAD^+ to NADH. The electrons from the captured hydrogen are then fed into a chain of electron carriers and ultimately transferred to molecular oxygen to form water. ATP is generated by capturing energy during electron transport by a process known as oxidative phosphorylation. The TCA cycle and oxidative phosphorylation can generate up to 38 molecules of ATP per glucose molecule, whereas glycolysis only produces two molecules of ATP per glucose molecule. Nonetheless, the blood-stage of mammalian malaria parasites do not exhibit a complete TCA cycle. An explanation for this inefficiency is the abundance of glucose in the mammalian blood stream. In contrast, the parasite does appear to exhibit a TCA cycle in the glucose-poor environment of the mosquito host.

The TCA cycle and oxidative phosphorylation are generally carried out in the mitochondria of eukaryotes. These processes are generally assumed to be non-functional in the blood-stage parasite as evidenced by the acristae mitochondria. However, recently a functional electron transport chain and oxidative phosphorylation have been demonstrated in the blood-stage parasite (14). In addition, the parasite mitochondria does have a membrane potential and cytochrome oxidase is present. The antimalarial drugs atovaquone has

been shown to inhibit electron transport and to collapse the mitochondrial membrane potential in malaria parasite. One possible function of mitochondrion during the blood-stage is for pyrimidine synthesis.

2.2 Fatty acids and lipids

Lipids are a major component of membranes. Membrane lipids are composed of a glycerol (3-carbon unit) backbone which has a polar head group and two long chain fatty acids. The rapidly growing parasite requires large amounts of lipids for this increase in parasite surface area and volume of internal membranes. The parasite is incapable of synthesizing fatty acids *de novo*, but utilizes preformed fatty acids and lipids from the host. Several parasite enzymes involved in lipid synthesis from glycerides and fatty acid, as well as enzymes involved in the remoldeling of lipid polar head groups have been identified. An enzyme capable of activating fatty acids (necessary for incorporation into lipids) has been localized to membranous structures found within the cytoplasm of the infected erythrocyte (15).

2.3 Proteins and amino acids

Proteins are composed of linear chains of amino acids which fold into 3-dimensional structures. Through their roles as enzymes or structural proteins. Proteins are responsible for cellular structure and function. The blood-stage parasite obtains amino acids for protein synthesis from three sources: 1). Degradation of ingested hemoglobin, 2). Uptake of free amino acids from the host plasma (or cells) and 3). *De novo* synthesis. The most abundant source of amino acids is the ordered degradation of hemoglobin.

Several amino acids are taken up by infected erythrocytes at accelerated rates (16) and *in vitro* culture studies indicate that *P. falciparum* requires seven exogenously supplied amino acids: isoleucine, methionine, cysteine, glutamine, proline, tyrosine (17). The parasite is also able to fix carbon dioxide and thereby synthesize alanine, aspartate and glutamate. However, the amino acids formed via carbon dioxide fixation and some of the exogenously added amino acids are not readily incorporated into proteins. Many of these amino acids (through transamination reaction) can interact with payhways involved in energy production and possibly serve as fuel sources. In addition, some amino acids serve as precursors or components of biosynthetic or other metabolic pathways. Of particular note is the proposal that glutamate dehydrogenase provides the reduced NADPH needed for glutathione reductase (18) which presumably functions in redox metabolism.

Ribosomes are supramolecular complexes composed of ribosomal RNA and proteins. Their function is to translate mRNA into protein. The mechanism of protein synthesis is presumably typical of other eukaryotes. Interestingly, different rRNA molecules are expressed during the vertebrate and invertebrate stages of the parasite's life cycle (19). The functional significance of stage specific ribosomes is not known.

2.4 Nucleotides and nucleic acids

DNA and RNA are polymers of nucleotides. Nucleotides consist of a ribose sugar group linked to either a purine (adenine and guanine) or pyrimidine (cytosine, uracil and thymine) base. These bases can either be

obtained via *de novo* synthesis or from the environment by the salvage pathway. The malarial parasite obtains preformed purines by the salvage pathway and synthesizes pyrimidines *de novo*. Since the host can obtain both types of bases by either pathway, it may be possible to exploit the parasite's limited capability in nucleotide metabolism.

The primary purine salvaged by the parasite is hypoxanthine which can be obtained from the host plasma. In addition, adenosine in the host plasma can be converted to hypoxanthine following deamination and dephosphorylation. Through a series of reactions the hypoxanthine is converted into ATP and GTP (or deoxy-ATP and GTP) and incorporated in RNA (or DNA).

The parasite cannot utilize preformed pyrimidins and must synthesize them from bicarbonate and glutamine. One step of pyrimidine synthesis involves an electron transport in which dihydroorotate dehydrogenase transfers electrons to an electron transport chain involving ubiquinone, cytochrome and molecular oxygen (20). This activity is probably in the mitochondria and accouts for the microaerophillic requirements of the parasite. Pyrimidine synthesis also requires folates as co-factors.

2.5 Vitamins and co-factors

Many biochemical processes require co-factors which do not directly participate in growth processes as do the bulk nutrients. Instead, vitamins are usually required in smaller amounts and are usually recycled.

Pantothenate appears to be the only vitamin not supplied by the erythrocyte (17) and is probably needed for the formation of acyl-coenzymeA which is needed in lipid biosynthesis.

Folate and its derivatives are important co–factors in the synthesis of nucleotides and amino acids and especially for the transfer of methyl (one carbon) groups. Especially important is the role of the dihydrofolate cycle in *de novo* pyrimidine synthesis. Dihydrofolate is reduced to tetrahydrofolate by dihydrofolate reductase (DHFR). Several antimalarials, such as pyrimethamine and cycloguanil, preferentially inhibit parasite DHFR. The tetrahydrofolate is mathylated by serine hydroxymethyl transferase and the resulting methylene tetrahydrofolate functions as a methyl donor. For example, thymidylate synthase catalyzes the formation of dTMP from dUMP by transferring the methyl group from methylene tetrahydrofolate. During this reaction the methylene tetrahydrofolate is converted back to dihydrofolate, which is then recycled.

Increased folates are needed to accommodate the demand for pyrimidines which are associcated with DNA replication. The parasite connot utilized preformed folate and must synthesize dihydrofolate from GTP, para-aminobenzoic acid and glutamate. Fansidar, a combination of sulfadoxine and pyrimethamine, inhibits folate metabolism at two distinct places in the pathway.

2.6 Redox metabolism

A by-product of metabolism and respiration are reactive oxygen intermediates (ROI) such as superoxide, hydroxyl radical and hydrogen peroxide. In particular, the digestion of oxy-hemoglobin results in the production of ROI. These ROI can damage lipids, proteins and nucleic acids and therefore need to be oxidized to oxygen and water. Parasite enzymes involved in redox matabolism have been identified. Superoxide dimutase (SOD), catalase, and glutathione reductase and the reducing equivalents of NADPH are probably generated through the pentose phosphate cycle. Glutamate degydrogenase is another potential source of NADPH. It has also been proposed that the parasite uses host catalase and SOD within the food vacuole. Interestingly, the malaria parasite may supply the host erythrocyte with glutathione which could participate in protecting the host cell from oxidative damage (21).

3. Enzyme uridine phosphorylase

Uridine phosphorylase (EC 2.4.2.3 ; uridine : phosphateα-D-ribosyl transferase) is an enzyme in the family of nucleoside phosphorylase that is an important biochemical reaction in the salvage pathway of pyrimidine nucleotide (22). Uridine phosphorylase was demonstrated in 1961 (6). It can be found in many organisms such as *E. coli, T. gondii, C. elegans, M. musculus* and *H. sapiens* (23-26).

Uridine phosphorylase play an important role in salvage pathway of uracil. It is one of the enzyme activities converting uracil to uridine

monophosphate (UMP), a precursor for nucleic acids biosynthesis. First, uridine phosphorylase adds ribose from ribose-1-phosphate to uracil, the products are P_i and uridine (27), according to the following ;

uracil + ribose-1-phosphate ⟷ uridine + Pi

Then, uridine is matabolized to uridine monophosphate by uridine kinase

uridine + ATP ⟶ UMP + ADP

Furthermore, uridine phosphorylase has an activity to inter-convert uracil, uridine and deoxyuridine (22).

Most human cells, except erythrocytes, can obtain uracil from salvage pathway by the activity of uridine phosphorylase and the gene encoding the enzyme is identified on chromosome 7 (26). In contrast to human cells, *P. falciparum* can only obtain pyrimidine from *de novo* process and several enzymes for the salvage of pyrimidine nucleosides were not detected (6,7) and there are no reported on the gene encoding the enzyme in the nearly complete genome sequencing project of the *P. falciparum* (28).

4. Background of the experiment approach

This study aimed to clone and heterologously express *P. falciparum* uridine phosphorylase gene in a bacterial system and to study

on the kinetics of the expressed enzyme. The DNA fragment of *P. falciparum* uridine phosphorylase gene form PCR amplifications would be cloned to pDrive cloning vector and allowed to multiply in *EZ* competent cells, then the positive clone would be subjected to DNA sequence and analyze by BLAST family of program. After sequencing, the DNA fragment carrying uridine phosphorylase gene would be subcloned into pQE30 expression vector and heterologously expressed in a SG13009 and M15 competent cells. Finally, the expecting enzyme would be purified and analyzed by SDS-PAGE and enzyme kinetics would be performed.

4.1 The pDrive cloning vector

The pDrive cloning vector use in this study is a linear form with a U-base overhang at each end, ready-to-use for direct ligation of PCR products. This vector allows ampicillin and kanamycin selection, as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognition sites around the cloning site, allowing easy restriction analysis of recombinant plasmids. The vector also contains a T7 and SP6 promoter on either side of the cloning site, allowing *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers. In addition, the pDrive cloning vector has a phage f1 origin to allow preparation of single-strand DNA.

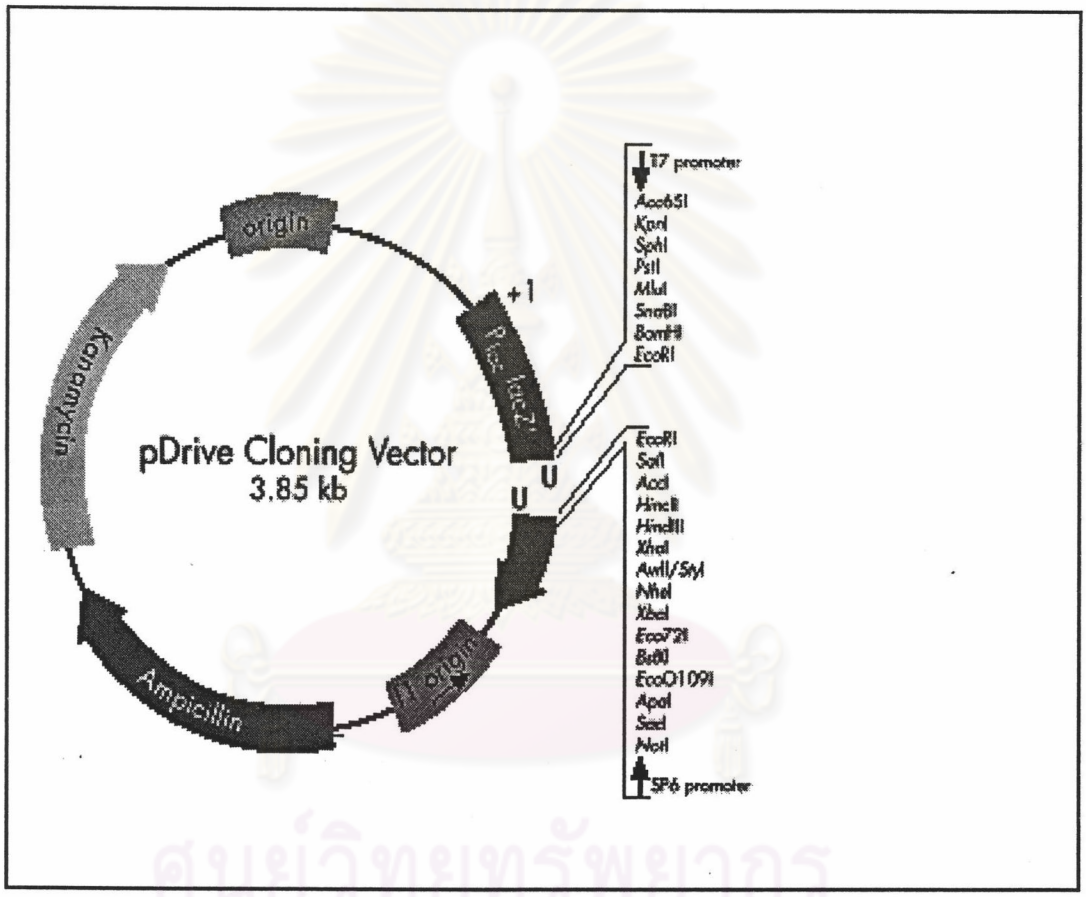

Figure 2-2 Map of the pDrive cloning vector.

(From QIAGEN® PCR Cloning Handbook)

4.2 The pQE30 expression vector

The pQE30 expression vector is belong to the pDS family of plasmid (29). These low-copy plasmid have 6xHis-tag coding sequence either 5' or 3' to the cloning region and have the synthetic ribosomal binding site, RBSII, for high translation rates. The pQE30 expression vector allows ampicillin selection. The vector obtains several unique restriction-endonuclease recognition sites around the cloning site allowing easy restriction analysis of recombinant plasmids.

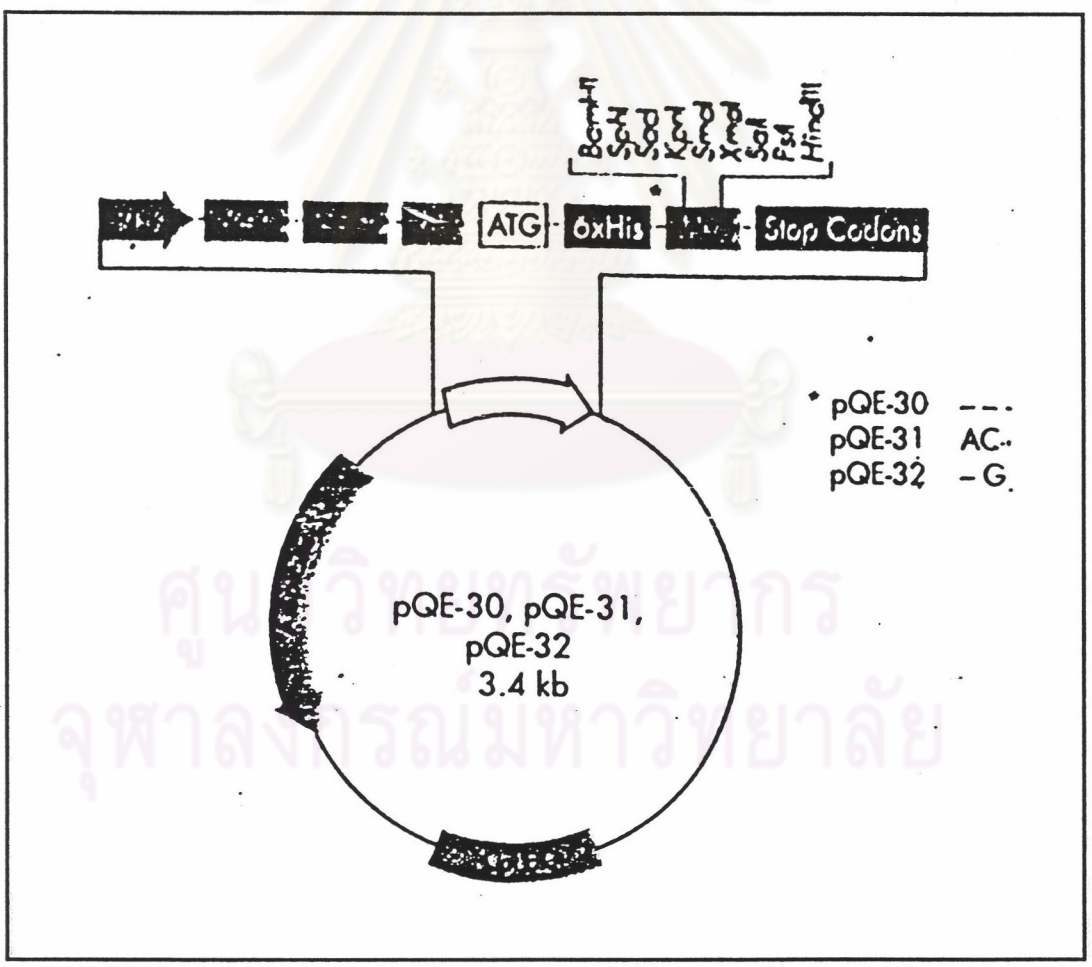

Figure 2-3 Map of the pQE30 expression vector.

(From *http://www.qiagen.com*)

4.3 The *EZ* competent cells

The *EZ* competent cells are capable of high-efficiency transformation (> 1.0 x 10^8 colony forming units per microgram DNA). The cells are compatible with ampicillin and kanamycin selection and blue/white screening following transformation with pDrive cloning vector.

4.4 The M15 and SG13009 competent cells

The *E. coli* strains M15 and SG13009 contain the low-copy plasmid pREP4 which confers kanamycin resistance and constitutively express the lac repressor protein encoded by the lacI gene (30). The pREP4 plasmid is derived from pACYC and contains the p15A replicon. Multiple copies of pREP4 are presence in the host cells that ensure the production of high levels of the lac repressor protein which binds to the operator sequences and tightly regulates recombinant protein expression. The pREP plasmid is compatible with all plasmids carrying the ColE1 origin of replication, and is maintained in *E. coli* in the presence of kanamycin at a concentration of 25 μg/ml. *E. coli* strain M15 (pREP4) is permits high-level expression and strain SG13009 (pREP4) (31) may be useful for the production of proteins that are poorly expressed in M15 (pREP4). Both the M15 and SG13009 strains derived from *E. coli* K12 and do not harbor any chromosomal copy of the *lacI*^{q} mutation, so pREP4 must be maintained by selection for kanamycin resistance.