National Science Foundation Research Proposal, Example 1

Objectives: The objective of this study is to use insertional transposon mutagenesis to identify genes in *Helicobacter pylori* which when inactivated confer resistance to the antibiotic Metronidazole. These genes will then be analyzed in strains isolated from patients where antimicrobial therapy fails. Finally, these genes will be characterized using bioinformatics to identify homologous genes with known function and the gene products will be biochemically tested for their ability to metabolize Metronidazole.

Background: *Helicobacter pylori* is a Gram-negative, microaerobic, spiral bacterium that colonizes the human stomach. Approximately 50% of the world's population carries *H. pylori*. While most cases are asymptomatic, *H. pylori* infection is the major causative agent of stomach ulcers and is a leading risk factor for gastric cancer (1).

The current treatment for *H. pylori* infections involve a "triple therapy" which consists of one of a variety of drugs that decrease the acidity of the stomach, and two antibiotics including Metronidazole (Mtz) (2). Mtz, used for treatment of many bacterial and parasitic pathogens, is administered as an inactive prodrug. The prodrug is converted to a cytotoxic form within the low intracellular redox potential environment of anaerobic and microaerobic organisms, but is unchanged in aerobic organisms, which is the basis for its selective toxicity. The active form of Mtz generates mutagenic oxygen radicals and nitroradicals leading to cell death.

The high use of antibiotics, including Mtz, both in humans as well as for domestic animals, has led to an increasing rate of resistance, as high as 80% in some areas of the world (3). In *H. pylori*, RdxA protein, a putative oxygen-insensitive NADPH nitroreductase, is thought to activate Mtz by reducing the nitroaromatic group of Mtz to hydroxylamine, forming a potent mutagen. Previously, it has been shown that inactivation of the *rdxA* gene is associated with Mtz resistant *H. pylori* (4). However, not all cases of Mtz resistance correlate to loss of RdxA protein (5). Furthermore, these resistant strains are still susceptible to Mtz if given in high enough doses. This suggests that there may be other genes responsible for activating Mtz. Metabolic enzymes such as pyruvate oxidoreductase (POR) and α-ketoglutarate oxidoreductase (KOR), for example, have been shown to activate Mtz in other anaerobes (6). POR and KOR activities have also been shown to be repressed in Mtz resistant strains of *H. pylori* cultured in the presence, but not in the absence, of Mtz (7). The focus of this study is to determine what genes, when inactivated in *H. pylori*, give rise to Mtz resistance.

Hypothesis: **Metronidazole resistance of *Helicobacter pylori* is mediated through loss of specific genes.**

Aim 1: Screen for *H. pylori* mutants that confer Mtz resistance and determine which genes were mutated.

A transposon insertion library of *H. pylori* has been constructed in the Salama Lab (unpublished data) with a mini Tn7 based transposon conferring resistance to chloramphenicol (Cm). Since screening for Mtz resistance in wild type background gives rise to many colonies that are resistant due to transposon insertion in the *rdxA* gene (Salama Lab, unpublished), *H. pylori* that are *rdxA*- due to a deletion will be transformed with genomic DNA from the transposon library and transformants selected on Cm media. These transformed cells will then be grown on blood plates that contain higher concentrations of Mtz than *rdxA*- mutants can tolerate, thus selecting for strains that have increased Mtz resistant due to inactivation of other genes.

The position of chromosomal insertion will be determined using a semi-random PCR method to amplify the DNA flanking the transposon (8). This product will then be sequenced to determine which gene was interrupted by the insertion of the transposon.

In order to determine that inactivation of the gene identified by the screen causes Mtz resistance, an independent mutant will be constructed using site directed mutagenesis on *H. pylori* that is *rdxA+*. This mutant will then be grown on plates containing Mtz to retest for Mtz resistance. If Mtz resistance is found, further confirmation will be obtained by introducing a wild type copy of the gene on a shuttle vector plasmid into the mutant strain to see if Mtz sensitivity is restored. Additionally, a wild type copy of the gene will be cloned into *E. coli*, which is naturally Mtz resistant, to test for Mtz sensitivity in order to determine the mechanism of action of the gene product.

If, however, the *H. pylori* mutant is not resistant to Mtz, the loss of both this gene and *rdxA* might be necessary to confer Mtz resistance. In this case, an independent mutant will be made in *rdxA- H. pylori*. Alternatively, the insertion of the transposon could cause a polar effect, disrupting genes downstream from the gene in question. Independent mutations will be made to these downstream genes, and then retested for Mtz resistance.

Aim 2: Determine if alterations of genes identified in the Mtz resistance screen are found in natural isolates of *H. pylori.*

To test whether mutation of these genes are found clinically, *H. pylori* samples will be taken from infected patients who fail to show eradication upon treatment with Mtz. The genes of interest will be sequenced from these strains and analyzed for alterations including stop codons, deletions and other defects.

Aim 3: Determine the function of these genes using sequence homology and electron spin resonance spectroscopy.

The sequences of these genes will be compared to other organisms to search for homologues with known function. The gene could share homology with known reductases, including *rdxA*. On the other hand, it could be involved in regulating reductase activity through transcriptional control or post-translational modifications. In either case, the gene's effect on reductase activity will be determined using *in vivo* electron spin resonance (ESR) spectroscopy. ESR detects electrons with unpaired spins, or free radicals, which are found in the activated, reduced form of Mtz (6). *H. pylori*, both wild type and mutant in the gene, will be treated with Mtz and tested independently for free radical formation. Detection of free radicals in the presence, but not the absence of the gene will verify the gene's role in reductase activation on Mtz, and explain how loss of this gene will give rise to resistant strains.

Relevance: Antibiotic resistance can be brought about through the gain of genes, as in efflux channels which pump out the antibiotic, or effector proteins which inactivate the antibiotic, as well as through loss of genes, as in the case of *rdxA* which codes for a seemingly nonessential metabolic enzyme. Identification of these resistance-conferring genes allow for methods of rapid detection of resistant pathogens to ensure proper treatment, as well as identifying targets for the development of new antibiotics. *H. pylori* provides an excellent model for studying the genetic basis of antibiotic resistance, as it was the first organism in which two different strains were fully sequenced (9,10). This invaluable tool, along with recent advancements in genomic tools developed for *H. pylori*, allow genetic manipulations and characterizations of genes of interest.

References:

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