Research Article

Point Mutations in the \textit{folP} Gene Partly Explain Sulfonamide Resistance of \textit{Streptococcus mutans}

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Received 5 November 2012; Accepted 24 January 2013

Academic Editor: Marco Gobbetti

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Cotrimoxazole inhibits dhfr and dhp5 and reportedly selects for drug resistance in pathogens. Here, \textit{Streptococcus mutans} isolates were obtained from saliva of HIV/AIDS patients taking cotrimoxazole prophylaxis in Uganda. The isolates were tested for resistance to cotrimoxazole and their \textit{folP} DNA (which encodes sulfonamide-targeted enzyme dhp5) cloned in pUC19. A set of recombinant plasmids carrying different point mutations in cloned \textit{folP} were separately transformed into \textit{folP}-deficient \textit{Escherichia coli}. Using sulfonamide-containing media, we assessed the growth of \textit{folP}-deficient bacteria harbouring plasmids with differing \textit{folP} point mutations. Interestingly, cloned \textit{folP} with three mutations (A37V, N172D, R193Q) derived from \textit{Streptococcus mutans} conferred substantial resistance against sulfonamide to \textit{folP}-deficient bacteria. Indeed, change of any of the three residues (A37V, N172D, and R193Q) in plasmid-encoded \textit{folP} diminished the bacterial resistance to sulfonamide while removal of all three mutations abolished the resistance. In contrast, plasmids carrying four other mutations (A46V, E80K, Q122H, and S146G) in \textit{folP} did not similarly confer any sulfonamide resistance to \textit{folP}-knockout bacteria. Nevertheless, sulfonamide resistance (MIC = 50 \mu M) of \textit{folP}-knockout bacteria transformed with plasmid-encoded \textit{folP} was much less than the resistance (MIC = 4 mM) expressed by chromosomally-encoded \textit{folP}. Therefore, \textit{folP} point mutations only partially explain bacterial resistance to sulfonamide.

1. Introduction

\textit{Streptococcus mutans} are commensal bacteria found in the oral cavity [1]. These bacteria which belong to the Viridans Streptococci Group (VSG) cause dental caries and in frequently give rise to extra oral infections like subacute bacterial endocarditis [1, 2]. Although dental caries is not usually treated by antibiotics, the VSG have attracted interest due to their potential to act as reservoirs of resistance to antibiotic determinants [3]. Additionally, in individuals taking antibiotics as prophylaxis, resistance of commensals to antibiotic determinants could be selected [4] and transferred to pathogenic organisms [5] such as \textit{Streptococcus pneumoniae} which kills over 1,000,000 children worldwide every year [4].

Cotrimoxazole (SXT) is a combination drug (sulfamethoxazole plus trimethoprim) that is commonly used as prophylaxis in HIV/AIDS patients [6]. Sulfamethoxazole is a long-acting sulphonamide. In addition to wide usage as prophylaxis, SXT is also a highly prescribed drug especially in Sub-Saharan Africa due to its low cost and easy availability. Sub-Saharan Africa is reputed for high antibiotic abuse [7]. In Uganda, SXT is not only highly prescribed in dental practice [8], but also selected for multiple antibiotic resistance in \textit{Streptococcus mutans} among HIV/AIDS patients [7]. Despite these findings, data on the mechanisms of SXT resistance in commensal bacteria such as \textit{Streptococcus mutans} is still scanty. In order to better understand the mechanism of \textit{Streptococcus mutans} resistance to SXT, we characterised the \textit{S. mutans folP} gene that encodes dihydropteroate synthase, the target enzyme of sulfonamides [9]. Previously, we reported [7] that \textit{folP} gene from the highly sulfonamide resistant \textit{S. mutans} isolate 797
did not confer sulfonamide resistance to *E. coli* folP knockout bacteria and that sequencing of the folA gene of trimethoprim (TMP) resistant isolates did not reveal any mutations. However, the folP gene is very polymorphic [7], and at least one of the variants of folP confers high sulfonamide resistance to *E. coli* folP knockout cells. In the current study, we report site-directed mutagenesis experiments in which we altered point mutations in *S. mutans* folP gene, inserting the mutagenized folP DNA in pUC19 plasmids, which were then transformed into folP deficient *E. coli* C600 cells. By assessing the growth of the transformant *E. coli* Delta folP cells on media containing different levels of sulfamethoxazole, the influence of individual amino acids on sulfonamide resistance in the folP gene knockout bacteria was determined.

2. Methods

The mechanism of resistance to sulfamethoxazole (SMX) in *S. mutans* was characterised as summarized in Figure 1.

2.1. Bacterial Strains and Plasmids. The bacterial strains used in this study (Table 1) were previously isolated [7] from oral specimens of HIV/AIDS patients taking cotrimoxazole as prophylaxis in Kampala, Uganda. The cloning vectors pJet1.2/blunt (Fermentas, Lithuania) and pUC19 [12] were used. *E. coli* top ten cells (Invitrogen, USA) and *E. coli* recipient strain C600 ΔfolP [13] which is a folP knockout strain were used in the transformation experiments. *Streptococcus pneumoniae* ATCC 49619 was used as a susceptible control when determining MICs.

2.2. Susceptibility Testing. Minimal inhibitory concentrations (MICs) were determined by the *E*-test method (AB Biodisk, Sweden) following the manufacturer's recommendations. All tests were done on Iso-Sensitest Agar (ISA, Oxoid, UK). Plates were incubated at 37°C in 5% CO2 for 24 h. For determination of sulfonamide resistance conferred by cloned folP genes, *E. coli* C600 bacteria were grown in Iso-Sensitest Broth (ISB, Oxoid, UK) to a cell density of 10^8/mL, diluted to 10^4/mL, and plated on ISA plates containing varying concentrations of sulfathiazole (Sigma Aldrich, USA).

2.3. DNA Extraction. Isolates of *Streptococcus mutans* were incubated at 37°C for 12 h on Iso-Sensitest Agar. Bacterial colonies were re-suspended in brain heart infusion broth (BioMérieux, France) and incubated at 37°C for 24 h in an atmosphere of 5% carbon dioxide. Chromosomal bacterial DNA from the cultured broth was then extracted using the Wizard Genomic DNA Purification Kit (Promega, USA).

2.4. Cloning. The PCR primer sequences used were based on the published sequence of the folP gene of *Streptococcus mutans* UA159 [10] (Table 1). FolP gene amplification was performed in 50-μL volumes containing 0.5 μM of each primer, 100 μM of the four deoxynucleoside triphosphates, 5 units of DNA polymerase (Pfu, Fermentas), 2 μL of template DNA (50–500 ng) preparation, and 1X reaction buffer (Pfu, Fermentas) containing 2 mM MgSO4. Amplification reactions were performed with the *Eppendorf* mastercycler gradient thermocycler (Eppendorf, Germany) using the following program: heating at 94°C for 2 min, followed by 25 cycles consisting of a denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and an extension at 72°C for 1.5 min. This was followed by a final extension of 72°C for 5 min and a holding step at 16°C.

The PCR products were cleaned by the PCR Cleanup kit (Omega, USA) and then used for cloning into the pJet vector using the blunt end pJet cloning kit protocol (Fermentas, Lithuania). The ligated products were introduced into *E. coli* top ten cells by heat-shock CaCl2 transformation method. Plasmids were prepared from the transformants using the plasmid preparation kit (Omega, USA) and prepared for sequencing.

2.5. Sequence Analysis. For sequencing the plasmids, the BigDye Terminator labelled cycle-sequencing kit (Applied Biosystems) and an ABI prism 310 Genetic Analyzer (Applied Biosystems) were used. The results of the folP gene sequence analysis were compared with database sequences of *Streptococcus mutans* UA159 [10] and NN2025 [11] using the BLAST programme at NCBI [14].

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**Figure 1:** Flow chart showing characterization of folP gene. Plasmids carrying folP gene were transformed in folP gene knockout bacteria to determine the effect of different mutations in plasmid encoded folP on bacterial resistance to sulfonamides.
Table 1: Characteristics of bacterial isolates used in the current study, the respective genes and susceptibility to Cotrimoxazole (STX), sulfamethoxazole (SMX), and trimethoprim (TMP) as determined by E-test.

| Isolate            | Accession number of folP gene nucleotide sequence used | Mutations in the folP gene | STX            | Sulfonamide susceptibility | Trimethoprim susceptibility | Mutations in the folA gene |
|--------------------|--------------------------------------------------------|-----------------------------|----------------|---------------------------|-----------------------------|---------------------------|
| S. mutans 8        | Not yet submitted to gene banks but previously published [7] | A37V, N172D, and R193Q<sup>*</sup> | >32 μg/mL | >1024 μg/mL (>4 mM) | >32 μg/mL | None |
| S. mutans 797      | HE599533.1                                             | A46V, E80K, Q122H, and S146G<sup>**</sup> | 0.5 μg/mL | >1024 μg/mL (>4 mM) | 2 μg/mL | None |
| S. mutans 135      | Not yet submitted but previously published [7]         | A63S, W174L, K175F, and M189I<sup>**</sup> | 8 μg/mL  | >1024 μg/mL (>4 mM) | 0.38 μg/mL | None |
| S. sobrinus 7      | HE 599535.1                                            | None                        | >32 μg/mL | >1024 μg/mL (>4 mM) | >32 μg/mL | None |
| S. downei 477      | Similar to ZP 0772557.1                                | None                        | 0.125 μg/mL | Not done | Not done | Not done |

* Mutations as compared to UA159 [10]. ** Mutations as compared to NN2025 [11].

Table 2: Primers used for cloning and site-directed mutagenesis.

| Primer name       | Nucleotide sequence                                      |
|-------------------|----------------------------------------------------------|
| Mutans DHPSph     | 5'-GAT CGA TCG CAT GCA CAT CAT AAC TAG GGA GCA AGC-3' |
| mutansDHPSBam     | 5'-GAT GGA TGG GAT CCA AAA TAATCT TAT CCA TAA CAC CCT CA-3' |
| dhpsfwpheco       | 5'-AAC CTA CTG CAT GCA TAA GAA TCA G-3' |
| downeinolpfwp     | 5'-GCA TGG CAA AGA CAG GAA TTA CGT AC-3' |
| Downeifolpreps    | 5'-CTG CAG CCA AAA TTT GCC CCA GAC-3' |

Primer names for changing specific amino acids in isolate 797

| Primer name       | Nucleotide sequence                                      |
|-------------------|----------------------------------------------------------|
| DHPS46AVfw        | 5'-TGA AGC CAT GTT AGT AGC AGG AGC GGC TA-3' |
| DHPS46AVrev       | 5'-TAG CCG CTC TGG CAT TAA ACA TGG CCT CA-3' |
| DHPS80aEKfw       | 5'-TCC TTC CAA TTA AAG CTA TTA GGA AA-3' |
| DHPS80aEKrev      | 5'-TTG CAC TTC TTT AAT AGC TAA TGG GA-3' |
| DHPS122QHfw       | 5'-CCTT TAT GAT GGG CAC ATG TTT CAA TAA GC-3' |
| DHPS122QHrev      | 5'-GCT AAT TGA AAC ATG TGC CCA TCA TAA AG-3' |
| DHPS146SGfw       | 5'-GTT AAG AAG TTT ATG GAC AGA TAA CAA AA-3' |
| DHPS146SGrev      | 5'-TTG TGT TCG TAT ATG GCC ATA AAC TTC TTG AC-3' |

Primer names for changing specific amino acids in isolate 8

| Primer name       | Nucleotide sequence                                      |
|-------------------|----------------------------------------------------------|
| V37Afw            | 5'-AAC CAA TCG ATC AGG CTC TAA AAC AGG TTA A-3' |
| V37Arev           | 5'-TCA ACC TGT TTT AGA GCC TGA TCG ATG TTT T-3' |
| DI72Nfw           | 5'-GGA GTT AAA AAA AAA AAT ATG TGG CTT GAT G-3' |
| DI72Nrev          | 5'-GAT CAA GCC AAA TAT TTT CTT TTT TAA CTC C-3' |
| Q193Rfw           | 5'-ACA TCG AAC TTC TAC GAG GCT TAG CCG AGG T-3' |
| Q193Rrev          | 5'-ACC TCC GTC AGG CCT GCT AGA GTG ATG TTG ATG-3' |

2.6. Site-Directed Mutagenesis. Mutagenesis was carried out using a 50 μL reaction mix (Fermentas, Lithuania) containing 1X Pfu buffer with MgSO<sub>4</sub>, 2.5 units Pfu DNA Polymerase (Fermentas, Lithuania), 0.1 mM dNTPs, 10–100 ng of template DNA inserted in pUC19, and 1 μM of each primer (Table 2). The PCR program started with a heating step at 95°C for 30 s, followed by 18 cycles consisting of a denaturation step of 95°C for 30 s, annealing of 50°C for 30 s, and an extension of 68°C for 7 min.

Site-directed mutagenesis products (17 μL of the PCR product) were digested with 5 units of the restriction enzyme DpnI (Fermentas, Lithuania) in Buffer Tango to remove unchanged DNA. The mixture was incubated at 37°C for 4 h before incubating at 80°C for 20 min to inactivate DpnI.

2.7. Transformation. Ten μL of the digested mutagenesis product were transformed into CaCl<sub>2</sub>-treated folP knockout E. coli competent cells. Recombinant plasmids were prepared from part of a single transformant bacterial colony using the plasmid miniprep kit (Omega, USA). The plasmids were then sequenced as described above to confirm that site-directed mutagenesis had occurred. Bacteria from the same
transformant colony were then tested for growth at different SMX concentrations.

3. Results

The results of transformation of *S. mutans* folP gene into folP knockout *E. coli* are shown in Figure 2. As previously reported [7], *S. mutans* isolate 797 carries four point mutations (A46V, E80K, Q122H, and S146G) in folP gene, as compared to the control strain NN2025 [11] but harbours one such mutation (SI46G) if compared to control strain UA159 [12] (Table 1). Two other isolates with DHPS sequence differing from both reference strains were cloned using the same conditions as for 797. These were isolate 8 (A37V, N172D, and R193Q) and isolate 135 (A63S, W174L, L175F, and M189I). Of these, only the cloned folP gene from isolate 8 conferred sulfonamide resistance to the *E. coli* C600ΔfolP recipient. In the present paper, the above-mentioned four point mutations (A46V, E80K, Q122H, and S146G), and those in *S. mutans* isolate 8 (A37V, N172D, and R193Q) (Table 1), were successfully altered or removed from folP gene by site-directed mutagenesis. Nonmutagenized chromosomal DNA from isolates *S. sobrinus* 7 and *S. downei* 477 was individually inserted into *S. mutans* as well. Effects of the different folP gene constructs on resistance to sulphonamide were then investigated by transforming the recombinant pUC19 plasmids in folP knockout *E. coli* C600 and assessing the growth of transformant bacteria on media containing different concentrations of sulfamethoxazole (see Figure 1). Interestingly, plasmids harbouring triple mutant folP of *S. mutans* isolate 8 (bearing the mutations A37V, N172D, and R193Q) conferred (to folP knockout *E. coli*) intermediate level resistance (MIC: 50 μM) against sulfonamide (bar A in Figure 2) even though this is not equal to 4 mM, the MIC arising from the chromosomal folP gene in the natural isolate *S. mutans* 8 (Table 1). In addition, altering any of the three polymorphic amino acids (A37V, N172D, and R193Q) back to the UA159 sequence and transforming the double mutant folP gene into the *E. coli* Delta-folP cells produced reconstituted knockout *E. coli* cells of lower level (MIC: 30 μM) resistance to sulphonamide (Figure 2: bars B, C, D), while reversing two amino acid mutations (Figure 2 bar E) or all three amino acid mutations (Figure 2 bar F) to wild-type and transforming folP knockout *E. coli* likewise yielded transformant clones with low resistance to sulphonamide (MIC: 20 μM). On the other hand, as previously reported [7], cloned folP gene from 797 did not confer resistance of folP deficient *E. coli* C600 to sulphonamide. Moreover, changing the DNA encoding four amino acid mutations (A46V, E80K, Q122H, and S146G) of folP in *S. mutans* isolate 797 and transforming the mutant DNA in folP knockout *E. coli* to comply with either NN2025 or UA159 sequences (Figure 2 bar G) did not change susceptibility of folP knockout bacteria to sulphonamide. Controls consisting of folP knockout *E. coli* transformed with pUC19 plasmids encoding mutant folP from *S. mutans* isolate 135 (Figure 2, bar H), wild-type folP from *S. sobrinus* 7 (Figure 2 bar I), or wild-type folP from *S. downei* 477 produced minimal or reduced resistance.

![Figure 2: Comparing sulfamethoxazole minimal inhibitory concentrations (MICs) for folP knockout *E. coli* cells transformed with pUC19 plasmid carrying differing chromosome-encoded folP genes of streptococci. To determine the effect plasmid encoded mutant folP has on the sulfamethoxazole resistance of transformed C600ΔfolP *E. coli* bacteria, growths (in SMX containing media) were compared for folP-deficient *E. coli* transformed with pUC19 carrying triple-mutant folP (residues 37, 172, and 193: bar A), double-mutant folP (bars B, C, and D), single-mutant folP (residue 193: bar E), and wild-type folP (bar F) from *S. mutans* isolate 8. The sulfamethoxazole resistance of transformed folP deficient cells was notably increased by transformation with plasmid encoding triple-mutated folP (wild-type folP MIC = 20 μM, triple-mutant folP MIC = 50 μM). Note: The MIC (sulfamethoxazole) for chromosome-encoded folP in *S. mutans* isolate 8 was 4 mM (see Table 1). Controls comprising C600ΔfolP *E. coli* transformed with pUC19 encoding either mutant folP from *S. mutans* isolates 797 (bar G) and 135 (bar H) or wild-type folP from *S. sobrinus* isolate 7 (bar I) or *S. downei* isolate 477 (bar J) showed basal or less sulfamethoxazole resistance (MICs = 20–30 μM). **: *S. mutans* isolate 8 mutant folP; ***: *S. mutans* isolate 797 mutant folP; **: *S. mutans* isolate 135 mutant folP.](image)

4. Discussion

In the present study, we examined Ugandan *Streptococcus mutans* isolates from HIV/AIDS patients who were taking cotrimoxazole as prophylaxis [7]. The isolates were found to have different point mutations in the folP gene in relation to wild-type sequences found in databases. It should be noted that these same isolates lacked any mutations in the folA gene.

Resistance to sulphonamides in gram positive bacteria has been shown to be due to mutations in the folP gene that render the encoded dihydropterotate synthase insensitive to
the drug [15]. In the corresponding gene of *Plasmodium*, more point mutation combinations were previously found to be associated with higher resistance rates against sulfadoxine in *P. falciparum* [16]. In the present study, we assessed the influence of different combinations of mutations in *S. mutans* folP by performing mutagenesis experiments to remove mutations in the folP gene and transforming the changed DNA into folP knockout *E. coli* cells which were subsequently tested for growth in the presence of varying levels of sulfamethoxazole. We found that the cloned folP gene from isolate 8 conferred substantial sulfonamide resistance to folP knockout *E. coli* (MIC: 50 μM) (Figure 2) but not to the level observed for the natural isolate *S. mutans* 8 (MIC: 4 mM). Changes in any of the three divergent amino acids (residues 37, 172, and 193) of folP reduced the level of resistance to sulfonamide and the removal of all three polymorphisms totally abolished the resistance. In contrast, no combination of the mutations in folP from isolate 797 (A46V, E80K, Q122H, and S146G) led to differences in susceptibility of folP knockout bacteria to sulfonamide. In addition, we found that isolates 135, 7, and 477 with different mutation patterns in folP grew to the same resistance level (MIC: 20 μM) as isolate 797. This finding corroborates the previous report [7] that the cloned folP gene from 797 does not confer sulfonamide resistance to folP-gene knockout *E. coli* cells. However, that the cloned folP gene from isolate *S. mutans* 8 did not confer equally high sulfonamide resistance to folP as shown by the natural isolate *S. mutans* 8 (MIC: 4 mM) suggests that there is another mechanism of resistance to sulfonamide other than the point mutations. One possibility may be that DHPS synthesis and expression are increased as was found in *Streptococcus agalactiae* [17] or that there may be point mutations in other folate pathway genes. We could not rule out other causes of resistance to sulfonamide in *Streptococcus mutans* since sequencing the folA gene of *S. mutans* 8 and flanking regions including the promoter did not reveal any mutations (results not shown). Further experiments including whole genome sequencing of *S. mutans* 8 and other sulfonamide resistant strains may enhance the understanding of sulfonamide resistance in *Streptococcus*.

5. Conclusions

Point mutations are one of the explanations for the mechanism of resistance to sulfonamide in *Streptococcus mutans*. However, cloned folP gene did not confer full resistance to folP knockout cells compared to the original isolate 797, a result which does not rule out other possible mechanisms for the resistance to sulfonamides.

Conflict of Interests

The authors report no conflict of interests.

Authors’ Contribution

W. Buwembo carried out the preparation of bacterial isolates and polymerase chain reaction tests, participated in the microbiological antibiotic resistance tests, cloning, and sequencing analyses, and drafted the paper. S. Aery participated in the mutagenesis experiments. G. Swedberg designed the primers and participated in cloning and transformation experiments. F. Kironde, C. M. Rwenyonyi, and G. Swedberg conceived the study, participated in its design and coordination, and helped in writing the paper. All authors read and approved the final paper.

Acknowledgments

The authors are grateful to GS project students, namely, Mary MacRitchie, Soheila Rajabi, Marcus Andersson, and Fredrik Jonsson who assisted in this work. We sincerely thank the patients who donated clinical specimens, research associates, and administrators of TASO clinic. This work was financially supported by the Swedish Agency for Research Cooperation with Developing Countries. All the authors are responsible for the content and writing of the paper.

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