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In-vitro evolution of dihydropteorate synthase: Effect of amino acid changes on enzyme function and development of resistance.

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1. Abstract

The project has been divided into 2 parts. The first part was focused on the *Plasmodium* bi-functional enzyme PPPK-DHPS (7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase-dihydropteroate synthase) that has long been the target of antimalarial chemotherapy while the second part involved determining the possible resistance mechanisms in *Streptococcus mutans*.

Previous studies have been conducted on the functional importance of the PPPK part in a knockout *E. coli*. The present study was aimed at defining the DHPS part of the enzyme. Of the four *Plasmodium* specific insertions, PPPK-1 is essential for enzyme activity, while a major part of the PPPK-2 can be deleted without any effects. Similarly, DHPS-1 is crucial for enzyme activity while DHPS-2 is dispensable. Double mutants with deletions in the DHPS insertions were generated and the enzyme activity was tested as a measure of growth rate in a poor medium. The radioactive enzyme assay (which gives a direct measure of the enzyme activity) did not give results; hence, conclusive results could not be obtained. Further repeats of growth curve analysis could reveal greater details about the effects of mutations on enzyme activity, but from preliminary testing, it could be hypothesized that a smaller deletion of eight amino acids seems to be more deleterious to the enzyme activity than a longer deletion.

Streptococcus mutans (Viridans Group Streptococci) are commensal bacteria found in mouth and are the causative agents of dental caries. The main interest in the Viridans group has been because of their ability to act as potential reservoirs of antibiotic resistance determinants. These determinants can be transferred to related pathogenic species like *Streptococcus pneumonie*, which annually kills over one million children worldwide. Previous investigations found Cotrimoxazole (SXT) resistant *S. mutans* in clinical isolates. SXT is highly prescribed in dental practice in Uganda, where it is also used for HIV/AIDS prophylaxis. A previous

study in 2009 was aimed at determining the resistance mechanism in *S. mutans* by characterizing the *folP* gene. Isolate **797** showed high resistance *in vivo* but did not express any resistance *in vitro* in knockout *E. coli*. Only in case of isolate **8** did the cloned gene express resistance in knockout *E. coli*. Sequence analysis revealed three amino acid polymorphisms. The present study was aimed at changing these amino acids one by one and in relation to one another (using isolate **8** *folP* as a template) to study their role in resistance. Agar diffusion tests were carried out on all the mutants using 0.02-0.05 mM of Sulfathiazole in ISA. The triple mutant with sequence similar to isolate **797** showed a similar resistance pattern, suggesting the point mutations in *folP* gene as a possible resistance mechanism. Further studies need to be carried out using other isolates to confirm this hypothesis, including an examination of other possible resistance mechanisms involving intra-cellular pumps.

2. Introduction:

A class of drugs that have been highly successful as antibiotics and antiprotozoal agents are Antifolates. The drugs target various enzymes of the folate biosynthesis pathway, which is essential for the survival. Folates are necessary co-factors for various life processes like DNA synthesis, RNA repair. Humans are unable to synthesise folate de novo, unlike most microbes and parasites, which makes the folate biosynthesis an attractive target for antibiotics. Antifolates like pyrimethamine (PYR) and proguanil target the enzyme dihydrofolate reductase (DHFR), while sulfadoxine (SDX) and other sulfonamides target dihydropteroate synthase (DHPS).

The entire project has been divided into two parts. The first part of the project focuses on the DHPS enzyme in the Plasmodium parasite while the second part deals with the possible resistance mechanisms in Streptococcus mutans involving the dhps gene (folP).

2.1 Introduction to the *pf*PPPK-DHPS project

2.1.1 Malaria

The history of malaria predates the history of human evolution. Probably one of the oldest diseases, it remains one of the most widespread and lethal diseases in the developing world. WHO reported about 216 million documented cases, leading to 655,000 deaths in 2010[1], however, taking into account that many cases are unreported and undocumented, the estimated death toll is around 1.24 million[1,2].

Malaria is endemic to a very broad region around the Equator; however, it is in Sub-Saharan

Africa that most fatalities occur[3,4,5]. Children under 5 years of age[6], pregnant females and HIV patients are most vulnerable to the disease.

2.1.2 Vector Factor: Life cycle and Transmission

Malaria is a parasitic disease caused by the *Plasmodium* genus. Five species have been identified as disease causing in humans: *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*[7,8]. Amongst these, *P. falciparum* is the most common species identified and the cause of the majority of deaths[9], while *P. vivax* is the second most common. Currently, *P. knowlesi* is the only zoonotic species identified that causes disease to spread from macaques to humans[10].

Transmitted from the female *Anopheles* mosquitoes (Greek for 'good for nothing'), the signs and symptoms of malaria vary with the causative parasite. While in *P. vivax* and *P.ovale* infections, the symptoms cycle every 2-3 days, it can be as short as 36-48 hours in *P. falciparum* infections[11]. Also, severe malaria is restricted to the *falciparum* species[12], and recurrent malaria to *vivax* and *ovale* species[13].

Figure 1 illustrates the life cycle of the malaria parasite in the mosquito host and after infection, in the human host.

The life cycle of parasite can be divided into 2 parts:

1. The *Hepatic Stage of Infection*: During a blood meal, the mosquito injects the sporozoites into the blood stream of the human host. Sporozoites infect the liver, where they mature into schizonts in the hepatic cells and are released as merozoites upon rupture of the cells. The merozoites then invade red blood cells,

2. Initiating the *Blood Stage of Infection*, which manifests into the disease. The progress of the parasite is similar as the hepatic stage, except that some merozoites upon release mature into gametocytes.

The gametocytes are ingested by the mosquito during a blood meal, in which they mate and form zygotes, which mature into sporozoites in the mid-gut of the mosquito and travel to the salivary gland, ready to be injected again, completing the cycle.

In *P. vivax* and *P. ovale*, dormant schizonts can persist in the liver and relapse after infecting the blood stream weeks, or in some cases, years after the initial infection.

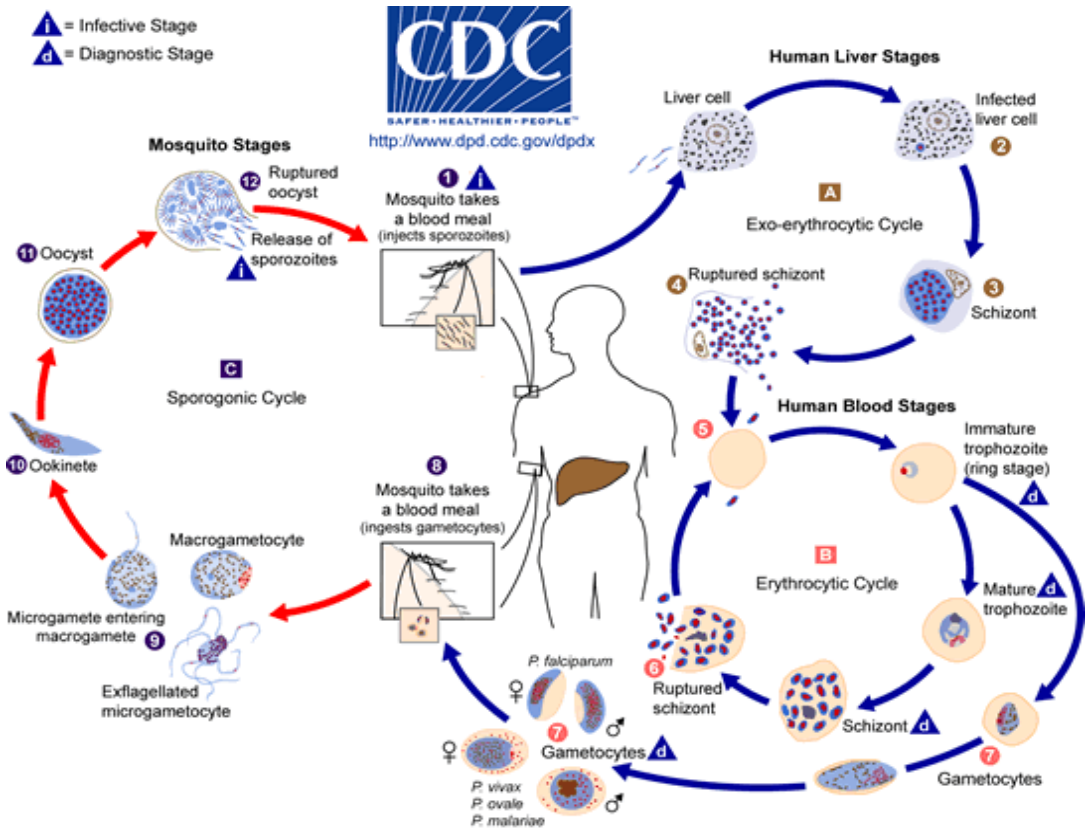

Figure 1: Life cycle of the *Plasmodium* parasite.

(http://dpd.cdc.gov/dpdx/html/Malaria.htm, DPDx, Division of Parasitic Diseases and Malaria, Centre of Disease Control, USA. Last update: 13th July 2009. Date visited: 26th July 2012)

2.1.3 Treatment: Past and Current Standards

Traditional remedies included *Cinchona succiubra* (Quinine) and *Artemisia annua* (Artemisinin) extracts, which have been used for centuries[14,15].

2.1.3.1 Chloroquine

The first synthetic antimalarial was chloroquine, which was synthesized as a substitute for quinine. It inhibits hemozoin production, which is a byproduct of hemoglobin proteolysis within the parasite. Since the drug target of chloroquine is host-derived, it took almost 19 years for *P. falciparum* to develop resistance against the drug[16-20]. It could also be that there are unknown mutations necessary to allow for resistance. The resistance is mostly related to mutations in the transporter genes (*pfcrt, pfmdr*)[16,21]. The first case of resistance was detected in South Asia in 1950's, after which the resistance spread rapidly to Africa and other parts and therefore quinine, its derivatives and substitutes were rendered useless against *P. falciparum* infections[22], although they still maintain some efficacy against *P. vivax* and *P. ovale* infections.

2.1.3.2 Antifolates and the Folic Acid Biosynthesis

Antifolates, which target the folate biosynthesis in the parasite, have also been highly successful antimalarials. Due to their selective action, low cost and ease of application, antifolates were very suitable for malaria endemic regions and were used extensively. Consequently, resistance to antifolates arose rapidly, after which they were successfully combined with sulfonamides (e.g. PYR-SDX). The result was targeting two different

enzymes of the pathway (DHFR and DHPS, respectively), which resulted in better clearing of the parasite[23-26]. The first cases of PYR-SDX resistance were detected in South-East Asia in 1960's, after which resistance spread rapidly, as in case of chloroquine. Resistance in *P. falciparum* is conferred mainly by key mutations in the *dhfr* gene (N51I, S108N/T, I164L) and the *dhps* gene (A437G, K540E, A581G)[27].

Figure 2 shows the transmission of the parasite and effect of the antimalarials on each stage of parasite development.

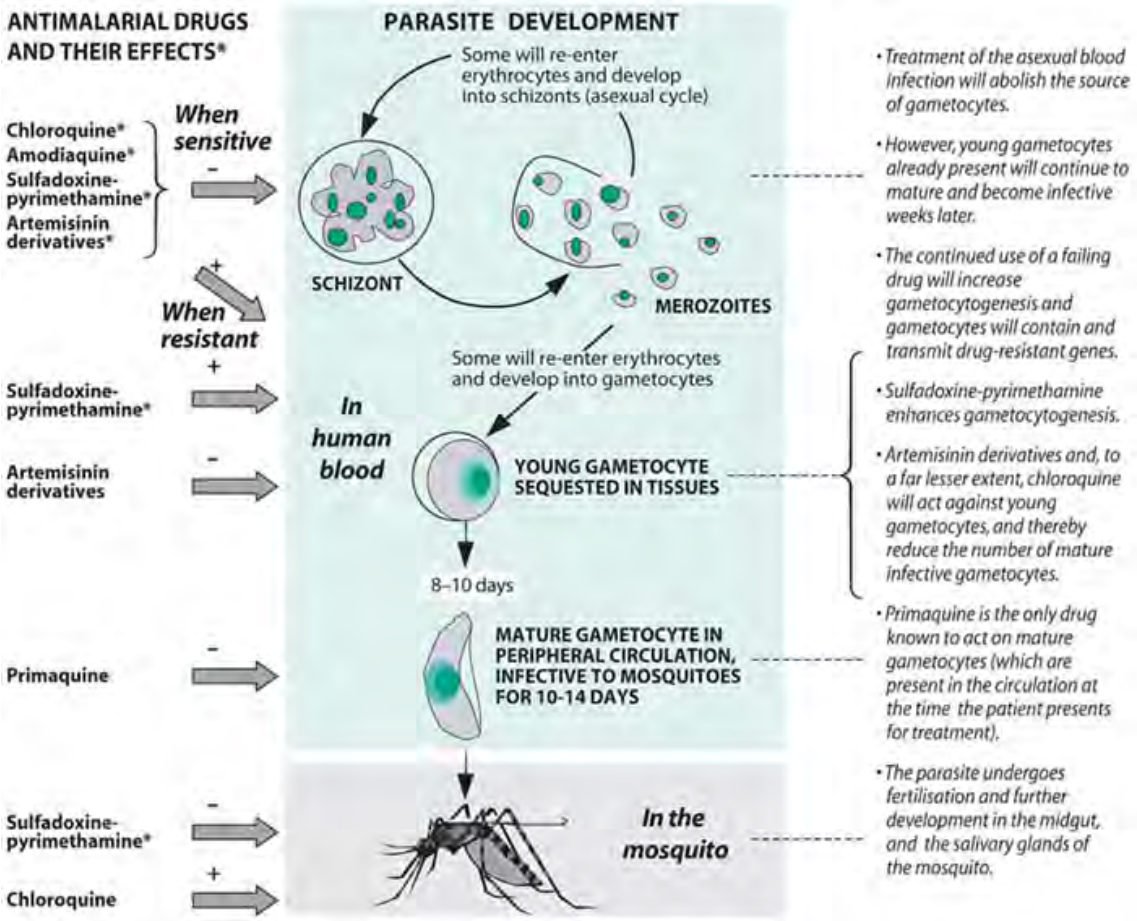

Figure 2: Transmission of Plasmodium falciparum and the effects of antimalarials.

(* When parasites are sensitive to the drug unless otherwise stated. Positive and negative arrows indicate the effect of the drug, enhancement (+) and suppression (-) respectively, on the parasite stage or its development.)

(Guidelines for treatment of malaria, 2nd Edition, 2010, World Health Organisation)

Folates are necessary co-factors needed for the survival of the *Plasmodium* parasite. Unlike their human hosts, which derive folates from their dietary intake, *Plasmodium* species are capable of synthesizing folates *de novo*. Due to this, the enzymes involved have been very attractive targets for antimicrobials since over half a century[23].

Most microorganisms synthesize folate by a simple pathway utilizing GTP (Guanosine-5'-triphosphate), p-ABA (p-Aminobenzoicacid) and L-glutamate[23]. However, unlike most folate synthesizing organisms, *P. falciparum* is capable of utilizing both routes, utilizing folate salvaged from the host plasma, or *de novo* synthesis[23,26,28], as shown in Figure 3.

Studies have shown that *in vitro*, the parasite is capable of depending almost completely on folate in culture medium, when the biosynthesis is blocked. However, the field data suggests that in normal infections, environmental folate cannot satisfy the parasite's requirements and it needs to rely on the biosynthetic pathway[23,26,29,30].

Ever since the folate pathway was deciphered in the early 1960's, the enzymes have been of special interest as attractive drug targets. Of special interest, is a bi-functional enzyme PPPK-DHPS (hydroxymethylpterin pyrophosphokinase-dihydropteroate synthase), which catalyzes two reactions in the pathway. This bi-functional enzyme is not just *Plasmodium* specific and has been reported in other organisms too[31].

The PPPK part catalyzes the ATP-dependent phosphorylation of 6-hydroxymethyl-7, 8-dihydropterin to 6- hydroxymethyl-7, 8-dihydropterin pyrophosphate, while DHPS catalyzes the conversion to Dihydropteroate using pABA as a second substrate, as shown in Figure 3 and 4.

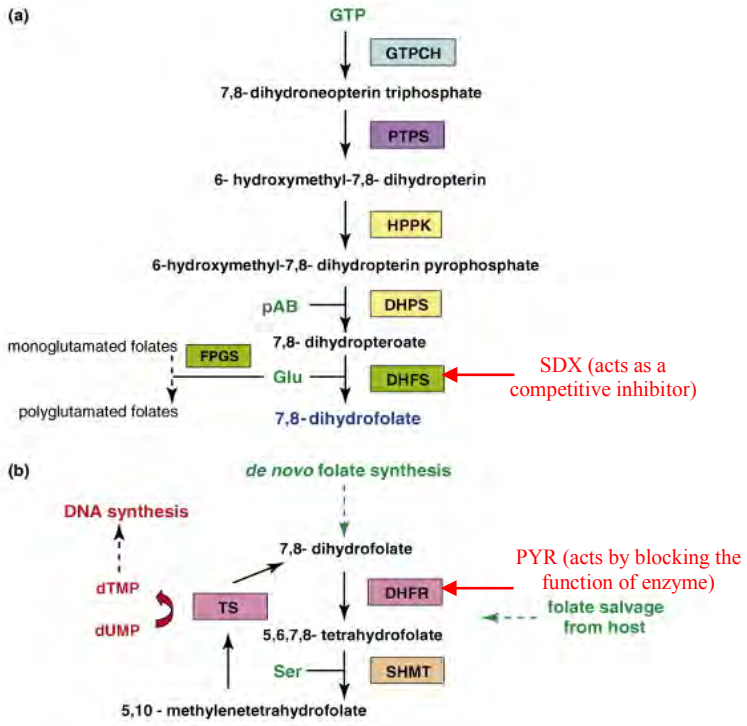

Figure 3: The folate biosynthesis and salvage pathway.

[(a) The folate biosynthetic pathway in *P. falciparum*. Conversion of GTP, *para*-aminobenzoate (pAB) and glutamate to dihydrofolate. (b) The thymidylate cycle. Enzyme activities in (a) and (b) that are encoded by a single bifunctional gene are indicated by boxes of the same color. Dashed arrows indicate multistep processes.]

(adapted from Muller I.B., Hyde J.E., Wrenger C. 2010. Vitamin B metabolism in *Plasmodium falciparum* as a source of drug targets. *Trends in Parasitology* **26** :35-43)

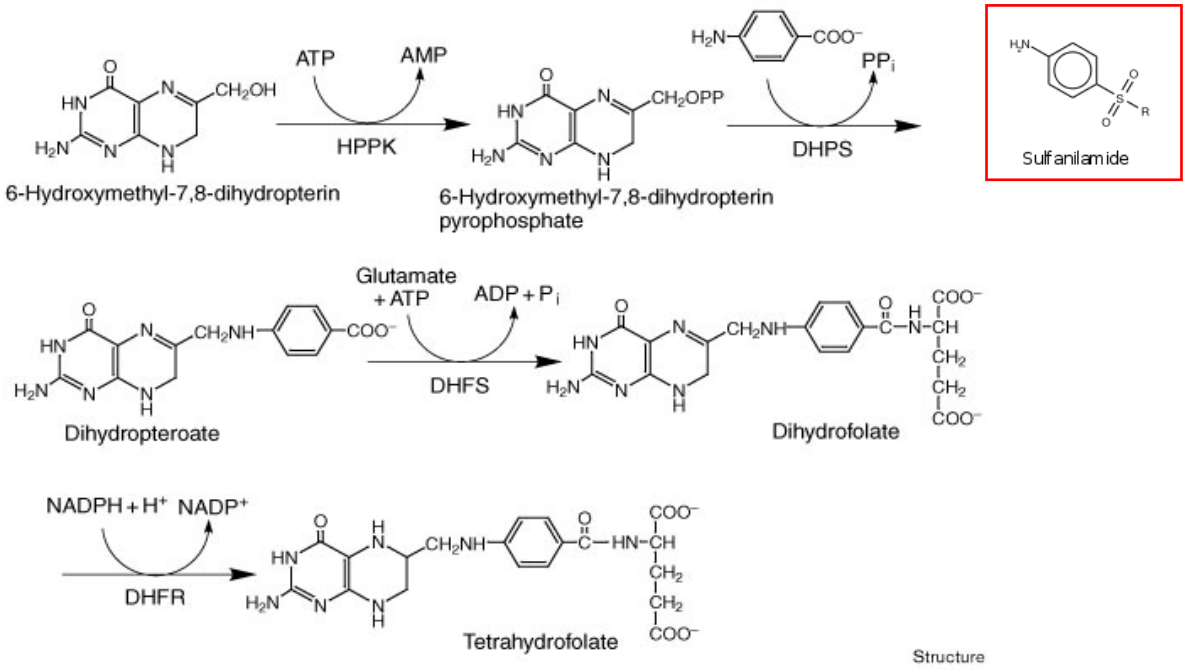

Figure 4: The folate biosynthetic pathway and the sulphanilamide functional group of sulfonamides.

(with permission from; Xiao B, Shi G, Chen X, Yan H, Ji X. 1997. Crystal structure of 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase, a potential target for the development of novel antimicrobial agents. *Structure* **7**: 489–496.)

2.1.4 The Bi-functional Target of Sulfonamides

pfpppk-dhps is encoded on chromosome 8. The primary structure of *pf*PPPK-DHPS was discovered in 1994 by Triglia *et al.*[31] and contains two domains, which have homologues to the PPPK and DHPS of other organisms. The molecular weight of *pf*PPPK-DHPS is 83 kDa[31]. Due to its large size, the crystal structure of the bi-functional enzyme has not been determined so far, but de Beer *et al.*[32] predicted the structure based on the crystal structure of *Saccharomyces cerevisiae* PPPK-DHPS (Figure 5)[32].

The PPPK part contains two *Plasmodium* specific insertions. PPPK-1 is highly conserved in all the *Plasmodium* species, while PPPK-2 shows low sequence conservation between species. The DHPS part also contains two species-specific insertions when compared to other

organisms. DHPS-1 is not conserved in all the species and shows differences in length and sequence. DHPS-2, appears to be highly conserved among all species, especially at the C-terminal, while in *P. falciparum* it shows five extra residues at the N-terminal.

All the *Plasmodium* specific insertions are shown in yellow in Figure 5[32].

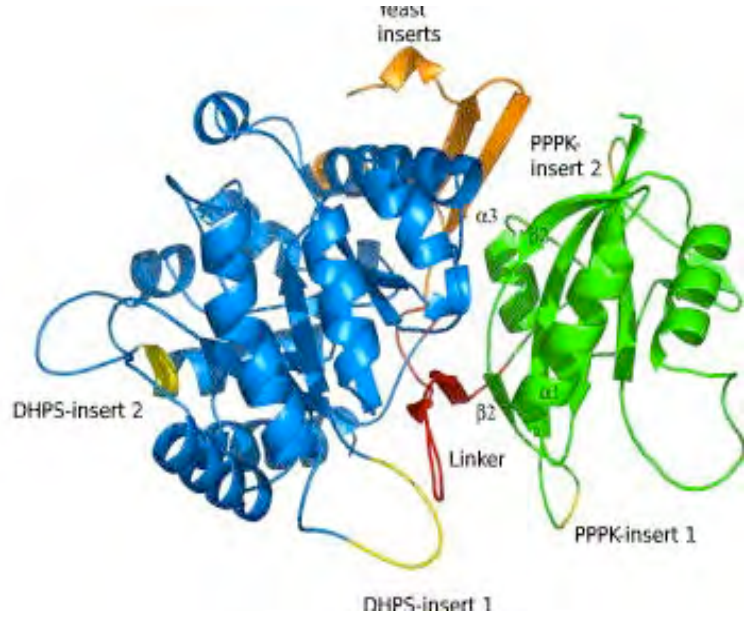

Figure 5: A steric view of the bifunctional *Plasmodium falciparum* PPPK–DHPS model.

(DHPS is colored blue, PPPK is colored green, parasite-specific inserts and locations are indicated in yellow, yeast specific inserts are colored orange, and the linker region is colored red. The substrates are shown in the active site with the metal ion colored gray. The yeast-specific insert was superimposed on the *P. falciparum* model to indicate its relative position.)

(with permission from; de Beer, T.A.P., Louw, A.I., Joubert, F. 2006. Elucidation of sulfadoxine resistance with structural models of the bifunctional Plasmodium falciparum dihydropterin pyrophosphokinase-dihydropteroate synthase. *Bioorganic and Medicinal Chemistry* **14:** 4433-4443)

2.1.5 Antimalarial therapy - Current standards and practices

The rapid resistance to antifolates made it necessary to look for alternate therapies. It was in 1972 that Artemisinin was 're-'discovered' by a Chinese researcher, Tu Touyou, in the

extracts of *Artemisia annua* (annual worm-wood)[33]. It took almost 34 years for Artemisinin to become the treatment choice of WHO, but with the condition that it would be administered in a combination therapy to reduce the risk of development of resistance[34]. The basic idea behind this remains the same as always: two drugs with different drug targets would, in a way, 'confuse' the parasite and thereby, delay (if not prevent) the generation of resistant mutants. Artemisinin is now combined with various drugs like Lumefantrine, Piperaquine and Pyronaridine[33].

Artemisinin is a very broad acting drug that acts on all the stages of parasite development (including the *P. falciparum* gametocytes, which only respond to Primaquine[16] and it is active against all *Plasmodium* species.

However due to its reckless and widespread use, it only took about 2 years for the parasite to develop resistance to the 'wonder drug'. The first case of resistance was reported and confirmed in a study in Cambodia in 2008[35,36]. It took another 4 years for the resistance to spread to neighboring Thailand[37]. The mechanisms for resistance are not clear as of yet.

2.1.6 Previous Investigation and Aim of the study

Despite the widespread resistance to antifolates, the folic acid biosynthesis pathway is one of the very few clinically proven targets in the parasite. Also, very little is known about the component enzymes, which makes it necessary to have a detailed evaluation of the pathway to identify new drug targets.

Previous studies on the bi-functional enzyme PPPK-DHPS, described the properties of the enzyme system by expressing the *Plasmodium* enzyme in a knockout bacteria strain and examining the enzyme kinetics[38]. During a previous study[38], mutants were created by deleting

amino acids in the *Plasmodium* specific inserts. Even very small deletions in PPPK-1 led to loss of PPPK activity, while the DHPS activity remained. Large deletions, however, resulted in complete inactivation of the enzyme. In PPPK-2, the entire *P. falciparum* sequence could be deleted without any effects. As for the DHPS insertions, it has been observed that DHPS-1 is crucial for enzyme activity and only a few amino acids could be deleted without the loss of enzyme activity. DHPS-2, on the other hand, seems dispensable and could be removed without damaging enzyme activity. Initial studies have also been performed on the 42 amino acid long linker region, where a small part (5 amino acids) could be deleted without the loss of any enzyme activity.

The present study was aimed at further defining the limits of amino acids that are essential for the enzyme activity. The deletions in the DHPS-2 were to be combined with DHPS-1 and the linker region. As the Δ247-306 deletion in the PPPK-2 insert did not disturb the enzyme function in Rattanachuen's study[38], this mutant named HD2A was used as a template for mutagenesis PCR (polymerase chain reaction) as well as for a positive control during the enzyme studies. The resulting mutants were to be analyzed by complementation experiments in knockout bacteria and by determining the enzyme activity. To date, a crystal structure of *pf*PPPK-DHPS has not been possible to elucidate because of its large size. Determination of a 'minimal enzyme' may therefore also help in crystallization studies.

2.2 Introduction to *S. mutans folP* project

The nasooropharynx is the first line of defense, microflora of which is established during the first week of life[39]. The dominant flora in the two cavities consists of *Staphylococci* and *Streptococci* (Viridans Group Streptococci [VGS]), respectively[39]. Out of the 18 recognized species of VGS, the Mitis group comprises the largest number of species[40]. These are commensal organisms that play a role in resistance of oral cavity to colonization by other bacterial species and are generally non-pathogenic. *S. mutans* is the cause of dental caries. They also act as a potential reservoir of antibiotic resistance determinants. These determinants could be selected in patients taking antibiotic prophylaxis and be transferred to related pathogenic species like *S. pneumoniae*, leading to emergence of resistant strains[41,42]. *S. pneumoniae* causes diseases like pneumonia and meningitis, resulting in death of more than 1 million children per year worldwide[43,44].

Cotrimoxazole (SXT) is recommended by UNAIDS and WHO for HIV/AIDS prophylaxis in Africa[43,45]. Apart from this, in Africa, SXT is also prescribed regularly in dental practice and for integrated management of childhood illness (IMCI). SXT is a combination of Trimethoprim and Sulfamethoxazole (sulfonamide), and is used as a broad spectrum antimicrobial. Due to the considerable side effects, its use is largely restricted to specific areas of the world where its efficacy has been sufficiently documented[45]. The combined therapy is considered to be more effective as it targets two successive enzymes in the folic acid biosynthesis, as shown in the Figure 6. This was based on a similar principle as the PYR-SDX combination for treatment of malaria (**2.1.3.2**). Sulfamethoxazole acts as a competitive inhibitor of DHPS, by competing with pABA, the secondary substrate and Trimethoprim acts by interfering the DHFR enzyme.

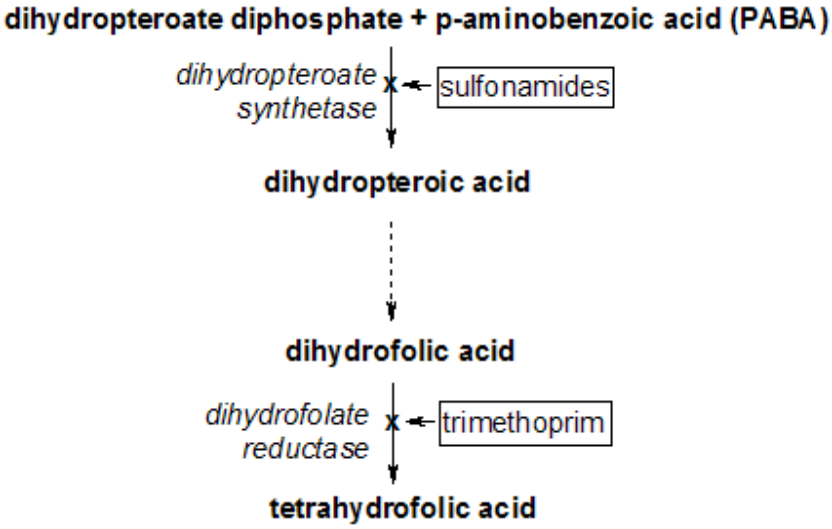

Figure 6: Drug targets for SXT combination therapy. Sulfonamides act as competitive inhibitors of DHPS, while Trimethoprim acts on the DHFR enzyme.

(http://www.onlinepharmacycatalog.com/co-trimoxazole-trimethoprim-sulfamethoxazole/, Last updated 26th June 2007, Date visited 7th August 2012)

As with all antibiotics, increasing use of SXT has lead to emergence of resistance globally[43]. Today, about 20-30% of *S. pnuemoniae* is multi-drug resistant, often to both SXT and penicillin[43,47,48]. Much higher levels of resistance are reported in Africa. In Uganda, for example, SXT has been shown to be highly prescribed in dental practice, and also to select for resistance in *S. mutans* among HIV/AIDS patients on SXT prophylaxis[49,50]. A clinical survey revealed resistance as high as 80% of *S. pneumoniae* to SXT[51]. Sequence analysis has revealed polymorphisms in the *folP* gene of *S. pneumoniae* and related commensals, suggesting a possible role of mutations in *folP* gene for the resistance in these organisms. There is, therefore, an urgent need for the characterization of the resistance mechanisms, not only due to reduced susceptibility to SXT and thus reduced usefulness of the drug among HIV patients[43], but also because of the risk of microbial cross-resistance[43,52]. It has also been reported that long term use of SXT may lead to increased resistance to antifolates among oral

bacterial flora[43,53]. Although extensive resistance in commensal *Streptococci* is clinically recognized[54], little or no data has been published on the mechanisms of resistance[43].

A study was conducted in 2009 to determine the mechanism of sulfonamide resistance in *Streptococcus mutans* by characterizing the *folP* gene encoding the DHPS enzyme, involved in the folate biosynthesis pathway[43]. The study was performed on a clinical isolate named **797**, which showed a high level of sulfonamide resistance *in vitro*. However, when the *folP* gene from the isolate was cloned into a plasmid vector, sulfonamide resistance was not expressed, despite the clone producing sufficient amount of active enzyme to complement the *E. coli* strain's lack of DHPS activity. Further sequence comparisons between different *S. mutans* isolates revealed large variations in the *folP* gene. Only in one case, isolate **8**, did the cloned gene express sulfonamide resistance. Sequence comparison showed that isolate **8** differed in three positions from isolate **797** suggesting the role of point mutations in generation of resistance (Figure 7). In order to gain more insight into the resistance mechanism of *S. mutans*, the project plan was to change these three amino acids one by one and in combination with each other to determine their role in resistance. The plasmid from isolate **797** was used as template for all mutagenesis reactions as well as control for the susceptibility tests.

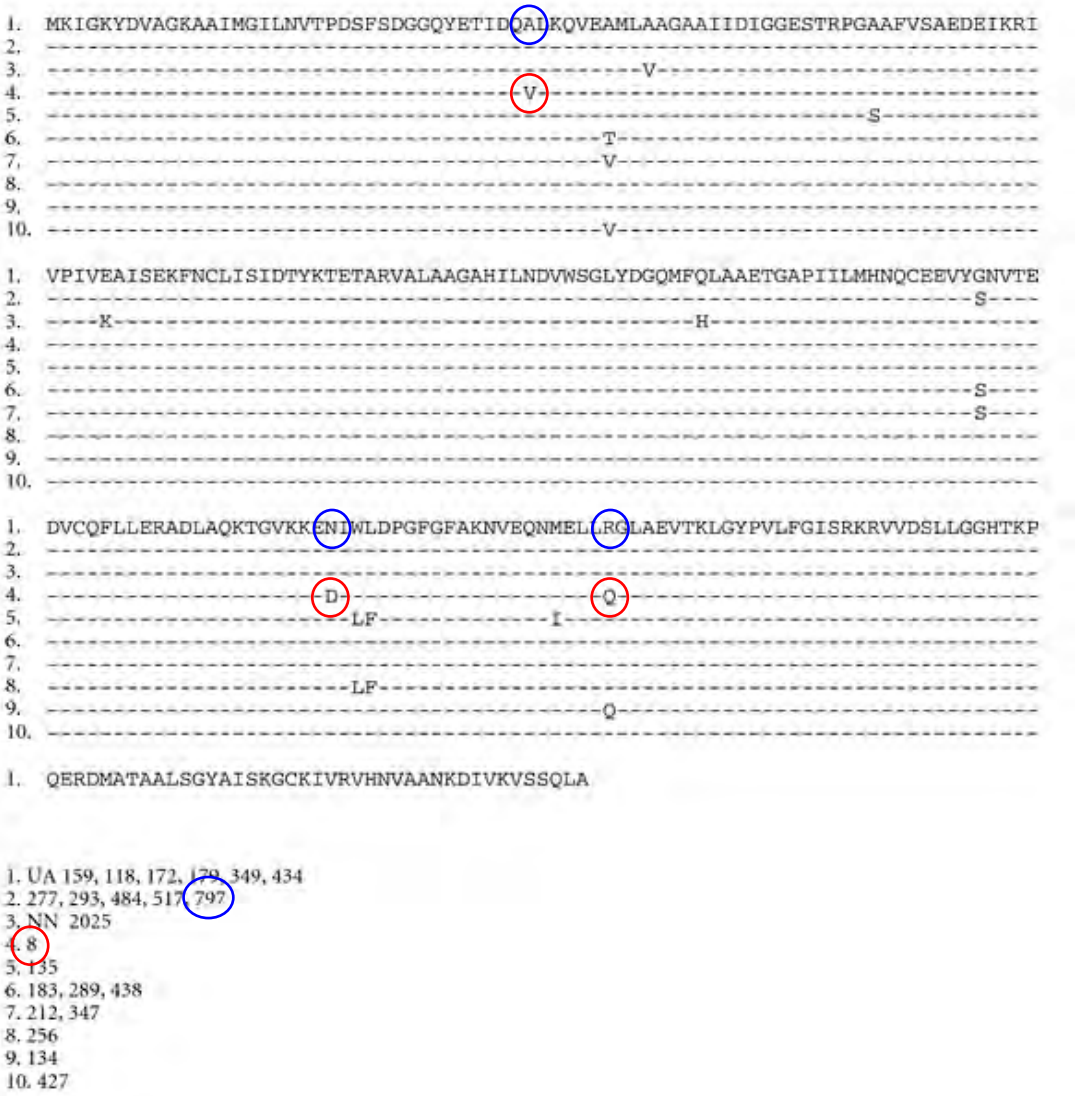

Figure 7: Variations in *S. mutans* DHPS amino acid sequence among various clinical isolates from Uganda. The circled positions represent the polymorphisms between isolate **797** and **8**. *folP* from isolate **8** is used as template for all mutagenesis reactions. The primers are designed so as to revert the polymorphisms in *folP* from isolate **8** to **797**.

(with permission from; William B, Rwenyonyi CM, Swedberg G, Kironde F. 2011, Cotrimoxazole prophylaxis specifically selects for Cotrimoxazole resistance in *Streptococcus mutans* and *Streptococcus sobrinus* with varied polymorphisms in the target genes *folA* and *folP*. *International Journal of Microbiology* **2012**: 1-10)

3. Materials and Methods

3.1 Bacterial Strains

DH5α

The DH5α strain was obtained from Invitrogen and used for routine cloning and transformation to obtain large copies of mutated plasmid.

The genotype of the cells was: F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1.

DH5α was cultured on ISA plates without antibiotics and grew very well at 37ºC.

BL21(DE3)ΔfolP::Km^R

To eliminate the background activity of E. coli *dhps*, a knockout strain was used. This strain was previously created by Fermér and Swedberg and carries Kanamycin resistance for selection[56].

C600ΔfolP::Km^R

To eliminate the background activity of E. coli *dhps*, a knockout strain was used. This strain was previously created by Fermér and Swedberg and carries Kanamycin resistance for selection[56].

3.2 Culture Media and Supplements

Table 1: Composition of Culture Media

LB Broth	12.0 g LB broth (Sigma)	to 600 ml demineralised water.
LB Agar	21.0 g LB Agar (Oxoid)	
Brain Heart Infusion Broth	28.2 g BHI broth (Oxoid)	
Brain Heart Infusion Agar	28.2 g BHI Agar (Oxoid)	
ISO-Sensitest Broth	14.04 g ISO-Sensitest Broth (Oxoid)	
ISO-Sensitest Agar	14.04 g ISO-Sensitest Agar (Oxoid)	
SOC Broth	12.0 g Tryptone (Sigma)	to 600 ml demineralised water
	3.0 g Yeat Extract (Sigma)	
	0.3 g Sodium chloride (Merck)	
	0.11 g Potassium chloride (Merck)	
	0.57 g Magnesium chloride (Merck)	
	1.44 g Magnesium sulphate (Merck)	

Table 2: Composition of Culture Supplements

Ampicillin 50 mg/ml	500 mg Doxtacillin (Meda AB)	9.5 ml sterile demineralised water
Kanamycin 50mg/ml	250 mg Kanamycin Monosulfate (Sigma)	
Thymidine 20 mg/ml	400 mg Thymidine (Sigma)	20 ml sterile demineralised water

Glucose 2M	18.0 g D-Glucose Anhydrous (VWR)	50 ml sterile demineralised water

- All culture media (broth and solid) was autoclaved at 121ºC and 2 bar pressure for 20 minutes. Broth media was stored at 4ºC until needed and the supplements were added just before use. Solid media was prepared a day before and stored at 55ºC. The supplements were added before pouring onto the plates. The plates were allowed to solidify overnight in the laminar airflow and then left at room temperature for 2 days before being stored at 4ºC. The plates with antibiotics were viable for about 6 weeks, and the ones without any supplements were viable for about 2 months.
- All the antibiotics were stored at -20ºC. Thymidine and Glucose were stored at 4ºC until needed.
- The antibiotics were used in the concentration of 50 µg/ml for all the experiments, unless specified otherwise.

For SOC medium, the medium was prepared and autoclaved without any glucose in it (SOB medium). 5 ml of glucose was added just before use by filter sterilising using a 0.2 µm syringe filter.

3.3 Vectors

The *pfpppk-dhps* was previously cloned into a pET-19b expression vector from Novagen, AMS Biotechnology, Oxford. pET-19b is an expression vector with a T7 promoter from bacteriophage T7, which is transcribes at a very high rate, due to which within few hours of induction, the gene product downstream becomes the dominant product in the culture. Also, the vector introduces a 6X N-terminal His tag on the protein, which enables the purification of the protein using Nickel-NTA affinity chromatography. The vector map of pET-19b is shown in Figure 8. *pfpppk-dhps* was cloned in the MCS (shown in black arrow) with NdeI and BamHI restriction enzymes.

The *folP* from *S. mutans* was previously cloned into a pUC19 vector, which is amongst the most commonly used vectors for routine molecular biology work. It contains one amp^R gene, which is used for selection of transformants. The vector map of pUC19 is shown in Figure 9.

After the generation of mutants, *folP* was to be introduced into an expression vector. For this a pET-19b vector system was used.

The pLATE bacterial expression vector used for Ligation Independent cloning was provided in the cloning kit. pLATE vector has similar features as a pET-19b expression vector (viral T7 promoter, *lac* operators for blue-white screening) but additionally, it also employs additional elements that control the basal expression of the cloned gene, providing a tight control of gene expression. The vector map of pLATE51 vector is shown in Figure 10.

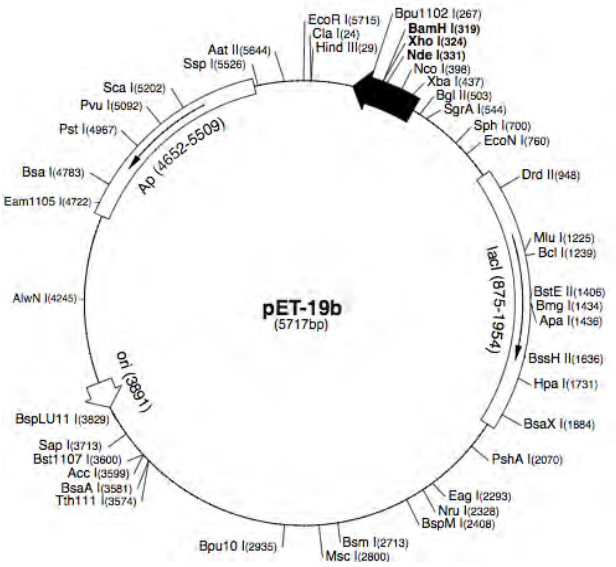

Figure 8: Vector map of pET-19b (Novagen)

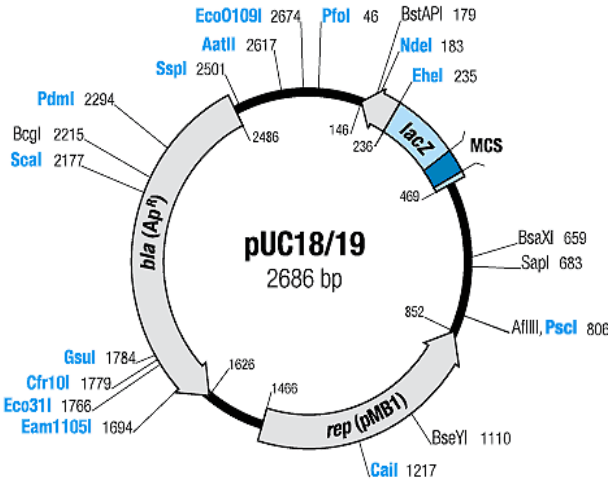

Figure 9: Vector map of pUC19 (Lofstrand)

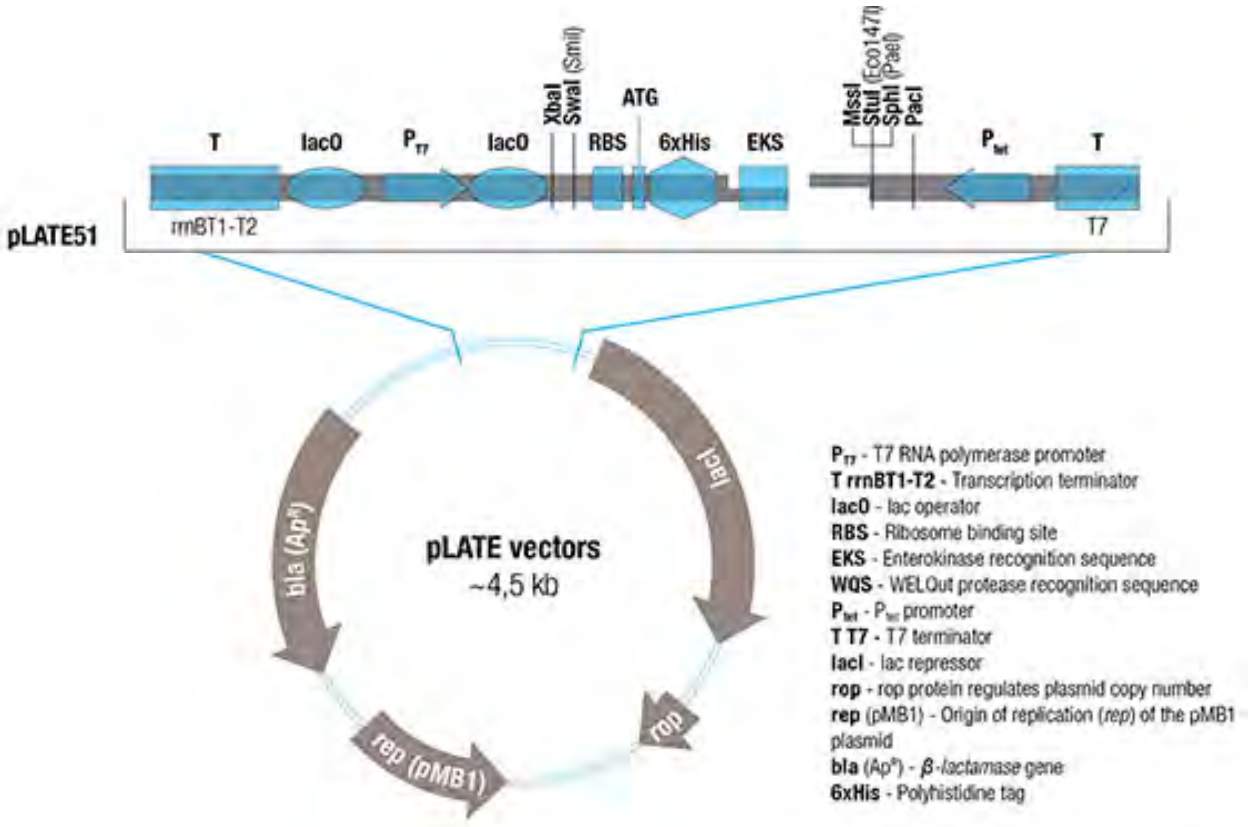

Figure 10: Vector map of pLATE51 (Fermentas)

3.4 Primers

The primer details used for two projects are given in Table 3 and Table 4 respectively. All the primers were synthesised by Eurofins MWG Operon, Ebersberg, Germany and were delivered in a lyophilised form, which was reconstituted with sterile demineralised water prior to use.

Table 3: Primer sequences used for *pfpppk-dhps* mutagenesis and sequencing.

Primer	***Sequence (5'-3')***	***Comments***
del6cfw	TTATTGCCCATTGCATGAATGGATTAGCAATTG CTTCCTA	52 amino acid deletion of DHPS-2 in *pf*PPPK-DHPS.
del6crev	TAGGAAGCAATTGCTAATCCATTCATGCAATGG GCAATAA	
T7fw	TAATACGACTCACTATAGG	pET-19b vector specific primers. The *rev* was also used in sequencing.
T7rev	GCTAGTTATTGCTCAGCGG	
P7fw	TGACGAAATAATGAAAAATAATTTAAG	Used to sequencing of samples with *4e* template.

Table 4: Primer sequences used in the determination of role of point mutations of *folP* in conferring resistance to *S. mutans.*

Primer	***Sequence (5'-3')***	***Comments***
V37Afw	AAACAATCGATCAGGCTCTAAAACAGGTTGA	Used for generation of point mutations in *folP* from Isolate 8.
V37Arev	TCAACCTGTTTTAGAGCCTGATCGATTGTTT	
D172Nfw	GGAGTTAAAAAAGAAAATATTTGGCTTGATC	

D172Nrev	GATCAAGCCAAATATTTTCTTTTTTAACTCC	The number represents the position of amino acid and the alphabets represent the mutation (e.g.: change of V to A at position 37)
Q193Rfw	ACATGGAACTTCTACGAGGCTTAGCGGAGGT	
Q193Rrev	ACCTCCGCTAAGCCTCGTAGAAGTTCCATGT	
Universal fw	GTAAAACGACGGCCAGT	Used for amplification of the mutant *folP*. Universal primers flank the pUC vector carrying *folP* gene.
M13rev	AGCGGATAACAATTTCACACAGGA	
mutdhpssph	GATCGATCGCATGCACATCATAACTAGGGAGCAAGC	Used for sequencing of the mutant *folP*.
mutansdhpsnde	GGAGCACATATGAAAATTGGTAAATATG	Used for generation of restriction sites on the *S. mutans folP* for pET cloning.
mutansdhpsbam	GATCGATCGGATCCAAAATAATCTTATCCATAACACCCTCA	
pJET1.2 Forward Sequencing Primer	CGACTCACTATAGGGAGAGCGGC	Used for testing the pJET clones. The primers flank the pJET1.2/blunt vector.
pJET1.2 Reverse Sequencing	AAGAACATCGATTTTCCATGGCAG	

Primer		
mutfolPfw	GGTGATGATGATGACAAGATGAAAATTGGTAAATATG	Used for generating overhangs on the *S. mutans folP* for LIC.
mutfolPrev	GGAGATGGGAAGTCATTATACTAACTGGCTGCTGAC	
LIC Forward Sequencing Primer	TAATACGACTCACTATAGGG	Used for testing LIC clones. The primers flank the pLATE51 vector and the expected product size is around 1166bp (900bp insert+266bp vector).
LIC Reverse Sequencing Primer	GAGCGGARSSCAATTTCACACAGG	

3.5 Buffers and Solutions

Table 5: Agarose Gel Electrophoresis

0.5M EDTA (pH 8.0)	186.1 g	Di-sodium ethylenediamine tetra-acetate (Merck)	to 1000 ml demineralised water
	24.6 g	Sodium Hydroxide (Merck)	
10X TBE	108.0 g	Tris base (Sigma)	
	55.0 g	Boric Acid (Merck)	
	40.0 ml	0.5M EDTA (pH 8.0)	
0.8% Agarose Gel	2.0 g	Ultrapure Agarose (Invitrogen)	
	200 ml	1X TBE	

- Agarose heated at 800W in a microwave for about 4 minutes till the agarose dissolved, giving a clear solution. About 4 drops of Ethidium Bromide 0.07% solution (Applichem) were added to the solution and it was stored at 55ºC.

Table 6: *KAPA HiFi™ PCR Mix*

14.0 µl	nuclease free water (Qiagen)
5.0 µl	5X KAPA HiFi Fidelity Buffer
0.75 µl	KAPA dNTP Mix(10mM each)
0.75 µl	Forward Primer (10µM)

0.75 µl	Reverse Primer (10µM)
1.25 µl	DMSO (100%)
0.50 µl	KAPA HiFi DNA Pol (1 U/µl)
2.0 µl	Template DNA

Table 7: DpnI Digestion Mix

25.0 µl	PCR product
1.0 µl	DpnI enzyme (10 U/µl) (Fermentas)

Table 8: *dNTP-Mix*

10.0 µl	ATP 100 µM (Fermentas)	960.0 µl nuclease free water (Qiagen)
10.0 µl	GTP 100 µM (Fermentas)	
10.0 µl	CTP 100 µM (Fermentas)	
10.0 µl	TTP 100 µM (Fermentas)	

Table 9: *DreamTaq™/Colony PCR mix*

17.25 µl	nuclease free water (Qiagen)
2.5 µl	10X DreamTaq™ Green Buffer (Fermentas)
2.5 µl	dNTP Mix (10mM each dNTP)
1.25 µl	Forward Primer (10µM)
1.25 µl	Reverse Primer (10µM)
0.25 µl	DreamTaq™ DNA Polymerase (5 U/µl) (Fermentas)

Table 10: Sequencing Mix

25-75 ng	DNA	to 18 µl nuclease free water (Qiagen)
1.0 µl	Sequencing primer (10 µM)	

Table 11: Double Digestion Mix

11.0 µl	Cleaned PCR product
6.0 µl	10X Buffer Tango (Fermentas)
1.5 µl	Nde I (Fermentas)
1.5 µl	BamH I (Fermentas)
10.0 µl	nuclease free water (Qiagen)

Table 12: Protein Purification

Binding Buffer (pH 8.0)	6.89 g	Potassium dihydrogen phosphate (50mM)(Merck)	to 1000 ml demineralised water	pH was adjusted with 2M Sodium hydroxide (Merck)
	29.2 g	Sodium Chloride (500mM)(Merck)		
	0.68 g	Imidazole (10mM)(VWR)		
	100 ml	Glycerol (10%)(Merck)		
Washing Buffer (pH 6.0)	6.89 g	Potassium dihydrogen	to 1000 ml demineralised	pH was adjusted with concentrated

		phosphate (50mM)(Merck)	water	Hydrochloric Acid (Merck)
	58.4 g	Sodium Chloride (1M)(Merck)		
	6.81 g	Imidazole (100mM)(VWR)		
	100 ml	Glycerol (10%)(Merck)		
Elution Buffer (pH 8.0)	6.89 g	Potassium dihydrogen phosphate (50mM)(Merck)	to 1000 ml demineralised water	pH was adjusted with 2M Sodium hydroxide (Merck)
	29.2 g	Sodium Chloride (500mM)(Merck)		
	3.40 g	Imidazole (500mM)(VWR)		
	200 ml	Glycerol (20%)(Merck)		

Table 13: SDS-PAGE Gel Electrophoresis

10X SDS Running Buffer	30.0 g	Tris base (Sigma)	to 1000 ml demineralised water	
	188.0 g	Glycine (Sigma)		
	100 ml	10% SDS (Serva Electrophoresis)		

2X SDS Loading Buffer	0.1 ml	Glycerol (Merck)	to 50 ml demineralised water	10% ß-merceptoethanol (Merck) was added just before use.
	0.2 ml	10% SDS (Serva Electrophoresis)		
	0.0125 mg	Bromophenol Blue (Merck)		
	0.125 ml	0.5 M Tris-HCl (pH 6.8)(Merck)		
15% Running Gel	2.3 ml	sterile demineralised water		4.0 µl TEMED (USB Corporation, Cleveland, USA) was added just before pouring the gel into the gel cast, as the polymerisation reaction starts immediately on the addition of TEMED.
	5.0 ml	30% Acrylamide/bis-Acrylamide (Bio-Rad)		
	2.5 ml	1.5 M Tris-HCl (pH8.8)(Sigma)		
	0.1 ml	10% SDS (Serva Electrophoresis)		
	0.1 ml	10% Ammonium persulfate (Sigma)		
5% Stacking Gel	2.1 ml	sterile demineralised water		3.0 µl TEMED (USB Corporation, Cleveland, USA) was added just
	0.5 ml	30%		

		Acrylamide/bis-Acrylamide (Bio-Rad)		before pouring the gel into the gel cast, as the polymerisation reaction starts immediately on the addition of TEMED
	0.38 ml	1.5 M Tris-HCl (pH8.8)(Sigma)		
	0.03 ml	10% SDS (Serva Electrophoresis)		
	0.03 ml	10% Ammonium persulfate (Sigma)		
Coomassie Solution	0.4 g	Coomassie Brilliant Blue R-250 (Eastman Kodak Co)		
	50.0 ml	demineralised water	to 200 ml demineralised water	
	15.0 ml	Glacial Acetic Acid (Sigma)		
	60.0 ml	95% Ethanol (Solveco)		
Destaining Solution	7 ml	Glacial Acetic Acid (Sigma)	to 100 ml water	

Table 14: Enzyme Assay

300 µM ^{14}C-pABA	72.2 µl	sterile demineralised water		
	24.8 µl	para-Aminobenzoic acid (Sigma)		
	3.0 µl	^{14}C-pABA (1.0 mCi) (Moravet Biochemicals)		
Radioactive Assay mixture	5.0 µl	1M Tris-HCl (pH 9.0)(Sigma)	to 50 µl sterile demineralised water	
	5.0 µl	Magnesium chloride (100mM)(Merck)		
	5.0 µl	ß-Merceptoethanol (1M)(Merck)		
	5.0 µl	ATP (Sigma)		
	5.0 µl	H_2-pteridine-phosphate (200µM)		
	5.0 µl	^{14}C-pABA (100µM)		
	2 to 4 µg	purified enzyme		

		extract		
Phosphate Buffer (pH 6.0)	5.30 g	Potassium dihydrogen phosphate (Merck)	to 1000 ml demineralised water	pH was adjusted with 2M Sodium hydroxide (Merck).
	10.85 g	Sodium hydrogen phosphate dihydrate (Merck)		

3.6 Plasmid Isolation

A single bacterial colony was suspended in 3 ml of medium and incubated over-night at 37 ºC with shaking. 1.5 ml of the over-night culture was transferred to an Eppendorf tube and centrifuged at 8000 rpm for 3 minutes. The supernatant was discarded and the remaining 1.5 ml of over-night culture was added to the resulting pellet, followed by another centrifugation step. Further steps were carried out using the GeneJet™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's protocol. The details of the culture media and supplements are given in Table 1 and 2.

For examining the purity of the plasmid, the samples were run on 0.8% agarose gel (Table 5) at 90 V for 50 minutes.

3.7 Mutagenesis PCR

3.7.1 for *pf*PPPK-DHPS:

Mutagenesis PCR was carried out with the *del6c* primers and different strains of HD2A single mutants. These included: Δ4e (previously introduced with a single mutation in the linker region by deleting 10 amino acids); Δ5a, Δ5b, Δ5d, Δ5f (with deletions in the *pf*DHPS-1 region spanning 10, 8, 8, 9 amino acids respectively).

The deletion primer pair was designed such that it would anneal partly to the template. This would result in a loop which would not be amplified and thus, deleted in the product.

Table 15: Template details for the mutagenesis PCR.

Template		***Deletion***
Δ5a	DHPS-1 insertion. Previous studies have shown that this region is important for enzyme activity and only a few amino acids can be deleted before the enzyme starts to loose activity.	10 amino acid deletion (IKNKIVKCDA)
Δ5b		8 amino acid deletion (NKIVKCDA)
Δ5d		8 amino acid deletion (NDIKNKIV)
Δ5f		9 amino acid deletion (NDIKNKIVK)
Δ4e	Linker region.	10 amino acid deletion (YVSRMKEQYN)

Primer details are given in Table 3 and the complete *pf*PPPK-DHPS sequence is shown in appendix (**6.1**). The mutagenesis PCR was carried out with the KAPA HiFi™ PCR Kit, following the manufacturer's guidelines with regards to both, the reaction mixture (Table 6) and the reaction conditions (initial denaturation at 95 ºC for 2 minutes; followed by 20 cycles of denaturation at 98 ºC for 20 seconds, annealing at 60 ºC for 30 seconds, extension at 72 ºC for 2 minutes; and a final extension step of 5 minutes at 72º C).

The PCR products were analyzed on 0.8% agarose gel.

3.7.2 for *S. mutans dhps*:

Mutagenesis PCR was also carried out for introducing point mutations in the Isolate **8** *dhps*

gene, which served as the template for all the mutagenesis reactions. For this, three primer sets; *37*, *172* and *193* (named for the position of the amino acid to be mutated in the Isolate 8) were used, generating mutants that were labeled by similar names. Primer details are given in Table 4 and the sequence of *S. mutans dhps* is shown in the appendix (**6.2**). The mutagenesis PCR was carried out with KAPA HiFi™ PCR Kit, following the manufacturer's guidelines with regards to both, the reaction mixture (Table 6) and the reaction conditions (initial denaturation at 95ºC for 2 minutes; followed by 20 cycles of denaturation at 98ºC for 20 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 2 minutes; and a final elongation step of 5 minutes at 72ºC). To analyze the PCR products, they were run on 0.8% agarose gel at 90V for 50 minutes and Generuler™ 1kb Plus DNA ladder (Fermentas) was used for size determination.

3.8 Digestion of PCR Products

The PCR products were digested with DpnI restriction endonuclease (Fermentas), which specifically digests methylated DNA (template) and thereby reduces the chances of false positives during transformation (which contain the template DNA). DpnI has proved to be just as efficient, without the use of a standard digestion mixture containing the buffer specified in the manufacturer's protocol. 1 µl of the enzyme was mixed with the 25 µl of PCR reaction mixture (Table 7) and was incubated at 37 ºC for 75 minutes.

3.9 Chemical Transformation

The PCR products or the plasmid was transformed into competent DH5α cells to obtain multiple copies of the mutated plasmid, while BL21(DE3)Δ*folP::Km*^{R} cells were used for protein expression. For antibiotic susceptibility testing, C600Δ*folP::Km*^{R} cells were used.

3.9.1 Preparation of Competent Cells

For preparation of DH5α cells, a single cell was suspended in 3 ml of LB broth medium and incubated over-night at 37 ºC with shaking. 1 ml of the culture was inoculated into 20 ml of LB broth medium the next day and the culture was grown at 37 ºC with shaking till OD_{600} reached 0.35-0.40. The suspension was then centrifuged at 4500 rpm for 7 minutes at 4 ºC. The supernatant was discarded and the resulting pellet was resuspended in 10 ml ice-cold 50 mM $CaCl_2$ solution, followed by incubation on ice for 5 minutes. After another centrifugation step, the pellet was resuspended in 2 ml of ice-cold 50 mM $CaCl_2$. The resulting cells were viable for 2 days when stored at 4 ºC. For stock preparation and long term storage, 15% glycerol was added to the 50mM $CaCl_2$ and care was taken to store the cells at -80 ºC immediately.

In preparation of BL21(DE3)Δ*folP::Km*^{R} and C600Δ*folP::Km*^{R}, a similar procedure was followed except that the cells were grown in BHI broth containing Kanamycin and 200 µg/ml of Thymidine.

3.9.2 Transformation Protocol

For transforming the plasmids, 5 µl of plasmid was mixed with 200 µl of competent cells, and incubated on ice for 30 minutes; followed by heat-shock at 42 ºC for 2 minutes. The cells

were then placed on ice for 2 minutes. Then 500 µl of SOC medium was added and the cells were incubated at 37 ºC with shaking to allow them to recover. After incubation for 60 minutes, the cells were centrifuged at 6000 rpm for 3 minutes. The resulting pellet was re-suspended in 100 µl of SOC medium and plated on LA+Amp plates. The plates were incubated over-night at 37 ºC. The resulting colonies were re-streaked on LA+Amp plates to eliminate the chances of false positives and analyse the growth pattern.

For transforming the digested PCR products, a similar protocol was used, except that the entire 26 µl of the reaction mixture (25 µl of PCR mixture+1 µl enzyme) was used for transformation with 200 µl of cells.

3.10 Electroporation

For plasmids that could not be successfully transformed with the chemical method, electroporation was used.

3.10.1 Preparation of Competent Cells

For preparation of competent cells, a single cell was suspended in 3 ml of appropriate broth medium and incubated over-night at 37 ºC with shaking. 1 ml of the culture was inoculated into 20 ml of broth medium the next day and the culture was grown at 37 ºC with shaking until the OD_{600} reached 0.3-0.4. The suspension was then centrifuged at 4500 rpm for 15 minutes at 4 ºC. The supernatant was discarded and the resulting pellet was re-suspended in 10 ml ice-cold de-ionized water. After another centrifugation step, the washing step was repeated 3 more times. After the final washing, the cells were re-suspended in 200 µl water.

The cells were used the same day. For stock preparation and long term storage, water with 15% glycerol was used for washing. After the final re-suspension in 200 µl water and glycerol solution, care was taken to store the cells at -80ºC immediately.

3.10.2 Transformation

The electroporation cuvettes (2 mm) were chilled on ice prior to use and care was taken to keep everything ice-cold until needed. 1 ml of SOC media per sample and LA+Amp selection plates were pre-warmed at 37ºC. 1 µl of the plasmid was carefully placed on the inside of the cuvette and 40 µl of competent cells were added on the top. The cuvette was gently tapped to ensure that there were no bubbles and the cells were in a thin layer. The mixture was electroporated using the following conditions for BTX ECM 630 and Bio-Rad GenePulser electroporators: 2.5 kV, 200 Ω and 25 µF; with a time constant of about 4.6 milliseconds.
Immediately after electroporation, 1 ml of pre-warmed SOC was added to the cells and the suspension was transferred to a fresh Eppendorf tube and incubated at 37 ºC with shaking to allow them to recover. After incubation for 60 minutes, the cells were centrifuged at 6000 rpm for 3 minutes. The resulting pellet was re-suspended in 100 µl of SOC medium and plated on LA+Amp plates. The plates were incubated over-night at 37 ºC. The resulting colonies were re-streaked on LA+Amp plates to eliminate the probability of false positives and to analyse the growth vigour.

3.11 Sequencing

Following transformation, it was necessary to confirm the presence of the deletion mutation, for which sequencing reactions were prepared. A single colony of the transformants was

suspended in a standard PCR reaction mixture containing DreamTaq™ polymerase (Fermentas) (Table 8 and 9) and the PCR reaction was carried out with an initial denaturation step of 2 minutes at 94 ºC; followed by 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 2 minutes; followed by a final extension step at 72 ºC for 5 minutes. The primers (*T7* primers and *mutdhpssh* respectively), flanking the DHPS gene were used for amplification. For each strain, the reaction was performed in quadruplets.

The products were analysed on a 0.8% agarose gel, which was followed by purification of the products using GeneJet® PCR Purification Kit (Fermentas) according to the manufacturer's guidelines, except that the product was eluted in 20 µl of Elution Buffer instead of 50 µl as specified in the kit. The concentration of the purified product was measured using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA) and water as blank. The sequencing mixture was prepared as described and the primer *T7rev* was used. For Δ4e mutants, an additional sample was prepared with primer *P7fw* (Table 10). The sequencing was performed at the Uppsala Genome Centre, Rudbeck Laboratories, using the Sanger Sequencing method. The resulting sequences were aligned and analysed using 4Peaks Bio-Edit Sequence Alignment Editor, from Tom Hall Ibis Biosciences (Carlsbad, USA).

3.12 Antibiotic Susceptibility Testing

To measure the susceptibility of the *folP* mutants to sulfonamides and for comparison with the wild type clinical isolate (Isolate 8), the Kirby-Bauer disk-diffusion test was carried out with Sulphamethoxazole 100 µg discs (Oxoid). However, the test did not give reproducible results, and therefore the Agar Dilution Test was carried out as well. The Agar Dilution Test is generally a preferred method for testing as it reports the susceptibilities in terms of Minimal

Inhibitory Concentrations (MIC) (as opposed to zone of inhibition value from the Disc Diffusion method) and is more reproducible.

3.12.1 Kirby-Bauer Disk-Diffusion Test

Competent C600Δ*folP::Km*^{R} were transformed with plasmids isolated from the single mutants of *folP* generated before. A single colony of each transformed strain was suspended in 3 ml sterile water. A pre-warmed ISA+Amp plate was inoculated with the suspension using a sterile cotton swab. This step was repeated three times, rotating the plate by an angle of 90º and 45º each time. The plate was dried for about 5 minutes, before placing an antibiotic disc in the centre of the plate using a pair of sterile forceps. Care was taken to ensure that the disc did not move after it touched the surface of the plate as the antibiotic starts diffusing into the agar immediately. The plate was sealed with parafilm and incubated over-night at 37ºC. The Zone of Inhibition (ZOI) was measured the following day, by measuring the diameter of the clear zone surrounding the disc to the nearest millimetre.

3.12.2 Agar Dilution Test

Transformed C600Δ*folP::Km*^{R} was cultured overnight in 5 ml of ISB with ampicillin. 100 µl of the overnight culture was plated on ISA plates with different concentrations of antibiotic. The antibiotic used was Sulfathiazole, which is a short acting sulfonamide. The antibiotic was incorporated in the concentrations 0.02, 0.03, 0.04 and 0.05 mM. The plates were incubated overnight at 37ºC and the resulting colonies were analysed the following day. The highest concentration of antibiotic that gave no colonies was reported as the Minimal Inhibitory Concentration (MIC) for the concerned strain.

3.13 Generation of Double and Triple Mutants

Plasmids isolated from the single mutants were used as template for generation of double mutants in various combinations of template and primer pair (37+172, 37+193, and 172+193). All the steps were similar as before. Following the generation of double mutants and antibiotic susceptibility testing, the entire procedure was repeated with double mutant acting as the template for the mutagenesis reaction.

3.14 Cloning of the mutated gene into expression vector

To check for the activity of the mutated enzyme, the *folP* gene had to be cloned into an expression vector, which would enable the expression of the mutated protein at a large concentration. For this, the pET19b vector was to be used, which contains a T7 promoter for expression of the protein (Vector map is shown in **3.3**). Furthermore, it also expresses the protein with a N-terminal 6x-His tag, which enables the purification of expressed protein by Nickel-NTA column affinity chromatography. The gene was to be cloned in between the NdeI and BamHI restriction sites on the pET vector.

3.14.1 Generation of restriction sites on the S. mutans folP

S. mutans folP was amplified with suitable primers to generate restriction sites. The primers used were: *mutansNdeI* and *dhpsbam*, the primer details are given in Table 5. Colony PCR was carried out by mixing a single colony of the mutant in PCR mix (Table 9). DreamTaq™ (Fermentas) kit with PCR conditions as specified in the manufacturer's guidelines was used (initial denaturation at 94ºC for 2 minutes, followed by 30 cycles of denaturation at 94ºC for

30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 90 seconds; ending with a final extension at 72ºC for 5 minutes)

The products were analysed on a 0.8% agarose gel as before.

3.14.2 pJET Ligation

The PCR products were cloned into a pJET1.2/blunt-cloning vector prior to cloning into pET19b vector. Previous experience in the lab has shown that it is easier to do a two step cloning for pET19b vectors than a single step direct cloning protocol which drastically reduces the probability of generating clones. DreamTaq™ kit generates PCR products with sticky ends and pJET is a blunt-end cloning vector. Because of this it was necessary to cleave the overhangs. The entire protocol was carried out according to the CloneJET™ PCR Cloning Kit (Fermentas). The only additional step was cleaning the PCR products prior to the start of cloning. The cleaning was done using GeneJet™ PCR Purification Kit (Fermentas) as before. After the ligation reaction, the mixture was transformed into competent DH5α cells as before. (The manufacturer specifies using 25 µl of commercially available DH5α cells)

To check for the presence of the ligated gene in the pJET vector and for amplification prior to cloning in the pET vector, colony PCR was run on the transformants with primers specific for the pJET vector (included in the cloning kit). The reaction was carried out with DreamTaq™ under the following conditions: initial denaturation at 94ºC for 2 minutes; followed by 25 cycles of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 3 minutes; ending with a final extension step at 72ºC for 5 minutes. The products were analysed on 0.8% agarose gel as before. The PCR products were cleaned using

GeneJet™ PCR Purification Kit (Fermentas) as before and the concentration measured using Nanodrop 1000 Spectrophotometer.

3.14.3 pET Cloning

For cloning into pET19b vector, both the PCR product and vector were digested with the same restriction endonucleases so as to have compatible sticky ends on both, which would make it easier to ligate. The restriction endonucleases were chosen such that:

1. they generated sticky ends,
2. the restriction sites were not present on the *S. mutans folP* i.e. the restriction sites would be primer generated sites, flanking the gene and
3. the restriction sites were present on the multiple cloning site (MCS) of the pET vector.

Two endonucleases, Nde1 and BamH1, were chosen. The digestion mix was prepared as described (Table 11) and was incubated at 37ºC for 2 hours and 15 minutes. The digested PCR products were incubated on ice, while the vector was dephosphorylated to remove any 5'-phosphate group from the vector, thus, reducing the chances for re-circularisation of the vector. Dephosphorylation was carried with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) according to the manufacturer's guidelines. The dephosphorylated, digested vector and the digested PCR products were run on 0.8% agarose gel and purified from the gel using the GeneJET™ Gel Extraction Kit according to the manufacturer's guidelines, except that the product was eluted in 20 µl of elution buffer, as opposed to 50 µl specified in the kit. The concentration of the purified product was measured using the Nanodrop 1000 Spectrophotometer. Ligation reaction was set up using the Rapid DNA Ligation Kit (Fermentas) and the manufacturer's guidelines were strictly adhered to. Post ligation, the entire mixture was used for transformation into competent DH5α cells as described before.

To check if the ligation reaction was successful, a colony PCR of the transformants was carried out using two sets of primers: *mutansNdeI* and *dhpsbam* (specific for the insert); and *T7 fw* and *T7 rev* (specific for the pET19b vector). DreamTaq™ and the following conditions were used: (for *mutansNdeI* and *dhpsbam* primers) initial denaturation at 94ºC for 2 minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 90 seconds; ending with a final extension at 72ºC for 5 minutes; (for *T7* primers) initial denaturation at 94ºC for 2 minutes; followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 2 minutes; ending with a final extension step at 72ºC for 5 minutes.

3.15 Ligation Independent Cloning (LIC)

When after repeated trials with the Rapid DNA Ligation Kit no results were obtained, we switched to aLICator™ Ligation Independent Cloning and Expression System (Fermentas). The kit contains a pLATE51 bacterial expression vector with a T7 promoter, a 6x N-terminal His tag and uses directional ligation independent cloning (LIC) cloning which does not depend on restriction and ligation steps.

The ligation was carried out strictly according to the manufacturer's guidelines. The original plasmids were used as template (diluted to 5 times with water) and were amplified using a new set of primers: *mutfolP*, which were designed according to the guidelines. The PCR reaction was carried out using KAPA HiFi™ PCR Kit with the following reaction conditions: initial denaturation at 95ºC for 2 minutes; followed by 20 cycles of denaturation at 98ºC for 20 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 2 minutes; and a final extension step of 5 minutes at 72ºC. The PCR products were run on 0.8% agarose gel,

purified from the gel as before and the concentration measured on Nanodrop. The reaction mixture was set up to generate the necessary 5' and 3' overhangs on the purified PCR product, followed by the annealing reaction as described in the manufacturer's protocol. The annealed mixture was directly used for transformation as described before. To check for the presence of the insert, colony PCR was run according to the kit's guidelines with *LIC Forward* and *Reverse Sequencing* primer pair and DreamTaq™ kit. The PCR products were analysed on 0.8% agarose gel as before and the expected size was 1166 bp (900 bp of insert + 266 bp of pLATE51 vector).

3.16 Enzyme Purification

To determine the activity of mutated enzyme, the crude extract was harvested using sonication and was then purified using Ni-NTA column, which enabled the isolation of the His-tagged enzyme from the crude extract.

3.16.1 Crude Extract Preparation

The strain to be analysed was grown over-night in 5 ml LB broth medium with 50 µg/ml Ampicillin at 37 ºC. This was then used to inoculate 600 ml LB broth medium with antibiotic. The culture was induced with IPTG at the final concentration of 1mM after it reached the OD_{600} of 0.5 and it was incubated over-night at 30 ºC with shaking. The culture was then centrifuged at 3000 rpm for 15 minutes at 4 ºC and the resulting pellet was re-suspended in 30 ml Binding Buffer. The suspension was transferred to a 50 ml centrifugation tubes and subjected to a similar centrifugation step as before. The pellet was re-suspended in 2 ml Binding Buffer. The suspension was then sonicated using a pulse mode with 13 cycles of 30 seconds sonication followed by 30 seconds of cooling, with the Biorupter UCD 3000

(Diagenode, Denville, USA). After the sonication, the suspension was transferred to 1.5 ml centrifugation tubes and centrifuged at 15,000 rpm for 30 minutes at 4 ºC. Supernatant was collected as crude extract and stored at -80 ºC until further use. (The composition of buffers has been listed in Table 12)

3.16.2 Enzyme Purification

Since the enzyme has an N-terminal 6x His-tag, after previously being cloned into the pET expression vector, it was possible to purify it by the principle of Affinity Chromatography using Nickel-NTA column. During this process, the tagged protein molecule binds to the Nickel ions in the column and after washing to remove the unspecific molecules, is eluted using an elution buffer with high concentration of Imidazole, which competes with the protein for Nickel ions. All the steps were carried out using the QIA*expressionist*™ system from Qiagen and at 4 ºC to ensure the stability of the protein

The frozen crude extract was thawed on ice and added to 0.5 ml of Ni-NTA Agarose and gently shaken for 30 minutes at 4 ºC to enable the protein-ion interaction. Subsequently, the agarose was pelleted by centrifuging at 2300 rpm for 10 seconds. Supernatant with the unbound proteins was collected in a fresh tube and 1 ml of Washing Buffer was added to the pellet. After a similar centrifugation step, the supernatant was collected and the pellet was re-suspended in Washing Buffer. The washing step was repeated 5 times. After the 6^{th} wash, the pellet was re-suspended in 0.5 ml Elution Buffer, centrifuged and the supernatant was collected. The elution step was repeated twice. (The composition of buffers has been listed in Table 12)

For analysing the purity of the eluted protein, all the samples collected during the purification were run on 15% polyacrylamide gel. 10 µl of sample was mixed with 10 µl of SDS-loading

buffer containing 10% β-mercaptoethanol, boiled at 95 ºC for 5 minutes and loaded on the gel. PageRuler Prestained Protein ladder (Fermentas) was used for size determination (Table 13). The gel was run for 60 minutes at 200 V, after which it was stained over-night using Coomasine Blue staining solution and destained the following day for 4 hours using 7% acetic acid as destaining solution. The gel was stored in demineralised water when needed.

3.16.3 Measurement of Protein Concentration

The concentration of eluates was measured using Nanodrop 1000 Spectrophotometer and was performed in triplets using Elution Buffer as blank. According to the Beer-Lambert Law, the protein concentration was calculated using the average of the three measured values of the molar extinction coefficient, which was calculated using ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics).

Table 16: Molar extinction coefficient and molecular weight of HD2A, calculated using ExPASy Bioinformatics Resource Portal, Swiss Institute of Bioinformatics.

Enzyme	HD2A
Extinction Coefficient	0.932
Molecular Weight (kDa)	77.1

3.17 Enzyme Assay

To determine the specific activity of the DHPS enzyme, an enzyme assay was carried out based on the reaction shown in blue:

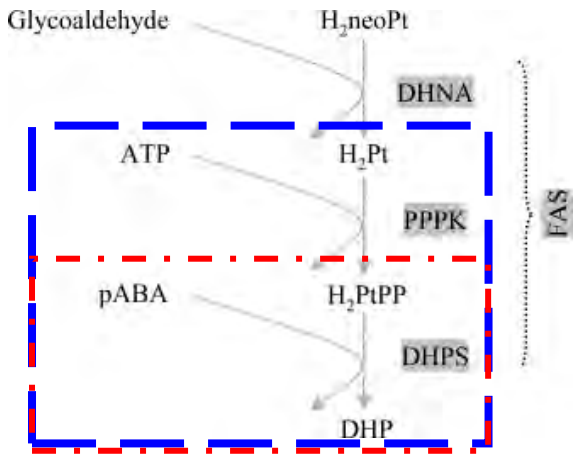

Figure 11: Folate biosynthetic pathway. The multidomain (trifunctional) enzyme referred to as FAS includes: DHNA (dihydroneopterin aldolase), PPPK (hydroxymethyldihydropterin pyrophosphokinase) and DHPS (dihydropteroate synthase). Substrate abbreviations are: H_2neoPt (2-amino-4-hydroxy-6-(d-erythro)-trihydroxypropyl-dihydropteridine), H_2Pt (2-amino-4-hydroxy-6-hydroxy-methyl-dihydropteridine), H_2PtPP (2-amino-4-hydroxy-6-hydroxy-methyl-dihydropteridine pyrophosphate), and DHP (dihydropteroate). (adapted from Iliades P, Walker DJ, Castelli L, Satchell J, Meshnick SR, Macreadie IG (2004b) Cloning of the *Pneumocystis jirovecii* trifunctional FAS gene and complementation of its DHPS activity in *Escherichia coli. Fungal Genetics and Biology* 41:1053–1062.)

Dihydropteridine pyrophosphate was used as the primary substrate for the enzyme and ^{14}C-pABA as the secondary substrate. The enzyme, DHPS, catalyzes the reaction using ATP, converting one molecule of ^{14}C-pABA into one molecule of dihydropteroate, releasing the ^{14}C which could be detected by a scintillation counter. Thus, the radioactivity detected per molecule is equal to one molecule of the reaction product formed, which gives a direct correlation to the enzyme activity.

A defined amount of enzyme (2 to 4 µg) was added to the standard reaction mixture (Table 14) and was incubated at 37 ºC for 10, 20 or 30 minutes. Water was used instead in the negative control and each reaction was performed in triplets. After the incubation, the reaction was stopped by boiling the mixture at 95 ºC for 1 minute, followed by centrifugation at 8000 rpm for 3 minutes.

The supernatant was spotted onto a 3M Whatman Chromatography paper in a 1.5 cm x 1.5 cm square. After drying the spots, descending paper chromatography was performed for 90 minutes using phosphate buffer (pH 6) as the mobile phase. After drying the chromatography paper at 43 ºC, the squares were excised and transferred to 1.5 ml tubes and 2 ml of Optiphase 3 scintillation liquid (Perkin Elmer, Waltham, USA) was added. The radioactivity was detected by Beckman LS6500 scintillation counter (Beckman Coulter).

3.18 Determination of Growth Rates

To examine the effect of the deletions on the growth of *pf*DHPS mutants, the doubling time of the transfected knockout bacteria was measured using BioScreen C and EzExperiment program (Oy Growth Curves Ab Ltd, Finland).

The mutated plasmid was transformed into BL21(DE3)Δ*folP::Km*^{R} as described before. One of the transformed colonies was inoculated in 1.5 ml ISB containing Kanamycin and incubated over-night at 37 ºC. The over-night culture was diluted 1000 times (2 µl culture + 1998 µl fresh ISB without antibiotics). 300 µl of the diluted culture was transferred to one well of a BioScreen Honeycomb plate. For each mutant strain, four wells were used. The plate was incubated at 37 ºC for 16 hours with continuous shaking and the OD_{600} was measured every 4 minutes. 300 µl of ISB in two wells served as blank for each plate measured. The data was analyzed using Microsoft® Excel® 2004 for Mac. The average of the values from the blank wells was reduced from values of the sample wells and only the values between 0.04 and 0.08 were used for creating a XY Scatter plot, with a logarithmic time scale (Y-axis).

The equation resulting from the exponential trend-line ($y=a*e^{bx}$) was used for calculating the generation time, by substituting in the formula: ($t=ln\ (2)/b$), where t, is the generation time.

HD2A was treated as wild type for all the runs and the generation time calculated was

converted to relative generation time with respect to the wild-type generation time. A bar graph was plotted with all the values to analyze the growth rate.

4. Results

*4.1Results for the pf*PPPK-DHPS *project*

4.1.1 Protein Purification using Nickel-NTA Agarose

The project was started with the plasmid isolation and determining of the enzyme activity of mutants Δ6a and Δ6c, containing deletions in the DHPS-2 insertion. These mutants had been generated by previous students. Δ6c was also to serve as one of the controls of future mutagenesis experiments. The mutant plasmids were extracted and transformed into competent BL21 (DE3) Δ*folP::Km*^{R} cells for expression of mutated protein. The expression of the protein was induced in a 600 ml LB broth culture with IPTG as described in section **3.16.1**. The resulting cells were harvested and the protein was extracted by lysis and sonication. The protein extract was purified using Nickel-NTA affinity chromatography (**3.16.2**). The mass and purity of the resulting eluate was verified on a SDS-PAGE gel.

Figure 12 shows the results of the protein purification. The first supernatant well and the first wash show multiple bands of unbound protein, which get clearer with consecutive washes. The three wells after the protein ladder show the three elutions. One sharp band can be observed at the size of 70 kDa, which corresponds to the expected size of the protein. Faint bands are also seen around the protein band.

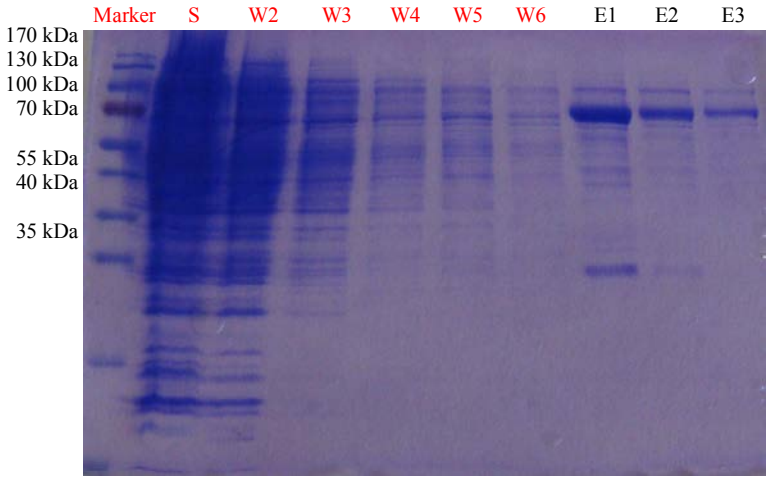

Figure 12: Purification profile of HD2A after Ni-NTA affinity chromatography. (The first well from the marker represents the supernatant with all the unbound proteins (S), the five wells after that show the 5 washes with Washing Buffer (W1-W5) and the last three wells show the elutes after elution with Elution Buffer)

4.1.2 Determination of Enzyme Activity using radioactive substrate

The eluates were tested for specific enzyme activity of the purified DHPS, the details of which are given in section **3.17**. Radioactive ^{14}C-pABA was used as secondary substrate, along with Dihydropteridine pyrophosphate. The reaction equation is shown in blue in Figure 11 (**3.17**). Radioactive ^{14}C-pABA is soluble in Phosphate buffer, due to which it was possible to separate the unused ^{14}C-pABA from the insoluble reaction products by descending paper chromatography. The results from the scintillation counting would thus, correspond to amount of reaction product formed and which would allow for the calculation of the specific activity of the enzyme. However, when the assay was carried out, samples showed scintillation count as low as the negative control. The assay was carried out numerous times using various conditions (incubation for shorter/longer time, incubation at a lower temperature, duplicates, and triplicates) with no results. Each time, the readings would be as low as the negative control (which contained water and no enzyme) or slightly more.

It was then decided to try the assay with a stored and pre-tested extract from HD2A to check if all the reaction components were still active. The results from the assay showed that the enzyme had lost considerable activity, which could either be because of storage or because some reagent was limiting the reaction. The assay was then repeated once more with a fresh extract of HD2A, and the values obtained were as much as the negative control, while the positive control (5 µl of ^{14}C-pABA) was almost 110 folds more. The next trial was done with preparing a fresh stock of the substrate and using Sodium dithionate as a reducing agent. Further, all the other reagents were also prepared fresh, with no results from the assay. Eventually, a fresh batch of 6-hydroxymethylpterindiphosphate was ordered, which took about 2 months to arrive. Enzyme assay was carried out with it under various conditions, with no results. The reaction is shown in red in Figure 11(**3.17**). The enzyme assay was then disregarded in the favor of growth rate experiments in which the mutants would be grown in a nutritionally poor medium like ISB. This would directly correlate to how active the mutated enzyme was (see below).

4.1.3 Generation of mutants using deletion PCR

Mutagenesis PCR was carried out to generate double mutants. Four mutants with deletions in the DHPS-1 insertion and one with a deletion in the linker region were selected as templates and a deletion was to be introduced in the DHPS-2 insertion, which previously has been shown to be dispensable. The primer pair was *6c* and five mutants were selected to act as templates, as shown in Table 15.

Mutagenesis was carried out using KAPA HiFi™ PCR kit and the manufacturer's guidelines were followed in setting up the PCR reaction. The PCR products were analysed on a 0.8% agarose gel and digested with DpnI to remove any trace of template DNA. The digested

products were transformed into competent DH5α cells and LA+Amp plates were used for selection. Colony PCR was done on the resulting colonies with *T7* primers that flank the pET-19b vector. The product was sent for sequencing using *T7rev* primer. For the (Δ4e+Δ6c) mutant, an additional sequencing reaction was set up using *P7fw* primer, since *T7rev* primer was not able to read too deep into the *pfpppk-dhps*. In this manner it was possible to construct the double mutants (Figure 13).

Δ5a+Δ6c

```
Query 853 YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPN  674
          YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPN
Sbjct  48 YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPN  107
Query 673 PKISERDLVVPVLQLFQKEWND----------KPIISIDTINYNVFKECVDNDLVDILND  524
          PKISERDLVVPVLQLFQKEWND          KPIISIDTINYNVFKECVDNDLVDILND
Sbjct 108 PKISERDLVVPVLQLFQKEWNDIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILND  167
Query 523 ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV  344
          ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV
Sbjct 168 ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV  227
Query 343 LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN------  182
          LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN
Sbjct 228 LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMNDQNVVI  287
Query 181 ---------------------------------------------GLAIASYSYYKKVD  140
                                                        GLAIASYSYYKKVD
Sbjct 288 NTQQKLHDEQQNENKNIVDKSHNWMFQMNYMRKDKDQLLYQKNICGGLAIASYSYYKKVD  347
Query 139 LIRVHDVLETKSVLDVLTKIDQV  71
          LIRVHDVLETKSVLDVLTKIDQV
Sbjct 348 LIRVHDVLETKSVLDVLTKIDQV  370
```

Δ5b+Δ6c

```
Query 799 GGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEWN  620
          GGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEWN
Sbjct  69 GGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEWN  128
Query 619 DIK--------KPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFY  464
          DIK        KPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFY
Sbjct 129 DIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFY  188
Query 463 SVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKH  284
          SVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKH
Sbjct 189 SVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKH  248
Query 283 DQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN--------------------------  185
          DQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN
Sbjct 249 DQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMNDQNVVINTQQKLHDEQQNENKNIVDKS  308
Query 184 -------------------------GLAIASYSYYKKVDLIRVHDVLETKSVLDVLTKID  80
                                    GLAIASYSYYKKVDLIRVHDVLETKSVLDVLTKID
Sbjct 309 HNWMFQMNYMRKDKDQLLYQKNICGGLAIASYSYYKKVDLIRVHDVLETKSVLDVLTKID  368
Query  79 QV  74
          QV
Sbjct 369 QV  370
```

Δ5d+Δ6c

```
Query 971 NNTIRCLYNKYVSRMKEQYNINIKENNKRIYVLKDRISYLKEKTNIVGILNVNYDSFSDG 792
          NNTIRCLYNKYVSRMKEQYNINIKENNKRIYVLKDRISYLKEKTNIVGILNVNYDSFSDG
Sbjct  10 NNTIRCLYNKYVSRMKEQYNINIKENNKRIYVLKDRISYLKEKTNIVGILNVNYDSFSDG  69
Query 791 GIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEW--  618
          GIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEW
Sbjct  70 GIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPNPKISERDLVVPVLQLFQKEWND  129
```

```
Query 617 ------KCDAKPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFYS  456
                KCDAKPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFYS
Sbjct 130 IKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILNDISACTNNPEIIKLLKKKNKFYS  189
Query 455 VVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKHD  276
          VVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKHD
Sbjct 190 VVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLVLNGIPRYRILFDIGLGFAKKHD  249
Query 275 QSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN----------------------------  180
          QSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN
Sbjct 250 QSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMNDQNVVINTQQKLHDEQQNENKNIVDKSH  309
Query 179 ------------------------GLAIASYSYYKKVDLIRVHDVLETKSVLDVLTKXXX  72
                                 GLAIASYSYYKKVDLIRVHDVLETKSVLDVLTK
Sbjct 310 NWMFQMNYMRKDKDQLLYQKNICGGLAIASYSYYKKVDLIRVHDVLETKSVLDVLTKIDQ  369
```

Δ5f+Δ6c

```
Query 864 ISYLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVI  685
          ISYLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVI
Sbjct  46 ISYLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVI  105
Query 684 PNPKISERDLVVPVLQLFQKEW---------CDAKPIISIDTINYNVFKECVDNDLVDIL  532
          PNPKISERDLVVPVLQLFQKEW         CDAKPIISIDTINYNVFKECVDNDLVDIL
Sbjct 106 PNPKISERDLVVPVLQLFQKEWNDIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDIL  165
Query 531 NDISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNF  352
          NDISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNF
Sbjct 166 NDISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNF  225
Query 351 LVLNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN----  184
          LVLNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN
Sbjct 226 LVLNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMNDQNV  285
Query 183 ------------------------------------------------GLAIASYSYYKK  148
                                                           GLAIASYSYYKK
Sbjct 286 VINTQQKLHDEQQNENKNIVDKSHNWMFQMNYMRKDKDQLLYQKNICGGLAIASYSYYKK  345
Query 147 VDLIRVHDVLETKSVLDVLTKIXQV  73
          VDLIRVHDVLETKSVLDVLTKI QV
Sbjct 346 VDLIRVHDVLETKSVLDVLTKIDQV  370
```

Δ4e+Δ6c

```
Query1034 THRYSILLCLNDMIPEYKHNVLNNTIRCLYNK----------INIKENNKRIYVLKDRIS 885
          THRYSILLCLNDMIPEYKHNVLNNTIRCLYNK          INIKENNKRIYVLKDRIS
Sbjct 324 THRYSILLCLNDMIPEYKHNVLNNTIRCLYNKYVSRMKEQYNINIKENNKRIYVLKDRIS  383
Query 884 YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGXSSAPFVIPN  705
          YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGG SSAPFVIPN
Sbjct 384 YLKEKTNIVGILNVNYDSFSDGGIFVEPKRAVQRMFEMINEGASVIDIGGESSAPFVIPN  443
Query 704 PKISERDLVVPVLQLFQKEWNDIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILND  525
          PKISERDLVVPVLQLFQKEWNDIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILND
Sbjct 444 PKISERDLVVPVLQLFQKEWNDIKNKIVKCDAKPIISIDTINYNVFKECVDNDLVDILND  503
Query 524 ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV  345
          ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV
Sbjct 504 ISACTNNPEIIKLLKKKNKFYSVVLMHKRGNPHTMDKLTNYDNLVYDIKNYLEQRLNFLV  563
Query 344 LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN------  183
          LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMN
Sbjct 564 LNGIPRYRILFDIGLGFAKKHDQSIKLLQNIHVYDEYPLFIGYSRKRFIAHCMNDQNVVI  623
Query 182 ------------------------------------------------GLAIASYSYYKKVD  141
                                                           GLAIASYSYYKKVD
Sbjct 624 NTQQKLHDEQQNENKNIVDKSHNWMFQMNYMRKDKDQLLYQKKIWGGLAIASYSYYKKVD  683
Query 140 LIRVHDVLETKSVLDVLTKXDQV  72
          LIRVHDVLETKSVLDVLTK DQV
Sbjct 684 LIRVHDVLETKSVLDVLTKIDQV  706
```

Figure 13: Sequencing results of the mutant plasmids. The query line shows the detected sequence, the subject line shows the wild type sequence, and the middle line (blue) is the agreement between the subject and the query. The deleted amino acids are shown in red. The sequencing was performed at Uppsala Genome Centre, Rudbeck Laboratory and the chromatograms were analysed by 4 Peaks Bio-Edit sequence editor (Mekentosj B.V., Aalsmeer, The Neatherlands).

4.1.4 Growth complementation in knock-out bacteria

The mutated plasmids were transformed into bacterial cells lacking a functional *dhps* gene to see if the protein produced by the plasmid was active enough to compensate for the lack of a functional protein in the bacterial cell. The plasmids were transformed into BL21(DE3)Δ*folP::Km*^{R} cells. The transformed cells were plated on ISA+Amp plates to check for growth. ISA lacks Thymidine, because of which cells have to rely on the biosynthetic pathway to compensate for the lack of the same. The transformation of the cells was carried out using the standard chemical transformation protocol. It was not possible to transform (Δ5a+Δ6c) by chemical transformation, and hence electroporation was used. The transformants were plated on LA+Amp plates just after transformation to let them recover. All the transformants showed very good growth on LA plates. A single colony from each was re-streaked on ISA+Amp plates to check for the growth vigour. The resulting colonies showed good growth, although they were slightly smaller in size than the colonies on LA plates.

4.1.5 Measuring the generation time of the mutants

Since the enzyme assay did not give any results, it was decided to measure the growth rate of the transformed bacteria in a nutritionally poor medium to check for the activity of enzyme. The transformants were grown in ISB and the OD_{600} was measured every four minutes for 16 hours at 37 ˚C. All cultures were analyzed in quadruplets. The entire run was repeated twice and the average of the two runs was used to calculate the 'Relative Generation Time' with respect to HD2A. Figure 14 shows the average generation times of all the strains with respect to the wild type.

From analyzing the OD_{600} values for each well, one basic observation was that most of the strains had reached the same final OD_{600} values as HD2A (0.9). Δ5a showed a slightly lower final growth of around 0.7 OD_{600}, but as shown in Figure 14, the generation time was considerably lower than the wild type (25.46 and 23.51 minutes), which was quite surprising. Similar pattern was observed for (Δ5a+Δ6c), which had generation time as low as 26.40 and 24.15 minutes.

(Δ5b+Δ6c) seems to have had considerable effect on growth and showed the highest generation time (although with a significant standard deviation) (51.47 and 53.69 minutes).

Taking the average of two runs, it can be seen that most mutants, in general, had similar generation time as that of HD2A, albeit there were quite large differences between the two runs.

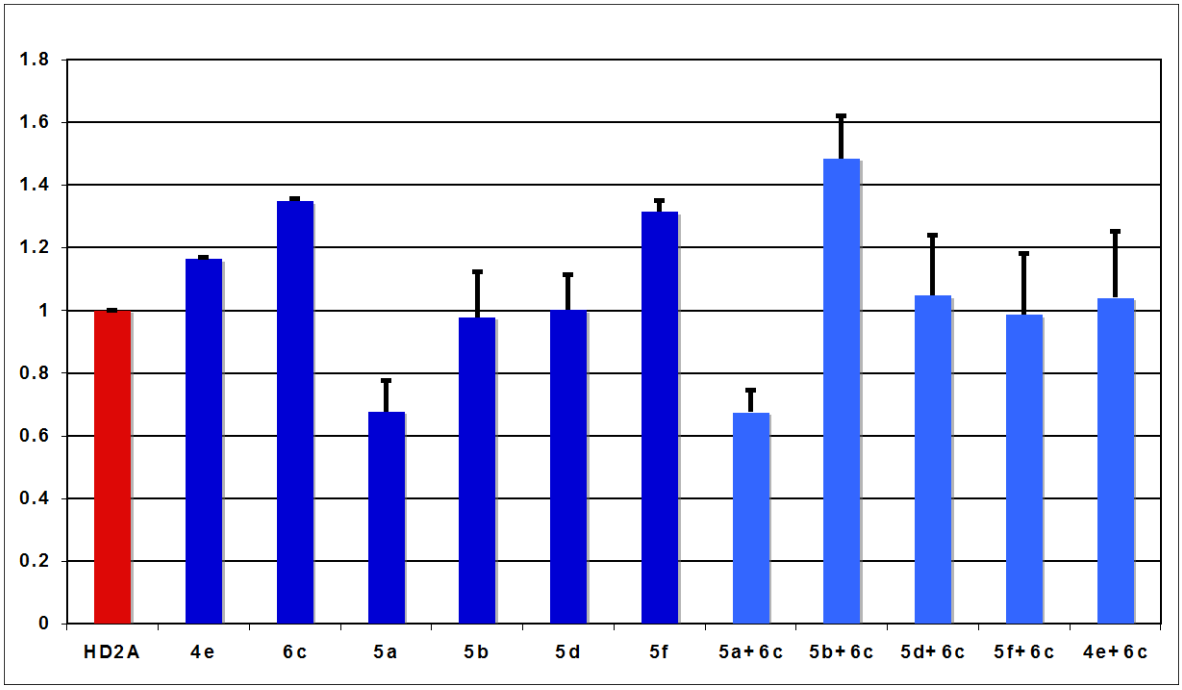

Figure 14: Average Relative Generation Times of all the mutants when compared to the generation time of HD2A (1). The error bars show the standard deviation between the two runs. The average of generation time for all the mutants from two Bioscreen runs was converted to Relative Generation Time with respect to HD2A as described in **3.18**.

4.2. Results for S. mutans folP project

4.2.1 Generation of mutants by mutagenesis PCR

In a previous study it was suspected that there were three amino acids that could be determinants of sulphonamide resistance in *S. mutans*. In order to decipher the role of these amino acids, mutants were to be generated with mutations in the 3 amino acids, one by one and in combination to each other. Mutagenesis PCR was carried out with primers designed to introduce single point mutations in the *folP* gene from isolate **8**. It was possible to create mutants with various combinations of changes in amino acids 37, 172 and 193 by using the KAPA HiFi™ PCR kit . Primer set *193* proved to be most difficult to generate mutants with. On several trials, the sequencing chromatograms showed double peaks at the position, due to which the mutagenesis had to be repeated several times. On some trials, there were no transformants on the plates, but when the remaining over-night culture was plated, several colonies with the concerned mutations were obtained. This was mostly attributed to the fact that the DH5α cells used were frozen stock, which markedly decreases their transformation efficiency and thereby the cells need a longer recovery time even in SOC media. The sequences of the mutants and template are shown in Figure 15.

37 AV

```
Query   23  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLaagaaiidiggxSTRPGAAFVS  202
            VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVS
Sbjct    8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVS  67
Query  203  AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA 382
            AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA
Sbjct   68  AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA  127
Query  383  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ 562
            AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ
Sbjct  128  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187
Query  563  NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV 742
            NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV
Sbjct  188  NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247
Query  743  RVHNVAANKDIVKVSSQL 796
            RVHNVAANKDIVKVSSQL
```

```
Sbjct  248  RVHNVAANKDIVKVSSQL  265
```

172 ND

```
Query   26  AGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLaagaaiidiggxSTRPGAAFVSA 205
            AGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVSA
Sbjct    9  AGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVSA  68
Query  206  EDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLAA 385
            EDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLAA
Sbjct   69  EDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLAA  128
Query  386  ETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQN 565
            ETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQN
Sbjct  129  ETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQN  188
Query  566  MELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVR 745
            MELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVR
Sbjct  189  MELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVR  248
Query  746  VHNVXANKDIVKVSXQLV  799
            VHNV ANKDIVKVS QLV
Sbjct  249  VHNVAANKDIVKVSSQLV  266
```

193 RQ

```
Query   26  VAGKAAIMGILNVTPDSFSDGGQYETIDQVLKQVEAMLaagaaiidiggxSTRPGAAFVS  205
            VAGKAAIMGILNVTPDSFSDGGQYETIDQ LKQVEAMLAAGAAIIDIGG STRPGAAFVS
Sbjct    8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVS  67
Query  206  AEDEIKRIVPIVEAISEKFNCLISIDTYXTXTARVALAAGAHILNDVXSGLYDGQMFQLA  385
            AEDEIKRIVPIVEAISEKFNCLISIDTY T TARVALAAGAHILNDV SGLYDGQMFQLA
Sbjct   68  AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA  127
Query  386  AETGAPIILMHNQCEXVYGNVTXDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVXQ  565
            AETGAPIILMHNQCE VYGNVT DVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNV Q
Sbjct  128  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187
Query  566  NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV 745
            NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV
Sbjct  188  NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247
Query  746  RVHNVAANKDIVKVSSQLV 802
            RVHNVAANKDIVKVSSQLV
Sbjct  248  RVHNVAANKDIVKVSSQLV  266
```

37+172

```
Query   25  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLaagaaiidiggxSTRPGAAFVS  204
            VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGG STRPGAAFVS
Sbjct    8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVS  67
Query  205  AEDEIKRIVPIVEAISEKFNCLISIDTYXTXTARVALAAGAHILNDVXSGLYDGQMFQLA  384
            AEDEIKRIVPIVEAISEKFNCLISIDTY T TARVALAAGAHILNDV SGLYDGQMFQLA
Sbjct   68  AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA  127
Query  385  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  564
            AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ
Sbjct  128  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187
Query  565  NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV 744
            NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV
Sbjct  188  NMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247
Query  745  RVHNVXANKDXVKVSSQLV  801
            RVHNV ANKD VKVSSQLV
Sbjct  248  RVHNVAANKDIVKVSSQLV  266
```

37+172+193

```
Query   28  VAGKAAIMGILNVXPDSFSDGGQYETIDQALKQVEAMLaagaaiidiggxSTRPGAAFVS  207
            VAGKAAIMGILNV PDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGG STRPGAAFVS
Sbjct    8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTRPGAAFVS  67
Query  208  AEDEIKRIVPIVEAISEKFNCLISIDTYXTXTARVALAAGAHILNDVXSGLYDGQMFQLA  387
            AEDEIKRIVPIVEAISEKFNCLISIDTY T TARVALAAGAHILNDV SGLYDGQMFQLA
Sbjct   68  AEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGQMFQLA  127
Query  388  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  567
```

```
            AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ
Sbjct  128  AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187
Query  568  NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV 747
            NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV
Sbjct  188  NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247
Query  748  RVHNVXANKXXVKVXSQL  801
            RVHNV ANK  VKV SQL
Sbjct  248  RVHNVAANKDIVKVSSQL  265
```

Isolate 8

```
Query  445   VKIGKYDVAGKAAIMGILNVTPDSFSDGGQYETIDQVLKQVEAMLaagaaiidiggESTR  624
             +KIGKYDVAGKAAIMGILNVTPDSFSDGGQYETIDQ LKQVEAMLAAGAAIIDIGGESTR
Sbjct    1   MKIGKYDVAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLAAGAAIIDIGGESTR  60
Query  625   PGAAFVSAEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYD  804
             PGAAFVSAEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYD
Sbjct   61   PGAAFVSAEDEIKRIVPIVEAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYD  120
Query  805   GQMFQLAAETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKEDIWLDPGFG  984
             GQMFQLAAETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKE+IWLDPGFG
Sbjct  121   GQMFQLAAETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFG  180
Query  985   FAKNVEQNMELLQGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAI  1164
             FAKNVEQNMELL GLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAI
Sbjct  181   FAKNVEQNMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAI  240
Query  1165  SKGCKIVRVHNVAANKDIVKVS  1230
             SKGCKIVRVHNVAANKDIVKVS
Sbjct  241   SKGCKIVRVHNVAANKDIVKVS  262
```

Figure 15: Sequencing results of the point mutations. The mutated amino acids are shown in bold, red. The query line shows the detected sequence, the subject line shows the wild type sequence, and the middle line (blue) is the agreement between the subject and the query. The deleted amino acids are shown in red. The sequencing was performed at Uppsala Genome Centre, Rudbeck Laboratory and the chromatograms were analysed by 4 Peaks Bio-Edit sequence editor (Mekentosj B.V., Aalsmeer, The Neatherlands)

4.2.2 Antibiotic susceptibility testing and determination of MIC of mutants

The mutant sequences generated, were transformed into *E. coli* cells lacking a functional *dhps* gene. For this purpose, the C600Δ*folP::Km*^{R} cell line was chosen. C600 is a standard cell line used in routine molecular biology work and the cell line used in this project has a Kanamycin resistance gene for selection. The resistance gene has been inserted into the bacterial *dhps* gene, creating a knock-out. The susceptibility of the transformed cells was measured by Kirby-Bauer disk-diffusion test. However, the results from the disc diffusion tests were not reproducible. It was previously thought that it could be because the discs were old or they had

been exposed to air for too long (which according to the manufacturer's guidelines degrades the antibiotics). However, upon testing with new discs, a similar pattern was observed. The results from 2 consecutive experiments are list in Table 17.

Susceptibility testing was then done with Agar Dilution method. ISA agar plates were made with different concentrations of antibiotics and a bacterial suspension was plated on to it. The highest concentration, at which no colonies were obtained on overnight incubation, was considered to be the Minimal Inhibitory Concentration (MIC) of the antibiotic for the organism. The results from the tests are shown in Table 18.

Table 17: Sulfathiazole susceptibility conferred by cloned *folP* gene, expressed in C600Δ*folP::Km*^{R} as determined by Kirby-Bauer Disk-Diffusion Test. A disc containing 100 µg of Sulphamethoxazole was placed on an ISA+Amp plate inoculated with bacterial suspension. The clear zone around the disc was measured after over-night incubation. The table shows values from two trials.

Origin of cloned DHPS gene	***Changes introduced***	***ZOI for Sulphamethoxazole (mm)***
S. mutans **8**	No change	1.7, 2.2
S. mutans **8**	37 AV	1.8, 2.0
S. mutans **8**	172 ND	2.2, 1.7
S. mutans **8**	193 RQ	1.7, 1.5
S. mutans **8**	37 AV + 172 ND	2.2, 1.5
S. mutans **8**	37 AV + 172 ND + 193 RQ	2.8, 1.8
S. mutans **797**	No change	2.6, 2.0

Table 18: Sulfathiazole susceptibility conferred by cloned *folP* gene, expressed in *C600ΔfolP::Km*^{R} as determined by Agar Diffusion Test. 100 µl of over-night bacterial suspension was plated on ISA plates containing 0.02, 0.03, 0.04, 0.04 and 0.05 mM of Sulfathiazole. The highest concentration that showed single colonies after over-night incubation was considered as the MIC of the strain.

Origin of cloned DHPS gene	***Changes introduced***	***MIC for sulfathiazole (mM)***
S. mutans **8**	No change	>0.05
S. mutans **8**	37 AV	0.03
S. mutans **8**	172 ND	0.03
S. mutans **8**	193 RQ	0.03
S. mutans **8**	37 AV + 172 ND	0.02
S. mutans **8**	37 AV + 172 ND + 193 RQ	0.02
S. mutans **797**	No change	0.02

4.2.3 Growth Complementation in C600Δ*folP::Km*^{R} on minimal media

All the mutants were transformed into knock-out bacterial cells lacking a functional *dhps* to see if the *S. mutans folP* could compensate for the gene activity. The transformed bacteria were grown on poor medium (in this case, ISA) without any Thymidine supplement. All the mutants were able to grow on minimal plates, suggesting that the *S. mutans folP* can compensate for the lack of bacterial *dhps*.

4.2.4 Cloning mutant sequences into a pJET vector

After introducing the mutations at all the three positions, the mutants were to be transformed into a pET expression vector to assess for the activity of the protein produced by the cells. For this, the gene was first cloned into a pJET vector. The entire protocol was followed as specified in the manufacturer's guidelines and it was fairly easy to generate pJET clones.

4.2.5 Cloning mutant sequences into an expression vector (pET 19b)

pET cloning, on the other hand, proved to be quite difficult. Different approaches were used for ligation with little success. The details of the protocol are described in section **3.14.3**. All the trials of ligation into pET vector resulted in about 5 colonies per sample and upon colony PCR with *T7* primers; no results were obtained. However, when colony PCR was run with *pJET* primers, sharp bands could be observed on the agarose gel, suggesting that the insert had re-ligated into the pJET vector. After the first few trials (which involved heat inactivation of enzymes at 80°C for 10 minutes and no gel extraction), gel extraction was carried out assuming that the single band excised from the gel would mean no contamination from previous vector (in this case pJET) or other sources. However, no results were obtained then either. It was then decided to use a third restriction enzyme for digestion of the pJET clones. The restriction enzyme was chosen such that it would cleave pJET vector once but not cut the *S. mutans folP*. Two restriction enzymes were chosen: Nco1 and Pst1. The pJET clones were cleaved with three restriction enzymes and the pET cloning protocol was repeated again, with no results again. Eventually various controls were set up to help determine the cause of problem, if any. Three controls were prepared, the details of which are given in Table 19.

Apart from the various controls, four different vector : insert ratios were also tested; 1:3, 1:6, 3:1, 1:1. During one trial, a new vial of ligation buffer was used (the manufacturer's guidelines specify that the ATP in the buffer tends to degrade upon repeated freeze-thaw cycles.

Table 19: Details of the controls used for pET ligation reaction. When several trials with Rapid Ligation Kit (Fermentas) gave no results, various controls were set up to identify the possible cause of failure.

Control	***Details***	***Shows***
1	Vector+Ligase+Water	Serves as the negative control, quantifies the un-digested and re-ligated vector.
2	Undigested Vector+Competent cells.	Verifies the transformation procedure, antibiotic and cell competency.
3	Pre-validated plasmid+Competent cells.	Similar reasons as Control 2.

All controls seem to work fine (Control 1 gave very few scattered colonies suggesting re-ligated vector, Control 2 and Control 3 gave numerous colonies suggesting that the transformation worked fine); however, no results were obtained from the ligation samples.

4.2.6 Ligation Independent Cloning (LIC)

Eventually, the pET ligation was disregarded in favour of Ligation Independent Cloning (LIC). LIC depends on generating 3' over-hangs on the insert, which are complementary to the over-hangs already present on the vector, which is included in the kit. The ligation takes

place in the cell to generate a circular plasmid. This eliminates the need for a T4 DNA Ligase and restriction endonucleases, which represent the main bottleneck in a ligation experiment. The manufacturer's guidelines were followed to the word, after which it took two trials to have the mutated *folP* in an expression vector (pLATE51). The clones were streaked on fresh plates and stored at 4˚C. It was decided to make the determination of enzyme activity as a separate project in the future.

5. Discussion

*5.1 pf*PPPK-DHPS and search of better drug targets

In order to determine the limits of amino acids that are necessary for a functional PPPK-DHPS enzyme, deletions in the various *Plasmodium* specific insertions were constructed. Four double mutants were generated with deletions in the two DHPS insertions (Δ5a+Δ6c, Δ5b+Δ6c, Δ5d+Δ6c, Δ5f+Δ6c) and one with a deletion in the linker region (Δ4e) and DHPS-2 insertion. It was possible to generate the mutants by mutagenesis PCR using a primer specific for the DHPS-2 insertion (Δ*6c*). From previous investigations it is known that this is a dispensable insertion. The primers were so designed that they would loop out the specific region, which would then be deleted in the amplification product. The mutagenesis PCR was not as tricky to perform, as it was to transform the mutated plasmids into competent cells. On various trials, the transformation gave no results on the first attempt. This is mostly attributed to the fact that frozen stocks of cells were being used for transformation, which lose considerable efficiency during storage and even more while thawing. Also, it could be that one hour of recovery was not enough for the cells. This was a likely scenario for DH5α transformation, as it was possible to get transformants from the overnight culture. However, for BL21(DE3)Δ*folP::Km*^{R} transformation with Δ5a and (Δ5a+Δ6c) plasmids, electroporation had to be used, which was successful at the first trial.

At the start of the project, radioactive enzyme assay was carried out with single mutants: Δ6a and Δ6c. The protein was extracted by lysis and sonication and the resulting extracted purified by affinity chromatography using Nickel-NTA resin. While running the SDS-gel to check for the quality of the purified protein, faint bands around the expected protein band were

observed. These could either be contaminant bands resulting from unspecific binding or protein degradation bands. Unspecific binding can be disregarded due to the fact that the Nickel-NTA resin would only bind to His-tagged proteins. The only explanation thus, was that these were protein aggregates or degradation products. Since the bands were quite faint with respect to the band of interest, this was not regarded much.

Radioactive assay was carried out using radio labeled substrate and the enzyme activity was to be calculated as a measure of the residual radioactivity. However, on multiple trials, samples showed scintillation count as low as the negative control. Various different approaches were tried, without any success. One possible explanation was that the enzyme was so sensitive, that it could not withstand the delay from the purification to the enzyme assay. It could also be that it was the degradation of the primary substrate (6-hydroxymethyl-7,8-dihydropterin pyrophosphate) that gave no results, but it seemed unlikely since Sodium dithionate was used as a reducing agent, as the reduced product is very stable.

Another possible explanation could be that since it is a *Plasmodium* enzyme complex, which has been transformed into a bacterial system, it just doesn't function there. To elaborate, it could be that either the enzyme complex doesn't fold well, or is degraded inside the cell once formed. However, complete degradation of the enzyme complex in the cell can be disregarded as the protein eluted after purification was of the expected size.

Eventually, it was decided to carry out growth rate measurements of the mutants. This was done in knock-out bacteria, which lack a functional *dhps* gene. This was based on the idea that in a poor media lacking Thymidine, the knock-out bacteria would have to reply on the biosynthetic pathway and thus, on the mutated *pf*PPPK-DHPS to fulfill its requirement. All the mutants showed good growth complementation in BL21(DE3)Δ*folp::Km*^{R} when plated on LA agar. The only difference observed on ISA plates was that the colony size was slightly smaller than the one on LA, which was expected.

Growth curve measurements were carried out using a Bioscreen C in ISB medium at 37 ˚C with continuous shaking for 16 hours. The results were analyzed using Microsoft Excel and a graph between the relative generation times for different strains was plotted. Significant differences between the two runs were found, but the only difference between the two was in the use of a different batch of medium.

All the cultures were observed to have grown as much as HD2A by the end of the run. Δ5a and (Δ5a+Δ6c) showed slightly less final growth but the generation time of the strains was much lower than that of HD2A. Except for (Δ5b+Δ6c), all double mutants showed a lower generation time than their single mutants. Considering the differences between the deletions, it can be observed that a smaller deletion (8 amino acids) seemed to be more pernicious than a longer one (10 amino acids).

Additional data from two more bioscreen runs would have probably given a better picture of the effects of deletions, but it was not possible because of lack of time. It would be interesting to continue this further so as to define the highest limits of the amino acid deletions that would result in the smallest, active bi-functional enzyme complex.

To date, it has not been possible to crystallize the *pf*PPPK-DHPS complex, mainly because of its large size. Defining the limits of essential amino acids, might also facilitate crystallization of the enzyme and help to determine the structure of the complex, giving greater insights into possible drug targets.

5.2 Dental caries: A potential storehouse of antibiotic resistance

In order to better understand the resistance mechanism to sulfonamides in *S. mutans*, a clinical isolate from Uganda, isolate **797**, was investigated. It was noticed that **797** showed high resistance *in vitro*, but when cloned into a plasmid vector it did not express any resistance. Only in case of isolate **8**, did the cloned gene confer resistance to the bacteria. Sequence analysis revealed point mutations at three positions in the *folP* gene between the two isolates (A37V, N172D, R193Q). The project started with the reversion of the three amino acids to the original **797** sequence. It was possible to generate the mutants by PCR using primers specific for individual amino acid positions. Following the manufacturer's guidelines, it was quite easy to generate mutants with all the combinations of amino acid changes. Primer *193* was the most tricky to work with, but since we already had single mutant 193, it was not used for generating double and triple mutants. As with the previous project, there were some problems with transformation, which were circumvented by switching to SOC medium for recovery of cells and a longer recovery time (90 mins/overnight).

The transformation of the mutated gene in knock-out bacteria gave good complementation suggesting that the *folP* from *S. mutans* is able to compensate for the lack of bacterial *folP*. All mutants showed good growth on ISA plates. The mutated plasmids transformed in knock-out C600Δ*folP::Km*^{R} were tested with 100 µg Sulphamethoxazole discs to test their antibiotic susceptibility. A very dilute bacterial suspension was inoculated on ISA+Amp plates and antibiotic disc was placed at the centre of the plate. After overnight incubation, the Zone of Inhibition was measured, which was to be used to calculate the MIC for the mutants. However, upon repeating the experiments, it was observed that there were significant differences between consecutive repeats. These were large enough to be disregarded as errors in measurements. It was first thought that the differences were due to the discs being old and

exposed to air for too long, but a similar pattern was observed upon testing with fresh discs. Moreover, there is a general disagreement among scientists about correlating the ZOI values (which are in mm) to MIC values (which should be in mM).

Eventually it was decided to use agar dilution test, which gives a more stable value of MIC. Sulfathiazole was dissolved in ISA before plating on 0.02, 0.03, 0.04 and 0.05 mM concentrations and 100 µl of overnight culture was plated on different concentrations. The highest concentration at which no colonies were observed after extended overnight incubation was taken as the MIC for that strain. From the results obtained by the agar dilution test, it can be seen that, isolate **8** gives the highest MIC with several colonies obtained on concentration as high as 0.05 mM. The MIC decreases as the number of mutations increase, with single mutants showing higher MIC of 0.03 mM and triple mutants (sequence similar to **797**) showing MIC as low as 0.02 mM (same as **797**). This gives an indication that the resistance mechanism in *S. mutans* might be point mutations in *folP* gene. However, since a bacterial system is used for testing, there might be a slight bias in the results, as it is still not known if the enzymes from *S. mutans* would function the same way in *E. coli*. Further tests would be needed to elucidate the resistance mechanism more clearly.

The mutants were to be cloned into pET expression vector to test for the activity of the enzyme produced. Previous experience had proved a two step cloning to be more efficient than a single step cloning when it came to pET vectors. For this, the mutated *folP* gene was first cloned into a pJET1.2/blunt cloning vector (Fermentas). The second step of cloning into a pET-19b vector proved to be very tricky. Several trials were made with different combinations of reagents, vector:insert and controls. However, no results were obtained and the cause could not be deciphered either since all the controls seem to work fine.

LIC seemed like an easier way to generate clones in an expression vector and this was indeed found to be the case, as it took only one trial, following the manufacturer's guidelines, to get

all the clones, which were preserved as frozen stocks and plasmid extracts. It was decided that the measurement of enzyme activity would be made a separate project due to lack of time.

The project analyzed the possible resistance mechanism of *S. mutans*, which are important commensal species due to their ability of transfer their resistance determinants to pathogenic species. The results from the project suggest that *S. mutans* rely on generating point mutations of their drug targets to acquire the resistance. It would interesting to study this further by taking into account other isolates and test if there are any other possible mechanisms like intra-cellular pumps or longer mutations that confer resistance.

6. Appendix

6.1 *pf*PPPK-DHPS amino acid sequence

M E T I Q E L I L S E E N K T N I A V L N L G T N D R R N A V

L I L E T A L H L V E K Y L G K I I N T S Y L Y E T V P E Y I

V L D K K E S C E L I N K D C R I Y D V N Y I N E L M Q N L E

E S K Y E E N K E L I D K C E E Y E T F L K N G K V D N S I L

K E V N V E N Y L L E C N N I I V K N D E I M K N N L S K Y K

D K Y Y T S Y F Y N L T V V V K T F V N D P L S M L V V I K Y

I E E L M K R E N V K E K E K F E N R I I D I D I L F F N D F

T I F M K N I K L E K N M I Y K I L S K Y I H L E R D I K N G

N D N M S K V N M D K D I N L N N N N N I K K K N N N D I D C

D C V D Q K M N N H V N N K N Y I N S F R D P Q E I I N N M V

D N I E F L S I P H V Y T T H R Y S I L L C L N D M I P E Y K

H N V L N N T I R C L Y N K Y V S R M K E Q Y N I N I K E N N

K R I Y V L K D R I S Y L K E K T N I V G I L N V N Y D S F S

D G GI F V E P K R A V Q R M F EM I N E G A S V I D I G G E S

S G P F V I P N P K I S E R D L V V P V L Q L F Q K E W N D I

K N K I V K C D A K P I I S I D T I N Y N V F K E C V D N D L

V D I L N D I S A C T N N P E I I K L L K K K N K F Y S V V L

M H K R G N P H T M D K L T N Y D N L V Y D I K N Y L E Q R L

N F L V L N G I P R Y R I L F D I G L G F A K K H D Q S I K L

L Q N I H V Y D E Y P L F I G Y S R K R F I A H C M N D Q N V

V I N T Q Q K L H D E Q Q N E N K N I V D K S H N W M F Q M N

Q M R K D K D Q L L Y Q K N I C G G L A I A S Y S Y Y K K V D

L I R V H D V L E T K S V L D V L T K I D Q

Figure 16: *pf*PPPK-DHPS amino acid sequence. The *Plasmodium* specific inserts of the bi-functional enzyme have been colour coded. PPPK-1 is shown in green, is highly conserved between all *Plasmodium* species, is essential for enzyme activity as previous investigation has shown that only a few amino acids can be deleted before the enzyme complex starts losing activity. PPPK-2 is shown in yellow, has low sequence conservation between species and is non-essential to an extent; about 100 amino acids can be deleted without any significant loss. HD2A has a deletion in PPPK-2 and was used as wild-type for all experiments. DHPS-1 is shown in pink, is not conserved between species and is essential, only a few amino acids can be deleted before the enzyme starts loosing activity. Various Δ5 deletions were in this part. DHPS-2 is shown in blue and is highly conserved between all *Plasmodium* species, although is non-essential; the entire insertion can be deleted without any loss of activity; Δ6c deletion. Red shows the Linker Region. The amino acids deleted have been made bold and underlined.)

6.2 *S. mutans* DHPS amino acid sequence

(a)

M K I G K Y D V A G K A A I M G I L N V T P D S F S D G G Q Y

E T I D Q A L K Q V E A M L V A G A A I I D I G G E S T R P G

A A F V S A E D E I K R I V P I V K A I S E K F N C L I S I D

T Y K T E T A R V A L A A G A H I L N D V W S G L Y D G H M F

Q L A A E T G A P I I L M H N Q C E E V Y G N V T E D V C Q F

L L E R A D L A Q K T G V K K E N I W L D P G F G F A K N V E

Q N M E L L R G L A E V T K L G Y P V L F G I S R K R V V D S

L L G G H T K P Q E R D M A T A A L S G Y A I S G C K I V R V

H N V A A N K D I V K V S S Q L A

(b)

M K I G K Y D V A G K A A I M G I L N V T P D S F S D G G Q Y

E T I D Q V L K Q V E A M L V A G A A I I D I G G E S T R P G

A A F V S A E D E I K R I V P I V K A I S E K F N C L I S I D

T Y K T E T A R V A L A A G A H I L N D V W S G L Y D G H M F

Q L A A E T G A P I I L M H N Q C E E V Y G N V T E D V C Q F

L L E R A D L A Q K T G V K K E D I W L D P G F G F A K N V E

Q N M E L L Q G L A E V T K L G Y P V L F G I S R K R V V D S

L L G G H T K P Q E R D M A T A A L S G Y A I S G C K I V R V

H N V A A N K D I V K V S S Q L A

Figure 17: *S. mutans*DHPS amino acid sequence from clinical isolate **797** (a). The *folP* gene from clinical isolate **8** (b) was used as template for mutagenesis reaction. The polymorphisms between **797** and **8** are shown in red and blue respectively.)

6.3 References

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