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Sulfonamide resistance in *Streptococcus mutans* strain 797 is not caused by single amino acid changes in the dihydropteroate synthase

Marcus Andersson

Degree project in biology, Master of science (1 year), 2011
Examensarbete i biologi 30 hp till magisterexamen, 2011
Biology Education Centre and Department of Medical Biochemistry and Microbiology, Uppsala University
Supervisor: Göte Swedberg

Abstract

In Uganda high use of Co-trimoxazole as a prophylaxis among HIV-patients have selected for sulfonamide resistance among the bacteria in the oral microflora. Among those bacteria that proved to be highly resistant towards sulfonamides was *Streptococcus mutans*, which is known to cause dental caries. A strain of *S. mutans* named 797 was found to have four amino acid changes in dihydropteroate synthase (DHPS), which is an important catalyst for a specific step of the folic acid synthesis and the target of sulfonamide inhibition. It was suspected that these changes might be the cause of the sulfonamide resistance and in order to test this hypothesis the DHPS gene from *S. mutans* 797 was isolated, inserted into a vector and subjected to directed mutagenesis using specially constructed primers in order to change these four amino acids to resemble those of *S. mutans* strain NN2025 and create a set of each one of the original amino acids by themselves. The mutated DHPS was transformed into *E. coli* C600ΔfolP which then was tested for resistance towards different sulfonamide concentrations. The test showed a very weak resistance towards sulfonamides in the bacteria with the wild type DHPS as well as in the different DHPS that had undergone mutagenesis. This indicates that the resistance is not linked to amino acid changes in the DHPS sequence in *S. mutans* 797.

Content:

Introduction

Project background

This project is a continuation of a study in Uganda where the prevalence of resistant commensal bacteria from the oral microflora towards different drugs was investigated. Co-trimoxazole (trimethoprim/sulfamethoxazole) was highly used as a prophylaxis among HIV patients. Samples were collected from saliva in several dental clinics around Kampala in order to check the resistance in the oral microflora.

Among the bacteria checked towards co-trimoxazole resistance was *Streptococcus mutans* that was found to have a high prevalence of resistance towards this drug (Buwembo, unpublished data).

S. mutans

S. mutans is a gram-positive bacterium that is part of the normal oral microflora and was pointed out as the main cause of dental caries (Chhour *et al*, 2005). It adheres to the surface of teeth and there starts to produce a large amount of polymers that is used to form an enclosing matrix, also called biofilm, that works as an protective barrier for the bacteria inside it protecting them from harmful substances from the outside. The biofilm, called plaque, is often composed of several different bacteria working together and differentiating among themselves to adapt to the need of the complete biofilm like a bacterial community. This is often referred to as resembling the structure of tissues in higher eukaryotes (Costerton *et al*, 1999; Hall-Stoodley *et al*, 2004).

S. mutans causes dental caries when it degrades carbohydrates, like sucrose, glucose, fructose, and some starch variants as nutrients. Unfortunately, this process produces acids that rapidly lower the pH in the proximity of the biofilm, causing a rapid drop from around normal pH 7 to about pH 4 in a few minutes. This leads to damage to the tooth surface by dissolving it, because the enamel can only handle a pH value above 5.7 and the underlying dentin only above 6.2.

The saliva works as a neutralization media by washing away the carbohydrates from the plaque and raising the pH back to its original state. It also contains calcium and phosphate ions that remineralize the damage caused by the acids.

If the dental caries is untreated *S. mutans* can enter the bloodstream and cause several severe infections of which some can be fatal if untreated, for example bacteremia, infective endocarditis and infection of the heart valve (Kazuhiko*et al*, 2008).

Sulfonamides: Use and mode of action

Sulfonamides are antimicrobial drugs that have been used for a long time, but due to the high frequency of resistance that developed the usage of them are limited today.

In the folic acid biosynthesis the dihydropteroate synthase (DHPS) catalyzes the formation of dihydropteroate (DHP) from *p*-aminobenzoic acid (PABA) and dihydropteroate diphosphate (DHPPP). After this step dihydrofolate synthetase catalyzes the adding of glutamate to the DHP which lead to the production of dihydrofolate, that in turn is reduced by dihydrofolate reductase to produce folic acid (Figure 1).

In the folic acid biosynthesis sulfonamides targets the DHPS catalyzed step. Here the sulfonamide due to its structural similarity to PABA binds to the DHPS catalyst. This leads to the use of the sulfonamide instead of PABA to binding to DHPPP. Binding of sulfonamides to DHPPP creates a molecule that cannot be utilized in later steps of the folic acid biosynthesis. This ultimately leads to depletion of folic acid and inhibited growth in the affected microorganism (Brown, 1962; Vinnicombe & Derrick, 1999; Sköld, 2000; Patel *et al*, 2003; Valderas *et al*, 2008) (Figure 1 & 2).

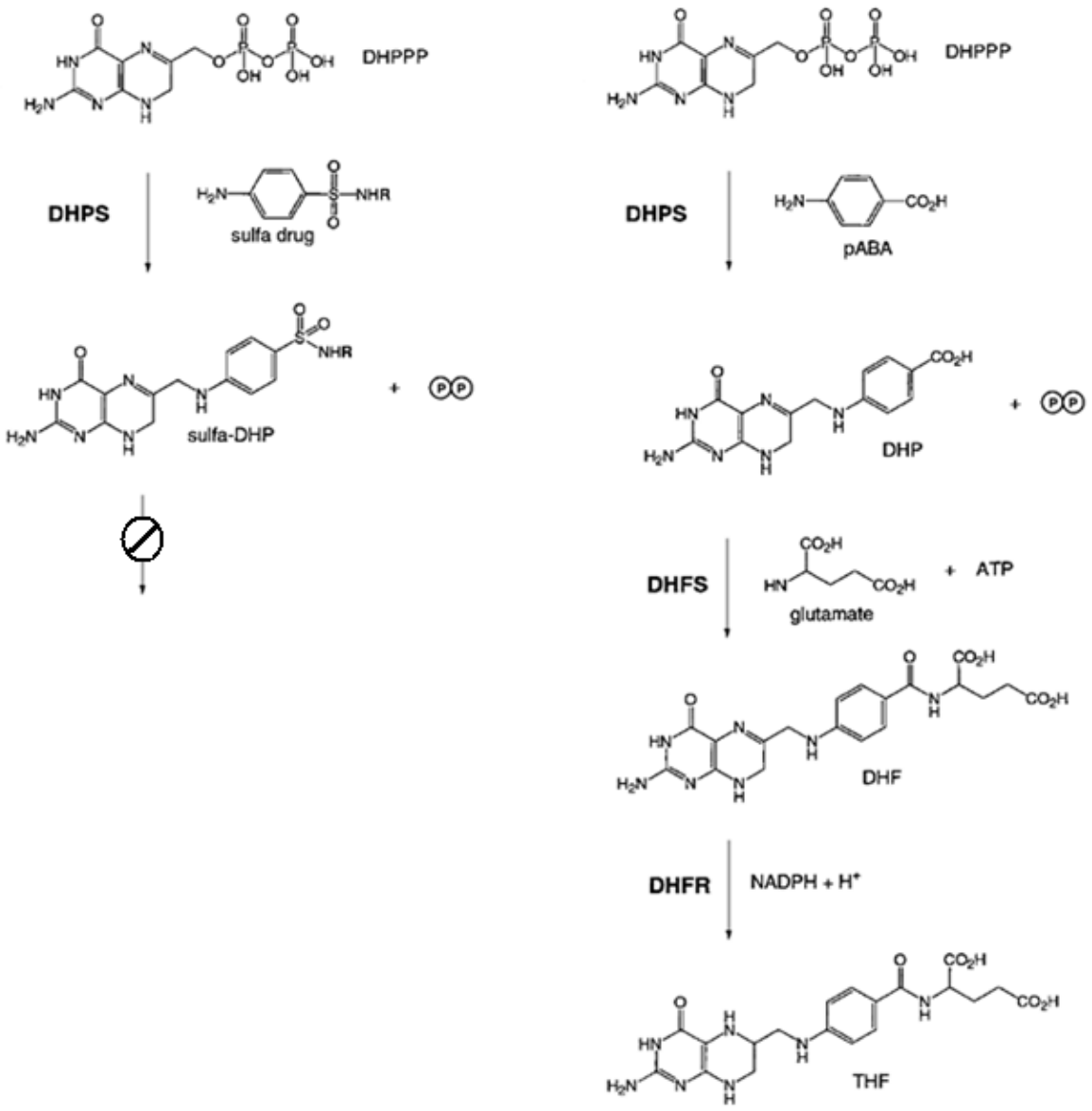


Figure 1. The vital formation of dihydropteroate (DHP) from p-aminobenzoic acid (PABA) and dihydropteroate diphosphate (DHPPP) catalyzed by the DHPS enzyme (closer details in the left square) from the folic acid synthesis and the alternative formation of sulfa-DHP that inhibit this synthesis by creates an unusable DHP molecule (based on Patel *et al*, 2003 and Levy *et al*, 2008).

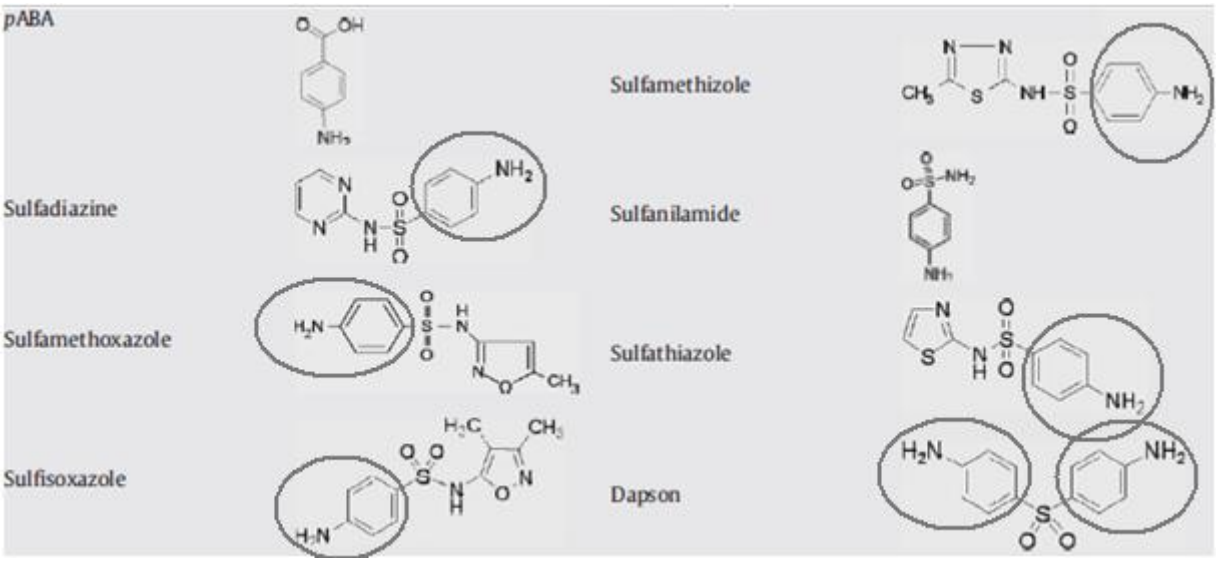


Figure 2. Comparison between different sulfonamides and the pABA molecule they mimic, observe encircled structures on the sulfonamides (based on Valderas *et al*, 2008).

Because higher eukaryotes can use folic acid taken up through food they are not dependent of internal synthesis of folic acid like bacteria and normally lack DHPS. In bacteria and some parasites, on the other hand, the DHPS is well preserved making sulfonamides perfect as a broad spectrum antimicrobial (Baca *et al*, 2000).

Because of this, sulfonamide-containing drugs is often used as prophylaxis in HIV patients to prevent opportunistic bacteria and parasites from growing out of control (Fuchs & Elsner, 2003; Zachariah *et al*, 2007).

Among the parasites treated with combination drugs containing sulfonamides is *Toxoplasma gondii*, a small parasite that is spread via feces from cats. In humans this parasite can infect cells but cannot grow under normal circumstances due to the immune system. Unfortunately, it can reactivate in immunosuppressed patients such as those with HIV. After reactivation the *T. gondii* divide rapidly and depending on where in the body this occur, this can be fatal (Ho-Yen, 2005).

Sulfonamide resistance

Because sulfonamides targets DHPS it is sensitive towards sequence changes in this enzyme. During the catalytic step DHPS firstly binds firstly to DHPPP, which causes a conformational change in the DHPS that forms a binding cleft for pABA. Sulfonamides interrupt the formation of DHP and instead lead to the formation of a sulfa-containing DHP (Levy *et al*,

2008). Alterations in DHPS can lead to resistance towards sulfonamides by impacting on its binding affinity to DHPS compared to pABA.

In *E. coli*, spontaneous mutations in the DHPS gene causing resistance towards sulfonamides can easily form in strains cultivated in the laboratory. In one such mutant the affinity towards sulfonamides was found to have dropped more than a 100 fold in comparison to that of pABA that only dropped around 10 times the normal affinity. This affinity loss towards pABA leading to an activity loss compared to normal DHPS activity but is probably a viable trade of for the bacteria to acquire sulfonamide resistance. But an additional downside was that this specific mutant also was heat sensitive and did not grow at temperatures above 32^{o}C (Swedberg *et al,* 1979). After sequencing this *E. coli* DHPS it was found to have a single base pair substitution in position 28 in the amino acid sequence were a Phe residue had been changed to an Ile (Swedberg *et al,* 1993).

Sulfonamide resistance can also be caused by other factors than mutations in the indigenous DHPS gene. For example in isolates of *Streptococcus pyogenes* the DHPS was found to have most of its amino acids changed with a big difference in the sequence between resistant and susceptible strains. This suggests that it is possible that a foreign gene variation of DHPS have been inserted via transformational recombination (Swedberg *et al,* 1998).

There are also cases were the bacteria naturally can utilize the sulfonamides as alternative substrates without any negative effects on the folic acid synthesis. One such case is *Bacillus anthracis* (Valderas *et al,* 2008).

In order to decrease the chances of resistance to develop most therapies today is combination of treatments where other drugs also are used. An example is trimethoprim that acts on the step after sulfonamide in the folic acid synthesis, the combination minimize the number of viable bacteria after treatment (Bushby & Hitchings, 1968).

Aim of project

The aim of this project is to verify if the 4 amino acid changes at position 46, 80, 122 and 146 in the DHPS sequence of *S. mutans* strain 797 from Uganda is the cause of its resistance towards sulfonamides.

This is done by isolating each amino acid change in the DHPS gene and testing them towards different sulfonamide concentrations in a suitable vector.

Materials

Bacteria & Plasmids

E. coli DH5α (Bethesda Research Laboratories, 1986). *E. coli* C600ΔfolP with deletion in the folP gene coding for DHPS (Fermer & Swedberg, 1997). Plasmid pUC19 coding for ampicillin resistance (Figure 3), DHPS gene from 797 wild type (WT).

Growth medium

OXOID CM1135 Brain hart infusion (BHI) growth media

OXOID LP001 Agar Bacteriological (Agar No.1)

OXOID CM0473 ISO-Sencitest™ Broth (ISB)

Kits

Fermentas GeneJet™ Plasmid Miniprep Kit #K0503

Fermentas GeneJet™ Gel Extraction Kit #K0692

Fermentas GeneJet™ PCR Cloning Kit #K1232

Fermentas Rapid DNA Ligation Kit #K1422

Methods

Growing bacteria for transformation

In preparation for transformation DH5α was grown in 20 ml BHI in a side arm E-flask that was incubated in a shaker at 37^{o}C. The absorption of the growing bacteria in the media was checked until the optic density reached a value corresponding to approximately 10^{8}-cells/ml.

The culture was transferred to a large centrifuge tube and centrifuged for 5 min, 4^{o}C at 4500 rpm and the supernatant was removed leaving only the pellet.

The remaining pellet was resuspended in 10 ml 50 mM $CaCl_2$ and put on ice for 5 min before it was centrifuged again for 5 min, 4^{o}C at 4500 rpm. The supernatant was again removed and the pellet was resuspended in 2 ml 50 mM $CaCl_2$ and put on ice and stored in a coldroom were the cells was viable for an additional 2 days after the preparation.

The preparation of C600ΔfolP was done in the same way as with DH5α but with the exception of adding 0.2 ml thymidine and 20 µl kanamycin to the BHI to enrich the media enough to let the C600ΔfolP grow properly.

Transformation of plasmid to bacteria

200 µl of prepared bacteria was mixed with 5 µl plasmid in an eppendorf tube and packed on ice for 30-40 min before the mix was heat shocked for 2 min at 42^{o}C.

1 ml ISB was added and the mix was incubating for 1 hour at 37^{o}C before it was centrifuged for 3 min at 8000rpm. Most of the supernatant was removed leaving 50-100 µl to resuspend the pellet in. After this the resuspension was spread out on ampicillin containing agar plates and incubated at 37^{o}C overnight.

Plasmid purification

Bacteria containing the plasmid to be purified were grown in 3 ml ISB for 3 hours or overnight.

The overnight culture was centrifuged for 3 min at 8000 rpm in an eppendorf tube, the supernatant was removed and the pellet was resuspended with 250 µl resuspension solution with added RNase (from the GenJET^{TM} plasmid mini preparation kit) and vortexed.

250 µl lysis solution was added and the tube inverted 4-6 times before 350 µl neutralization solution was added and the tube inverted again 4-6 times. The tube was centrifuged for 5 min at 11 000 rpm.

The supernatant was the moved to a spin column and centrifuge for 1 min at 11 000 rpm before it was treated two times with 500 µl wash solution and centrifuged for 30-60 sec at 11 000rpm. The flow trough was removed between the washings.

The empty spin column was centrifuged 1 min at 11 000 rpm before the spin column filter was moved to a new eppendorf tube and 50 µl elution buffer was added to filter and the incubated at room temperature for 2 min before it was centrifuged for 2 min. The flow through containing the purified plasmids was then stored in a freezer.

Gel electrophoresis

A gel was prepared using 0.8% liquid agarose with one drop of ethidium bromide 0.07% added for every 50 ml used. The agarose was poured into a mold of appropriate size and wells were placed into the agarose. The solidified agarose gel was placed in an electrophoresis bath and 5 µl of the products to be controlled was mixed with 1 µl loading dye was pipetted in to each well, 1 µl Gene Ruler 1 kb DNA ladder (Fermentas) was also added to a separate well to

be used as size reference. A small gel containing 50 µl was run at 90 V for 1 hour. A large gel of 150 µl was run at 125 V for 1 hour. The band size on the gel was then controlled under a UV-light.

PCR preparations

The PCR mix was prepared by mixing 5 µl Pfu buffert + $MgSO_4$ (Fermentas), 5 µl dNTP 1 mM, 1 µl Pfu DNA polymerase 2,5 u/µl (Fermentas) and 29 µl H_2O with 2,5 µl of each forward and reverse primer and 5 µl template DNA used in the mix. Totally this leads to 50 µl in each PCR tube.

PCR-programs

Mutagenesis: 95^{o}C for 30 sec, 95^{o}C for 30 sec, 50^{o}C for 1 min, 64^{o}C for 10 min, repeated for 25 cycles.

PJetlong: 95^{o}C for 3 min, 94^{o}C for 30 sec, 60^{o}C for 30 sec, 72^{o}C for 1.3 min, repeated for 30 cycles (used for each PCR not done for mutagenesis).

Sulfonamide resistance testing

The different sets of DHPS with introduced new point mutations was transformed into C600ΔfolP and allowed to grow overnight in 2 ml ISB before being diluted 10 000 times and spread out on sulfonamide containing ISA plates to test for sulfonamide resistance. An ISA plate was used as a control and sulfonamide plates with concentrations of 0.01-0.05 mM was used and plates containing 0.1 mM sulfonamide was used as an extreme. The plates were incubated overnight at 37^{o}C before the growth was controlled.

Making plates

The plates used were created by mixing 4.8 g Agar Bacteriological (Agar No.1), 9,36 g ISO-Sencitest^{TM} Broth (ISB) and 400ml deionized H_2O in a 500 ml flask. This was the autoclaved for 20 min at 120^{o}C.

For sulfonamide containing plates sulfonamide was added to the flask to a final concentrations of either 0.02 mM, 0.03 mM, 0.04 mM, 0.05 mM or 0.1 mM. For ampicillin plates the drug was added to the flask to a final concentration of 50 µg/ml instead of sulfonamide and for ISA plates nothing was added. The agar was poured on agar plates and allowed to solidify.

Results

All work in this project was using a DHPS gene isolated from the sulfonamide resistant *S. mutans* strain 797 from Kampala Uganda, containing four amino acid differences compared to *S. mutans* NN2025 sequence from the sequence database at positions 46, 80, 122 and 146.

Isolated *S. mutans* strain 797 DHPS gene sequences from previous trials were inserted into plasmid pUC19 (Figure 3) and amplified in DH5α and purified before being validated via gel electrophoresis.

One of the previously cloned DHPS genes was deemed viable for continual use (after sequencing it was verified as the wild type DHPS from the *S. mutans* strain 797).

The resistance towards sulfonamides for the 797 WT was tested by streaking out pUC19; 797 WT containing DH5α on sulfonamide containing ISA plates with 0.01mM-0.05 mM and 0.1 mM concentrations. There was no growth of the DH5α on the sulfonamide plates.

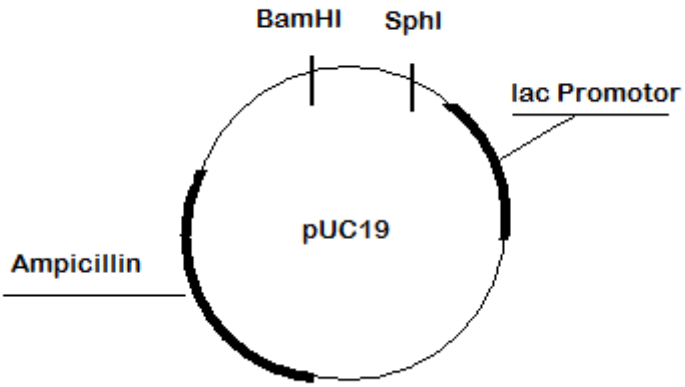


Figure 3. Schematic representation of plasmid pUC19. DHPS inserted between BamHI & SphI.

To exclude any unfavorable interactions between the natural DHPS gene from DH5α and the inserted *S. mutans* DHPS in the pUC19 plasmid the 797 WT-containing plasmid was inserted into *E. coli* strain C600ΔfolP that lacks DHPS activity. After spreading out pUC19; 797 WT containing C600ΔfolP on sulfonamide plates and using the same plate concentrations of sulfonamide the results was that in these case the bacteria did grow, but at a dilution of 10 000 times the growth was only located to the ISA plate and 0.02-0.03 mM sulfonamide. This shows that the DHPS is expressing in the C600ΔfolP as it cannot grow at all on these media without a DHPS inserted.

In order to check whether all or no one of the four amino acid changes that the 797 strain DHPS contains is causing the resistance towards sulfonamides the changes was altered one at a time to resemble those of the *S. mutans* NN2025 sequence from the sequence database with PCR using forward and reverse primers individually designed for the differing DHPS positions and the Mutagenesis PCR program (see table 1).

Table 1. The individually designed forward and reverse primers used to perform directed mutagenesis for the 4 positions in the *S. mutans* 797 DHPS gene.

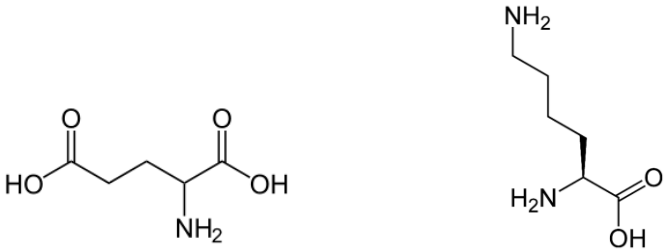
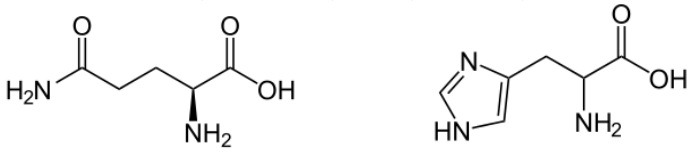
Primer	Sequence
mutansDHPSSph	5'-GAT CGA TCG CAT GCA CAT CAT AAC TAG GGA GCA AGC-3'
mutansDHPSBam	5'-GAT CGA TCG GAT CCA AAA TAA TCT TAT CCA TAA CAC CCT-3'
DHPS46AVfw	5'-TGA AGC CAT GTT AGT AGC AGG AGC GGC TA-3'
DHPS46AVrev	5'-TAG CCG CTC CTG CTACTA ACA TGG CTT CA-3'
DHPS80aEKfw	5'-TCG TTC CAA TTG TTA AAG CTA TTA GCG AA-3'
DHPS80aEKrev	5'-TTC GCT AAT AGC TTT AAC AAT TGG AAC GA-3'
DHPS122QHfw	5'-CTT TAT GAT GGG CAC ATG TTT CAA TTA GC-3'
DHPS122QHrev	5'-GCT AAT TGA AAC ATG TGC CCA TCA TAA AG-3'
DHPS146Gfw	5'-GTG AAG AAG TTT ATG GCA ATG TAA CAG AA-3'
DHPS146Grev	5'-TTC TGT TAC ATT GCC ATA AAC TTC TTC AC-3'

First one single mutation of each of 46, 80, 122 and 146 was attempted to be introduced into 797 WT on their own. The results from the sequencing were compared to the NN2025 to see if the intended amino acid had been changed. If a change could be verified it was then used in later mutagenesis with other primers to produce DHPS sequences with more reversed point mutations. After the sequence results from these were obtained changes for 46 and 80 were verified. These single mutations were then used to produce double mutations by attempting changes of additional mutations. In this cases for 46; 80,122 and 146 was attempted to be added and for 80; 46,122 and 146. After sequencing DHPSs containing changed 46+80 and 80+146 were verified.

Again these were subjected to directed mutagenesis to create triple mutants so that a set of all possible triple mutations was created containing 80+146+122, 46+146+122, 46+80+146 & 46+80+122. This resulted in isolation of each one of the original suspected mutations from 797 (see appendix).

These in turn were used for creating a quadruple mutant that in all positions resemble NN2025. The specific changes at each amino acid position can be seen in Table 2.

Table 2. The amino acid changes for each of the 4 positions in the *S. mutans* 797 compared to the amino acids for the same position in *S. mutans* NN2025.

Mutation Position	Amino Acid Change NN2035 797
46	A (alanine) → V (Valine) 
80	E (Glutamic Acid) → K (Lysine) 
122	Q (Glutamine) → H (Histidine) 
146	S (Serine) → G (Glycine) 

After transformation to C600ΔfolP this set of DHPS variants was grown in liquid cultures and diluted 10 000 times before being spread out on sulfonamide plates with concentrations 0.02-0.05 mM, 0.1 mM and ISA plates for each of the sets with three amino acid positions changes as well as the sequence with four changes and for those with two changes that were collected. Also untransformed diluted C600ΔfolP was used to compare with the transformed ones.

The results for the different point mutations, the four changes, three changes and the two changes was the same as for the 797 WT DHPS sequence, colonies was growing up to a sulfonamide concentration 0.03 mM (Table 3). The untransformed C600ΔfolP did not grow on either concentrations of sulfonamides, or the ISA control.

Table 3. The results obtained after testing the resistance towards different sulfonamide concentrations for the different DHPS sequences created in *E. coli* C600ΔfolP.

Sample	Sulfonamide Concentration				
	0.02mM	0.03mM	0.04mM	0.05mM	0.1mM
46:2 (WT)	+++	++	-	-	-
46+80	+++	++	-	-	-
80+146	+++	++	-	-	-
46+80+122	+++	++	-	-	-
46+80+146	+++	++	-	-	-
80+146+122	+++	++	-	-	-
46+146+122	+++	++	-	-	-
46+80+146+122	+++	++	-	-	-

Stability of resistance

In order to control the stability of the sulfonamide resistance *S. mutans* 797 were grown on ISA plates in a CO_2 rich environment for two days and restreaked once a week. After 12 weeks they were restreaked on to 0.1 mM sulfonamide plates and incubated in a CO_2 rich environment for two days. The results were that the *S. mutans* 797 could still grow on sulfonamide containing plates.

Discussion

According to the results of the sulfonamide resistance test the resistance towards sulfonamides was the same in the wild type, the four different single point mutations and the NN2025 reverse. Because of this and due to the low grade of resistance, resistance only up to 0.03 mM sulfonamide, the conclusion is that the sulfonamide resistance in *S. mutans* 797 is not caused by single point mutations.

This is contradictory to previous results obtained during an earlier project to control the sulfonamide resistance for the 797 WT DHPS done summer 2010. There results a correlation between the number of amino acid differences between 797 and NN2025 and the level of sulfonamide resistance was obtained. That the results are not the same in these two projects can in great part be due to the fact that the dilution used for the previous one was less than in this project and might have affected the growth of the bacteria. Also the possibility exists that the dilutions was not carefully done which might have given these results.

It would also be suggested that in the future test these set of changed 797 DHPS sequences again to verify the results from this project and perhaps even try to introduce the changed

DHPS into a bacterium more similar to *S. mutans* like for example *Streptococcus pyogenes* to see if the DHPS behaves differently in a gram positive bacterium compared to the gram negative *E. coli*.

A quick examination of the distribution of the differing amino acid in the DHPS enzyme shows that they are all placed on the peripheral parts of the DHPS TIM-barrel structure, except for 146, thus further strengthening the claim that the differences have nothing to do with the interactions between the DHPS and the sulfonamide (Figure 4) and therefore no connection with the sulfonamide resistance in 797.

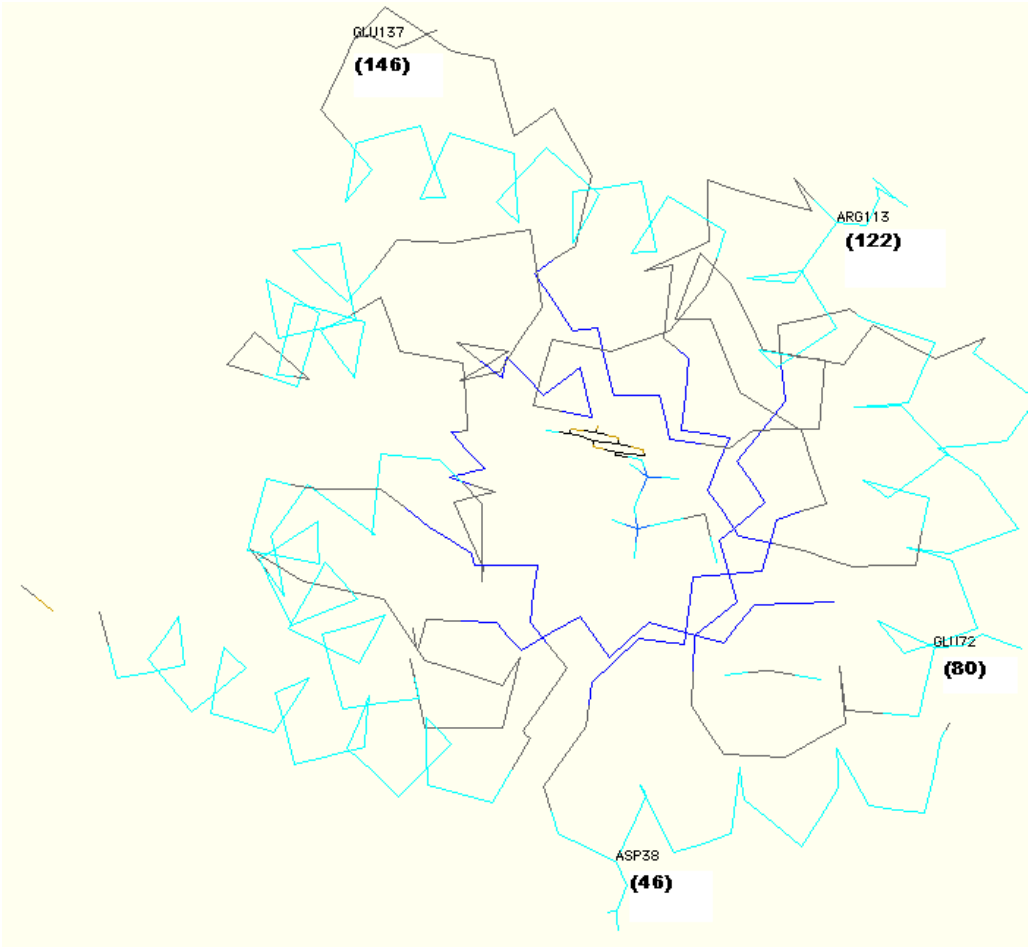


Figure 4. Distribution of the amino acid differences found in the *S. mutans* 797 DHPS enzyme. Position 46, 80 and 122 are positioned in the periphery of the DHPS TIM-barrel structure and position 146 is located in loop structure situated far from the binding site in the TIM-barrel center where pABA or sulfonamides can bind.

If the results from this project are correct, the cause for the resistance in 797 may be caused by other system differences compared to other nonresistant *S. mutans* like a duplication of the DHPS gene or a constantly active promoter. It might even be so that the resistance in *S. mutans* has nothing to do with the DHPS. It could be that an alternative pathway for the folic

acid synthesis has been created or that some kind of efflux pump system that transports the sulfonamides out from the cells has developed. This is for future experiments to decide.

Even if resistance towards sulfonamides is fairly large today the ability to target the DHPS in order to disrupt folic acid synthesis is a very effective method to cope with a broad spectrum of bacteria, parasites and fungi infections making the development of new drugs capable to target other parts of the DHPS a good way to continue to use the DHPS as the key target in future folic acid inhibitors (Hevener *et al*, 2010). In order to develop new drugs it is important to know how resistance was obtained towards the previously used ones in order to prevent rapid development of new resistance to the new drugs.

Conclusion

Although the *S. mutans* 797 DHPS from Uganda contains 4 point mutations in its amino acid sequence there is no difference in resistance towards sulfonamides any of these mutations on their own nor when they are all present in the sequence. This leads to the conclusion that the resistance present in the 797 is not caused by point mutations in the DHPS gene.

Acknowledgments

I want to thank my exam coordinator Lars Liljas and my supervisor Göte Swedberg for their efforts and guiding. To everyone at IMBIM for welcoming and helping me settle in.

Special thanks to Irem and Mariam that I welcomed to IMBIM during the course of this project.

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Appendix

Sequence of NN2025 in comparison to *S. mutans* 797 after preformed mutagenesis in position 46, 80 & 122 leading to isolation of the point mutation in position 146.

```
>  gi|290580578|ref|YP_003484970.1|  dihydropteroate synthase
[Streptococcus mutans NN2025]  gi|254997477|dbj|BAH88078.1|
dihydropteroate synthase [Streptococcus mutans NN2025] Length=266   GENE
ID: 8835689 folP | dihydropteroate synthase [Streptococcus mutans NN2025]
(10 or fewer PubMed links)   Score =  421 bits (1081),  Expect = 2e-115
Identities = 234/258 (91%), Positives = 234/258 (91%), Gaps = 0/258 (0%)
Frame = +2

Query  23 VAGKAXIXGILXVXPXSFSXGGQYXTIDQALKQVEAXLVagaaiidiggxSTRPGAAFVS 202
          VAGKA I GIL V P SFS GGQY TIDQALKQVEA LVAGAAIIDIGGSTRPGAAFVS
Sbjct  8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLVAGAAIIDIGGESTRPGAAFVS  67

Query 203 AEDEIKRIVPIVKAISEKFNCLISIDTYXTXTARVALAAGAHILXDXXSGLYDGHMFQLA 382
          AEDEIKRIVPIVKAISEKFNCLISIDTY T TARVALAAGAHIL D  SGLYDGHMFQLA
Sbjct  68 AEDEIKRIVPIVKAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGHMFQLA  127

Query  38 AETGAPIILMHNQXEXVYSNVTXXVCQFLLERADLAQKTGVKKXNIXLDPGFGFAKNVXQ  562
          AETGAPIILMHNQ E VY NVT  VCQFLLERADLAQKTGVKK NI LDPGFGFAKNV Q
Sbjct 128 AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187

Query 563 NMELLXGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGXKIV  742
          NMELL GLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKG KIV
Sbjct 188 NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247

Query 743 RVHNVAANKDIVKVSSQL  796
          RVHNVAANKDIVKVSSQL
Sbjct 248 RVHNVAANKDIVKVSSQL  265
```

Sequence of NN2025 in comparison to *S. mutans* 797 after preformed mutagenesis in position 46, 146 & 122 leading to isolation of the point mutation in position 80.

```
>  gi|290580578|ref|YP_003484970.1|  dihydropteroate synthase
[Streptococcus mutans NN2025]  gi|254997477|dbj|BAH88078.1|
dihydropteroate synthase [Streptococcus mutans NN2025] Length=266   GENE
ID: 8835689 folP | dihydropteroate synthase [Streptococcus mutans NN2025]
(10 or fewer PubMed links)   Score =  430 bits (1106),  Expect = 2e-118
Identities = 236/258 (91%), Positives = 237/258 (92%), Gaps = 0/258 (0%)
Frame = +3
Query  24 VAXKAAIMGILXVXPXSFSXGGQYETIDQALKQVEAXLVagaaiidiggxSTRPGAAFVS  203
          VA KAAIMGIL V P SFS GGQYETIDQALKQVEA LVAGAAIIDIGG STRPGAAFVS
Sbjct  8  VAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLVAGAAIIDIGGESTRPGAAFVS  67

Query 204 AEDEIKRIVPIVEAIXEKFNCLISIDTYXTXTARVALAAGAHILNDXXSGLYDGHMFQLA  383
          AEDEIKRIVPIV+AI EKFNCLISIDTY T TARVALAAGAHILND  SGLYDGHMFQLA
Sbjct 68  AEDEIKRIVPIVKAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGHMFQLA  127

Query 384 AETGAPIILMHNQXEXXYGNVTXXVCQFLLERADLAQKTGVKKXNIWLDPGFGFAKNVXQ  563
          AETGAPIILMHNQ E  YGNVT  VCQFLLERADLAQKTGVKK NIWLDPGFGFAKNV Q
Sbjct 128 AETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQ  187

Query 564 NMELLXGLAEVTKLGYPVLFGISXKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  743
          NMELL GLAEVTKLGYPVLFGIS KRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV
Sbjct 188 NMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIV  247

Query 744 RVHNVAANKDIVKVSSQL  797
          RVHNVAANKDIVKVSSQL
Sbjct 248 RVHNVAANKDIVKVSSQL  265
```

Sequence of NN2025 in comparison to *S. mutans* 797 after preformed mutagenesis in position 46, 80 & 146 leading to isolation of the point mutation in position 122.

```
>  gi|290580578|ref|YP_003484970.1|  dihydropteroate synthase
[Streptococcus mutans NN2025]  gi|254997477|dbj|BAH88078.1|
dihydropteroate synthase [Streptococcus mutans NN2025] Length=266   GENE
ID: 8835689 folP | dihydropteroate synthase [Streptococcus mutans NN2025]
(10 or fewer PubMed links)   Score =  412 bits (1058),  Expect = 7e-113
Identities = 216/248 (87%), Positives = 216/248 (87%), Gaps = 0/248 (0%)
Frame = +1

Query  52 LXVTXXXXXXGGQYETIDQXLXXXXAMLVAGAXXIDXGGXSXRPGAAFVSAEDEIKRIVP  231
          L VT      GGQYETIDQ L    AMLVAGA  ID GG S RPGAAFVSAEDEIKRIVP
Sbjct  18 LNVTPDSFSDGGQYETIDQALKQVEAMLVAGAAIIDIGGESTRPGAAFVSAEDEIKRIVP  77

Query 232 IVKAISEKFNCLISIDTYXTXTARVXLAAGAHILNDXXSGLYDGQMFQLAAETGAPIILM  411
          IVKAISEKFNCLISIDTY T TARV LAAGAHILND  SGLYDG MFQLAAETGAPIILM
Sbjct 78  IVKAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGHMFQLAAETGAPIILM  137

Query 412 HNQXEXVYGNVTXXVCQFLXERAXLAQKTGVKKXNIWLDPGFGFAKNVXQNMELLXGLAE  591
          HNQ E VYGNVT  VCQFL ERA LAQKTGVKK NIWLDPGFGFAKNV QNMELL GLAE
Sbjct 138 HNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKNVEQNMELLRGLAE  197

Query 592 VTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVRVHNVAANKD  771
          VTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVRVHNVAANKD
Sbjct 198 VTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGCKIVRVHNVAANKD  257

Query 772 IVKVSSQL  795
          IVKVSSQL
Sbjct 258 IVKVSSQL  265
```

Sequence of NN2025 in comparison to *S. mutans* 797 after preformed mutagenesis in position 80, 146 & 122 leading to isolation of the point mutation in position 46.

```
>  gi|290580578|ref|YP_003484970.1|  dihydropteroate synthase
[Streptococcus mutans NN2025]  gi|254997477|dbj|BAH88078.1|
dihydropteroate synthase [Streptococcus mutans NN2025] Length=266   GENE
ID: 8835689 folP | dihydropteroate synthase [Streptococcus mutans NN2025]
(10 or fewer PubMed links)   Score =  413 bits (1061),  Expect = 3e-113
Identities = 226/261 (87%), Positives = 226/261 (87%), Gaps = 0/261 (0%)
Frame = +2
Query  17 KYXVXGKAAIXXXLXVTPXXXXXGGQYETIDQALXXXXAMLaagaxxidiggXSXRPGAA  196
          KY V GKAAI   L VTP     GGQYETIDQAL    AML AGA  IDIGG S RPGAA
Sbjct  5  KYDVAGKAAIMGILNVTPDSFSDGGQYETIDQALKQVEAMLVAGAAIIDIGGESTRPGAA  64

Query 197 FVSAEDEIKRIVPIVKAISEKFNCLISIDTYXTXTARVALAAGAHILNDVXSGLYDGHMF  376
          FVSAEDEIKRIVPIVKAISEKFNCLISIDTY T TARVALAAGAHILNDV SGLYDGHMF
Sbjct  65 FVSAEDEIKRIVPIVKAISEKFNCLISIDTYKTETARVALAAGAHILNDVWSGLYDGHMF  124

Query 377 QLAAETGAPIILMHNQXEXVYGNVTXXVCQFLXERADLAQKTGVKKXNIWXDPGFGFAKN  556
          QLAAETGAPIILMHNQ E VYGNVT  VCQFL ERADLAQKTGVKK NIW DPGFGFAKN
Sbjct 125 QLAAETGAPIILMHNQCEEVYGNVTEDVCQFLLERADLAQKTGVKKENIWLDPGFGFAKN  184

Query 557 VXQNMELLXGLXEVTXLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAXLSGYAISKGC  736
          V QNMELL GL EVT LGYPVLFGISRKRVVDSLLGGHTKPQERDMATA LSGYAISKGC
Sbjct 185 VEQNMELLRGLAEVTKLGYPVLFGISRKRVVDSLLGGHTKPQERDMATAALSGYAISKGC  244

Query 737 KIVRVHNVAANKDIVKVSSQL  799
          KIVRVHNVAANKDIVKVSSQL
Sbjct 245 KIVRVHNVAANKDIVKVSSQL  265
```