Tetracycline Antibiotic Resistance Through Enzymatic Modification in *Bacteriodes* Strains

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Introduction

Tetracyclines are commonly used to treat infections caused by numerous microorganisms such Chlamydiae (Chlamydia) and Mycoplasma pneumoniae (Atypical pneumonia) in addition to combating moderate-severe acne. These antibiotics act through binding to the 16S part of the 30S ribosomal subunit, preventing the amino-acyl tRNA from binding to the A site of the ribosome and thus inhibiting protein synthesis. Tetracyclines originally exhibited a wide range of antibiotic action against both gram-negative and gram-positive clinically relevant bacteria, with each successive optimization of the core scaffold resulting in improved activity.

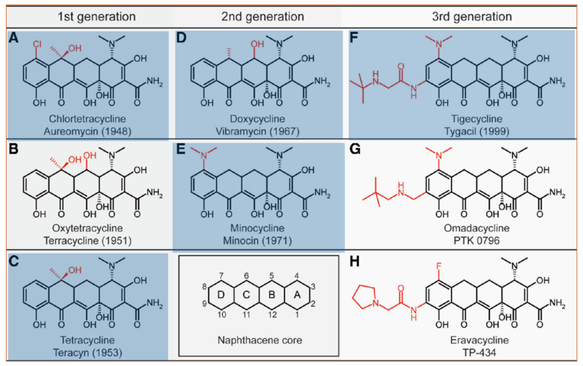


Figure 1. Chemical structures of various tetracycline antibiotics classified by generation. The blue-shaded compounds were utilized in this work (Nguyen F et al. 2014)

However, due in part to their vast popularity and lateral bacterial gene transfer, the rate of tetracycline resistance has increased dramatically, corresponding with a dissemination in common resistance mechanisms.[1] These include drug efflux pump activity and molecular target protection, which have been frequently investigated in bacteria as possible targets for combating widespread drug resistance. Our goal in this work is to focus on a third, less investigated mechanism utilizing enzymes to modify tetracyclines into inactive or unstable derivatives and thus limiting their clinical efficacy.

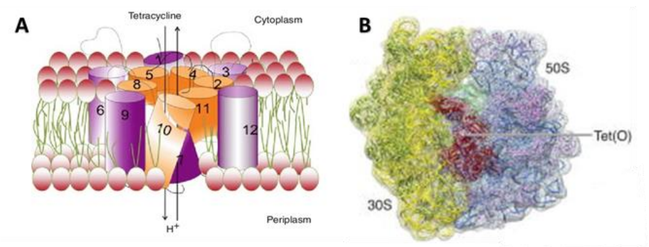


Figure 2. A. Model of TetA(B) efflux pump, used to pump out tetracycline from cell interior (Li W et al. 2013
B. Model of Tet(O) ribosomal protection protein, used to protect target from tetracycline activity (Thaker MN et al. 2009)

Bacteriodes Targets and Susceptibility Screening

Bacteriodes dorei and *Bacteriodes fragilis* are opportunistic pathogenic bacteria which take advantage of a weakened immune system to infect tissues and organs surrounding the human gut. Previous studies have found *B. fragilis* to be the most commonly isolated Bacteriodaceae in anaerobic infections.[2] These strains are part of the expansive human gut microbiome that is known to play essential roles in host physiology and human disease in addition to holding an accessible reservoir of antibiotic resistance. From initial susceptibility screening tests of 10 different anaerobic strains, *B. dorei* and *B. fragilis* were determined to hold resistance against most of the tetracyclines tested (Table 1).

Antibiotic activity was determined via two-fold drug dilutions in 100 µl reinforced clostridium medium (RCM) in a 96-well plate (in triplicate). Each well was inoculated with 50 µl of strain inoculum and incubated in 37° C for 20 hours before ODs were read via microplate reader.

Metabolism time courses were prepared for the following antibiotics

- Tetracycline (50 µm treatment in 5 mL media)
- Minocycline (12.5 µm treatment in 5 mL media)
- Doxycycline (25 µm treatment in 5 mL media)
- Chlortetracycline (25 µm treatment in 5 mL media)

Drug	Strain	
	B. fragilis	***B. dorei***
Tetracycline	88.89 µg/mL	88.89 µg/mL
Minocycline	5.72 µg/mL	11.44 µg/mL
Doxycycline	22.22 µg/mL	22.22 µg/mL
Chlortetracycline	23.94 µg/mL	23.94 µg/mL
Tigycycline	1.83 µg/mL	1.83 µg/mL

Table.1 Susceptibility of anaerobic strains *B. fragilis* and *B. dorei* against tetracycline antibiotics. An MIC value of ≥16 µg/mL in anaerobic strains is considered resistant (NCCLS 6th ed.)

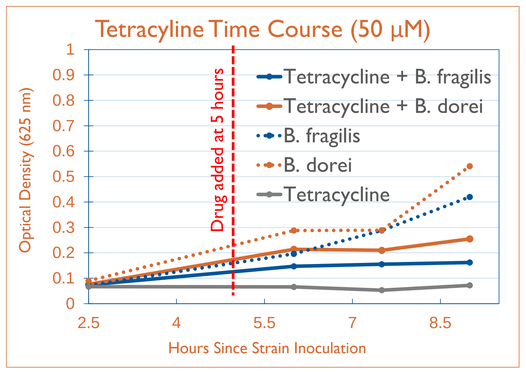


Figure 3. Strain originally inoculated in 5 mL of RCM in culture tube and allowed to grow in anaerobic incubator for 5 hours. 200 µl aliquots were taken at T=2.5,5,6, 7,5 and 9 hours since inoculation and ODs were read via microplate reader to quantify culture growth.

Additional 200 µl aliquots were recovered and 600 µl of cold 1:1 acetonitrile:methanol solution was added. The samples were incubated in -20°C freezer for 1 hour before being centrifuged for 20 minutes at 15000 rpm at 4°C. 200 µl of the supernatants were used in HPLC/LCMS analysis.

Mechanism of Tetracycline Destructases

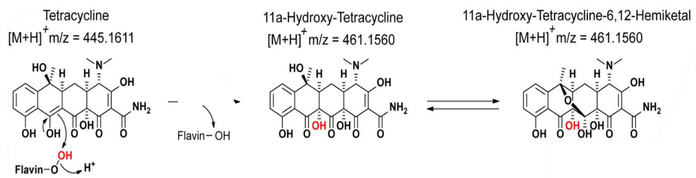
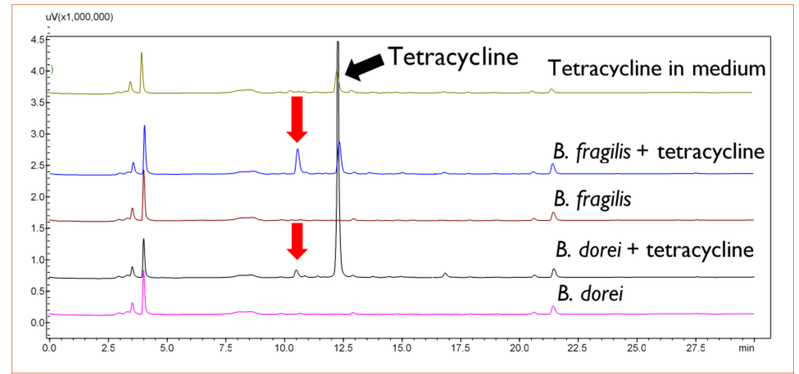


Figure 4. Hydroxylation of Tetracycline by TET(X) and TET (47)-TET(56) (Forsberg KJ et al. 2015)

Previously, genes encoding tetracycline resistance through destructase enzymes have been sequenced from *B. fragilis* and expressed in *E. coli*.[3,4] As a result, the enzymes TET(X) and TET(47)-TET(56) were determined to mediate the hydroxylation of Tetracycline into the inactive derivative. However, the destructase enzymes uncovered are flavin adenine dinucleotide (FAD)-dependent monooxygenases, signifying the need for O_2 during the hydroxylation reaction which is not available in the anaerobic environment of the human gut. Furthermore, Bioinformatic analysis via BLAST shows the *tet*(X) gene in *B. fragilis* 3_1_12 used in our tests sharing high identity (99.74%) with previously characterized *B. fragilis tet*(X) via *E. coli* transformation, while only sharing 44.44% identity with the tested *B. dorei* Tet(x). This suggests high variability within the tetracycline destructase gene that could effect resistant activity in anaerobic strains.

Potential Metabolism of Tetracycline



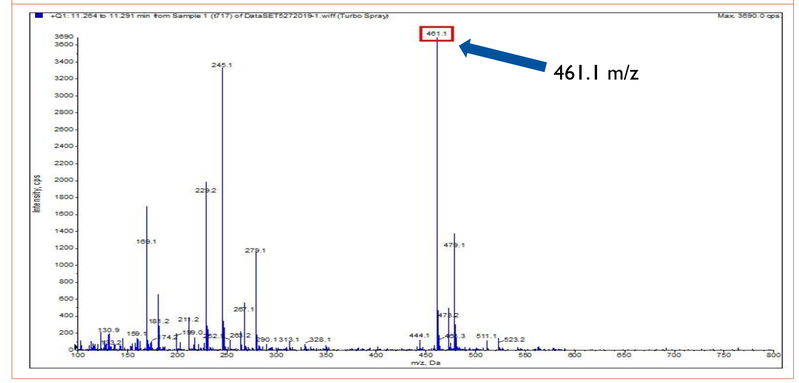


Figure 5. HPLC and mass spectrometer results from tetracycline metabolism time course sample. Aliquot correlating to T=4 hours after drug treatment was used as sample. The potential metabolite in the *Bacteriodes* treatment culture simples are marked by the red arrow with a retention time of 10.5 minutes. The mass spectrometer was utilized to determine the molecular weight of the possible metabolite peak, which at 461.1 m/z correlates to the metabolite 11a-Hydroxy-Tetracycline/ 11a-Hydroxy-Tetracycline-6,12-Hemiketal.

HPLC- Agilent Poroshell 120 EC-C18 column (2.7 µm, 4.6 x 50 mm), coupled with a PDA detector set at wavelength of 363 nm.

LC-MS- SymmetryShieldTM RP18 column (3.0 x 150 mm, 3.5 µm). Electrospray ionization was used for analysis of reaction products by mass spectrometry and ion counts for a particular m/z peak were determined by peak height.

Conclusion

The hydroxylated tetracycline metabolite product was formed after a 4-hour tetracycline treatment in anaerobic conditions, signifying the possibility of either resistance activity by a novel enzyme or possible Tet(x) variants that can function in these conditions. This metabolism product is also seen in *B. dorei*, which has a significantly different Tet(x) genetic profile than *B. fragilis*. Longer time courses performed saw increased nonenzymatic degradation of the antibiotic and the possible hydroxylated metabolite. Other tetracycline antibiotics tested did not induce this resistance response, although further screening is needed in different anaerobic strains. Further work is also needed on the potential bioactivities of the tetracycline metabolite and its possible impact on the relative inhibition of bacteria growth.

Acknowledgements and References

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