Host Range of the Doc Toxin

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Background:

-P1 plasmid TA system:
The Bacteriophage P1 lysogenizes host Escherichia coli cells via a plasmid addiction system that increases the apparent stability of a plasmid by killing plasmid-free daughter cells. The system is autoregulated by both an toxin, Doc, which causes Death On Curing (doc), and an antitoxin, Phd, that Prevents Host Death. (1). Phd is more unstable than doc due to its proteolytic breakdown by the host-encoded ClpXP protease, so in order to prevent host death it must be expressed at a much higher rate than is doc (1). Upon plasmid loss, Phd is no longer expressed and any free Phd is broken down, and the toxic effects of Doc kills the plasmid-free daughter cell(s). It is currently believed that the toxicity of Doc is due to a modification of a target protein, elongation factor Tu (EF-Tu).

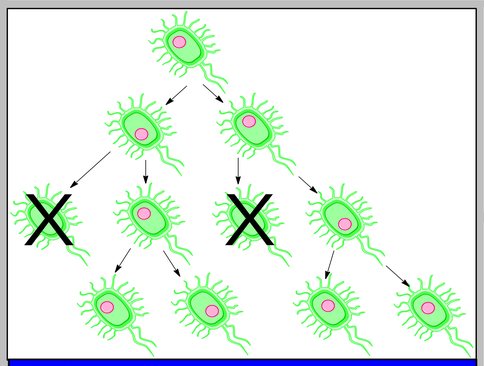

Figure 1 :
Diagram of the results of plasmid loss in bacteria with TA system

-Target: EF-Tu:
EF-Tu, a ~394 aa protein plays an integral role in the translation of proteins. EF-Tu mediates the entry of amino-acylated tRNAs (AARS) into the ribosome and regulates amino acid addition to the polypeptide chain via check point delays. Without EF-Tu, protein synthesis could not be possible. It is believed that Doc acts as a kinase and modifies EF-Tu via substrate level phosphorylation by a gamma phosphate from ATP. This phosphorylation causes a conformational change in EF-Tu that inhibits its ability to bind AARS, thus halting translation.

- Kinases:
Protein kinases are responsible for a variety of biochemical regulation mechanisms. They are ATP-dependent phosophotransferases that phosphorylate protein targets by transferring a γ-phosphate from ATP to the hydroxyl groups of their target amino acids (2). It is believed that Doc acts as a protein kinase by phosphorylating EF-Tu and inhibiting its ability to regulate and mediate protein translation.

This project was centered around analyzing the activity of the Doc toxin on its believed target, EF-Tu, found in species other than E. coli. If activity is found in these species, it may influence how the target is further studied and confirmed as well as indicate future alternatives to current antibiotic treatment of bacteria.

QUESTION: Is the target of the Doc toxin present in bacteria other than E. coli?

Species being tested:

1.	*Alcaligenes faecalis*	*11.*	*Mycobacterium smegmatis*	*21.*	*Staphylococcus epidermidis*
2.	*Bacillus cereus*	*12.*	*Neisseria lactamica*	*22.*	*Staphylococcus saprophyticus*
3.	*Bacillus subtilis*	*13.*	*Proteus mirabilis*	*23.*	*Streptococcus agalactiae*
4.	*Candida albicans*	*14.*	*Proteus vulgaris*	*24.*	*Streptococcus bovis*
5.	*Citrobacter freundii*	*15.*	*Pseudomonas aeruginosa*	*25.*	*Streptococcus equi*
6.	*Enterobacter aerogenes*	*16.*	*Pseudomonas fluorescens*	*26.*	*Streptococcus pneumoniae*
7.	*Enterococcus faecalis*	*17.*	*Saccharomyces cerevisiae*	*27.*	*Streptococcus mitis*
8.	*Escherichia coli*	*18.*	*Salmonella typhimurium*	*28.*	*Serratia marcescens*
9.	*Klebsiella pneumoniae*	*19.*	*Shigella flexneri*		
10.	*Micrococcus luteus*	*20.*	*Staphylococcus aureus*		

References:

1. Magnuson, Roy, et al. "Autoregulation of the plasmid addiction operon of bacteriophage P1." Journal of Biological Chemistry 271.31 (1996): 18705-18710.
2. Adams, Joseph A. "Kinetic and catalytic mechanisms of protein kinases." Chemical reviews 101.8 (2001): 2271-2290.
3. Subramaniam, S. (1998) The Biology Workbench--a seamless database and analysis environment for the biologist. Proteins, 32, 1-2.
4. Boxshade version 3.3.1, by Kay Hofmann and Michael D. Baron.
5. (Tree) Higgins, D.G., Bleasby, A.J. and Fuchs, R. (1992) CLUSTAL V: improved software for multiple sequence alignment. Computer Applications in the Biosciences (CABIOS), 8(2):189-191.
6. (Tree) Thompson J.D., Higgins D.G., Gibson T.J. "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic Acids Res. 22:4673-4680(1994).
7. (Tree) Felsenstein, J. 1989. PHYLIP -- Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.
8. CLUSTAL W: Julie D. Thompson, Desmond G. Higgins and Toby J. Gibson, modified; any errors are due to the modifications.
9. PHYLIP: Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Method:

1. Bioinformatics:
Using the National Center for Biotechnology Information (NCBI) database, the protein sequences for the various species were gathered. Sequences were abundant for many strains, so the sequences were chosen based off 3 factors:
   a. Sequences close to 394 amino acids long
   b. Sequences having large numbers of identical sequences in the database
   c. Sequences having both species name AND “elongation factor Tu” in title

Sequences were loaded into Biology Workbench (3), and alignments via ClustalW were done. From these alignments a rooted phylogenetic tree was made using PHYLIP.

2. Cell growth:
As samples cultures were grown, A growth curve was run on each to determine the optical density of each culture at the harvesting time for more accurate protein analysis.

3. Cell lysis:
Lysis of cell cultures was done using cup-horn sonication.

4. Protein quantification:
Protein content of crude lysates were quantified using the Bradford Assays, which uses a polyprotic dye, Coomassie Brilliant Blue G-250.

5. Activity Assay:
Incubation of crude lysates with and without Doc toxin, in the presence of Radioactive γ ^{32}P-ATP followed by a TCA precipitation to remove any contaminants and residual ATP. Proteins were separated by size by SDS-PAGE (sodium docecyl sulfate polyacrylamide gel electrophoresis, stained with Coomassie Blue and then autoradiographed to detect any radiolabeled proteins.

Results:

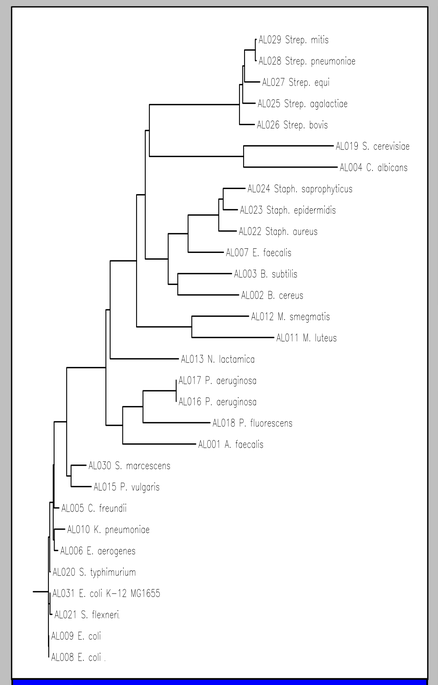

Figure 2 :
Phylogenetic tree of EF-Tu sequences based on Clustal alignment (5,6,7,8,9)

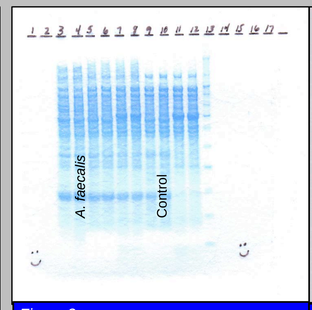

Figure 3a :
Coomassie-stained SDS-PAGE gel of *A. faecalis* and controls with and without Doc. Even lanes Doc+, odd lanes Doc-

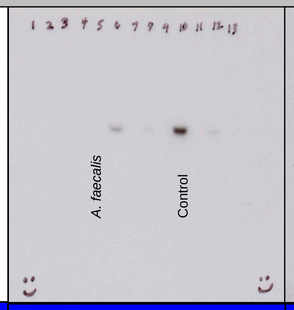

Figure 3b :
Autoradiography results of SDS-PAGE gel from figure 3a after a 3-day exposure

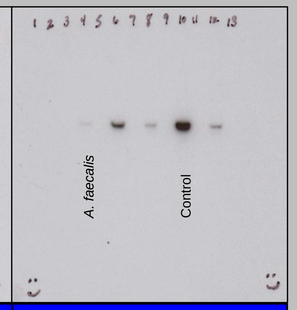

Figure 3c :
Autoradiography results of SDS-PAGE gel from Figure 3a after a 6-day exposure

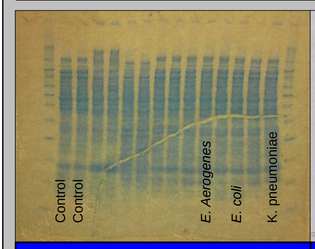

Figure 4a :
Coomassie-stained SDS-PAGE gel of *A. faecalis, B. cereus, C. freundii, E. aerogenes, E. coli, K. pneumoniae and controls* (lanes 1&2 Doc+) *with and without Doc. Even lanes Doc+, odd lanes Doc-*

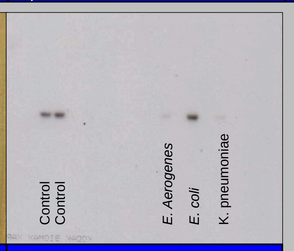

Figure 4b :
Autoradiograph of SDS-PAGE gel in figure 4a after a 3-day exposure (further exposure may result in positive results in other strains)

Future direction:

The results of this study indicate that, in some of the strains, partial modification of a target occurred. This activity appears to be on proteins with a molecular weight similar to that of the believed target. Depending on the species, varying degrees of target modification was observed. This variation could be due to less available target or possible inhibitory factors. Analysis of Doc activity on the remaining strains will be required to determine which strains should be looked into further. It may also be possible that a correlation between the amount of activity and the protein sequences exists. Detailed analysis of the aligned sequences will be required to confirm this correlation. So far, it appears that the range of the Doc toxin may in fact reach beyond the bounds of *Escherichia coli.*