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**Mapping the Toxic and Regulatory Domains of
Doc of the P1 Plasmid Addiction Operon**

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The addiction operon of the P1 bacteriophage encodes a toxin, Doc, and its antidote, Phd, under the control of a single promoter. This system kills all segregants from an infected cell that are cured of the plasmid in order to ensure its continuance in the population. Several *doc* mutations previously described were shown to retain their regulatory properties but lose their toxicity. In this study we used selective conditions to isolate non-toxic mutants and then assayed their regulatory properties by measuring transcription of the *lacZ* reporter gene. Twenty-five non-toxic mutants were isolated, confirmed, and tested for regulatory activity. These mutants are presently being sequenced in hopes that the sequence information they yield will saturate the areas in and around the residues previously reported to be necessary for toxicity and thus allow us to create a map of the domains of Doc both necessary and sufficient for toxic and regulatory activity.

There are many weapons in the plasmid's varied armory that help it fool the host's defenses and insert itself into the bacterium such that it can replicate to infect other cells. However, the competition for hosts can be fierce, so plasmids have developed means by which to help ensure their success. Among the most interesting of these molecular tools are the addiction operons, which are discrete genetic elements that function to increase the chance of vertical transmission of plasmid copies to each daughter cell during bacterial replication and segregation. Some systems encode both the enzyme necessary for the production of an antibiotic and another protein that provides protective measures against the antibiotic such that the bacteria carrying the plasmid creates its own selective advantage. (4) Bacteria lacking the plasmid are killed by the antibiotic produced by infected cells, which are protected from its affects by one or more other gene products from the same plasmid. This type of system may be treated as an addiction operon because cells that are cured of the plasmid or are segregated from the parent cell without receiving it are killed by accumulated antibiotic in the environment.

A far more elegant type of system does, however, exist in plasmids and operates entirely within the confines of the individual cell. Obviously, such a system can confer no selective advantage to the cells unless it encodes proteins or genetic elements that operate in some way outside the realm of plasmid

addiction since it uses up some of the cells resources but does not encode a secreted product. This does not necessarily mean that the addiction operon and the proteins it encodes must always be harmful; indeed, Jensen and Gerdes have speculated that several known addiction systems must have some other function or activity since their toxic aspect only results in something like a ten-fold increase in plasmid maintenance whereas other mechanisms-- most notably the *sop* of F partition system—easily yield a 1,000-fold increase in plasmid maintenance. (4) Nonetheless, the addiction operon is a recurring motif that we find in quite a few plasmids. Generally, the antidote and toxin are located on the same promoter. The antidote is always located upstream of the toxin and is expressed in greater amounts. (4) Also common to all known systems is the presence of proteases that degrade the antidote at a much greater rate than the toxin is degraded so that if the plasmid is lost and production of new antidote ceases, the toxin binds its target and the cell dies. This system is seen with only slight variation in *ccd* of F, *parD/pem* of R1/R100, *parDE* of RP4/RK2, and *phd/doc* of P1. (4)

The addiction operon comprised of *phd*, *doc*, and their promoter sequence on the P1 plasmid were the topic of this study. They follow the general pattern of proteic plasmid stabilization systems in that they encode Doc, a toxin, and Phd, a less stable antidote, that is degraded more rapidly than the toxin by ClpPX, an ATP-dependent serine protease specific to Phd and a very select few other substrates. (4,5,6) Following the conventions that other addiction operons seem to obey, *phd* and *doc* are situated with *phd* nearest the promoter and *doc* only a short distance downstream from its 'stop' codon. As in the other types, loss of plasmid results in the killing of the cell after segregation, thus explaining the P1 plasmid's loss rate of only 10^{-5} in *Escherichia coli*. (8) It has been claimed in a recent paper that Doc does not kill by directly binding a target protein in the cell, but rather inhibits the translation of *mazE*, part of a proposed chromosomal *E. coli* apoptosis system very similar to the Phd-Doc addiction operon. (It is not yet know what is the target of MazF-- the system's toxin-- but it is inactivated when bound to MazE, which is cleaved by ClpPA, a protease from the same family that ClpPX hails from. Necessarily, once free Doc has interrupted translation of *mazE*, MazE concentrations begin to fall due to proteolysis of the less stable antidote. MazE also represses translation of MazF, so reduction of the concentration of MazE results in more expression of the toxin, which ultimately overwhelms the available antidote and either kills the cell directly or sets off a chain of events or cascade that accomplishes the same effect. (3) These findings, however, are somewhat

questionable given that in another paper the same researchers link three dissimilar antibiotics to the same pathway by the same methodology.

As in the MazEF system, Phd represses translation of *doc* in a similar manner. (8) However, according to two separate groups, Phd and Doc exhibit a synergistic effect in the repression of their own operon. Phd alone repressed the operon 10-fold, but Phd and Doc together repressed it 40-fold to indicate a four-fold enhancement of Phd's level of repression alone. (2,7) In their study, Magnuson and Yarmolinsky published results characterizing the toxicity and regulatory activity inherent to ten separate and distinct strains expressing different mutants of Doc. Four of the ten mutations (shown below in figure 1) maintained their ability to regulate and lost toxicity, but the remaining six lost both regulatory and toxic activity.

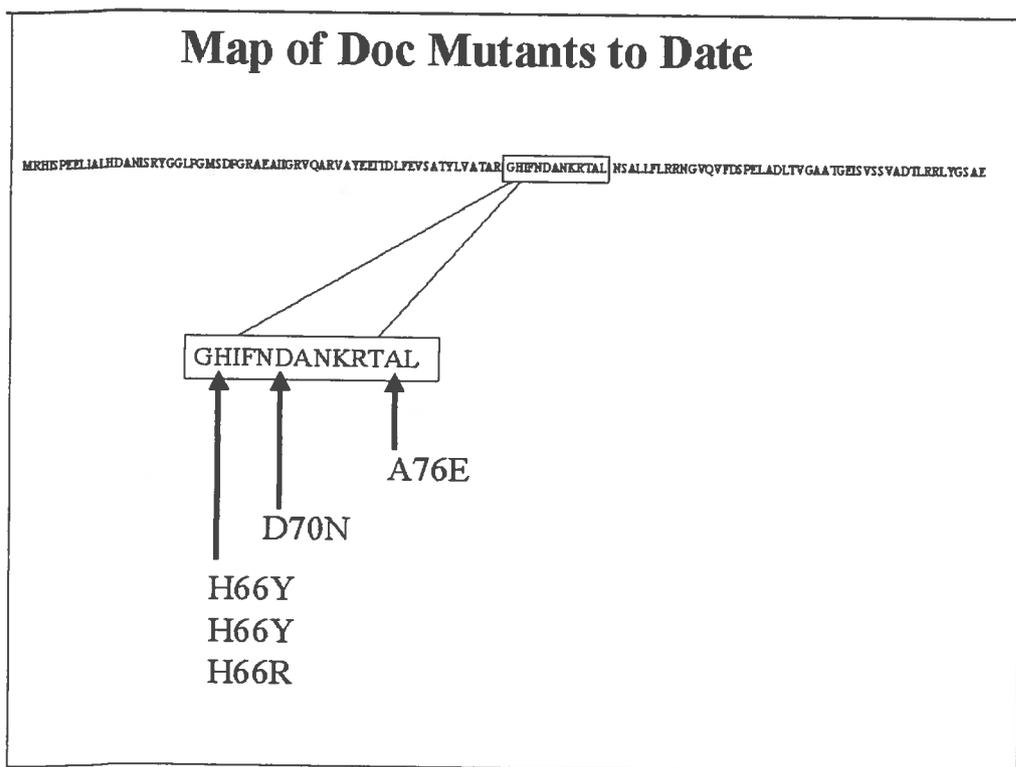


Figure 1

It was the goal of this project to provide further characterization of mutations that knocked out toxicity but did not have a significant effect on regulation. These mutations were to be mapped to the protein so that we can demonstrate the domains necessary and sufficient for toxicity and those also required for regulatory activity.

MATERIALS AND METHODS

Media. Cells were grown in Luria Broth (LB), on LB agar plates, or on Minimal Salts (MinA) agar plates with leucine, lactose, and vitamin B6 added. Cells were induced where indicated with either lactose or isopropyl- β -D-thiogalactopyranoside (IPTG.) Plasmids were selected for and maintained by use of ampicillin, spectinomycin, and kanamycin where necessary.

Strains. The experimental vectors BR6488 and BR 6472 carried *doc* on the heterologous lactose-inducible pTAC promoter along with the *lacZ* reporter. The control strains BR6487 and BR6486 lacked *doc*, but carried *lacZ* on the same promoter as the vectors in order to allow a comparison of transcriptional regulation by means of the β -galactosidase assay (discussed below.) All vector and control strains had *phd* integrated into the chromosome by λ .

Isolation of non-toxic *doc* mutants. Cells were grown either on MinA Lactose or LB IPTG agar plates for induction and selection as well as on LB agar plates as control. Single colonies were taken from the selective plates and were twice colony purified. The purified colony isolates were then grown overnight in LB on a roller rack and frozen away in 25% glycerol.

Confirmation that mutations were on *doc* in the plasmid. Strains were grown overnight in LB from the previously described freezer stocks. Cells were then pelleted, broken with an alkaline lysis method, and their plasmids were extracted and purified. Isolated plasmid DNA from each strain of mutant was transformed into competent cells and plated on both selective and non-selective media.

Quantification of regulation of transcription. β -Galactosidase assays were performed according to Miller in order to measure regulation of the promoter. The relative activity of each mutant and control strain was calculated by dividing the activity of β -galactosidase by the absorbance at 600nm. (9)

Extraction and affinity purification of plasmid DNA. Strains were grown 14 hours in LB at 30°C and then pelleted by centrifugation. DNA was extracted by use of the Wizard Miniprep System. (Promega)

Sequencing of plasmid DNA. Primers were designed based on the known plasmid constructs used. Samples are currently being sequenced. (Research Genetics)

RESULTS

Isolation of non-toxic mutants of *doc*. Twenty-five mutants were initially isolated, purified, and tested. The majority of mutants exhibited no toxicity whatsoever, but a few strains seemed to retain some toxicity. Comparison of growth under selective and non-selective conditions indicated a mutation frequency of approximately 1.4×10^{-7} mutants per colony forming unit (CFU).

Confirmation that mutations were on *doc*. All twenty-five mutants were shown to be located on the plasmid by isolation, purification, and subsequent transformation of the plasmid DNA of each strain into competent cells. Each transformed strain gained the appropriate antibiotic resistance and maintained its ability to grow in the presence of the inducers lactose or IPTG.

Quantification of regulation. β -Galactosidase assays showed that while most mutations did have some small negative effect on regulation, a few others acted nearly identical to the wild-type and therefore are presumed to affect regulation very little or not at all. Figure 2 shows the relative degree of repression of the promoter with respect to the wild-type.

β-Galactosidase Assay Results		
Isolate	Miller Units	Number of Folds Repression Relative to BR 6487
001	4200	5
002	3400	6.2
003	3900	5.4
004	4000	5.3
005	3900	5.4
006	3900	5.4
007	3900	5.4
008	4000	5.3
009	4200	5
010	4500	4.7
011	4000	5.3
012	3900	5.4
013	3800	5.5
014	3600	5.8
015	4000	5.3
BR6488	4000	5.3
BR6487	21000	--

Figure 2

Sequencing and mapping of the mutations generated. Sequencing of the discussed mutants is currently underway and is estimated to be completed before September 2001. The results of sequencing and mapping the mutations will be presented at a later date in a different forum.

DISCUSSION

We have shown that mutations on the Doc toxin of the P1 bacteriophage addiction operon may be selected for, tested, confirmed, and sequenced in large groups. This method will allow us to saturate the data points already known and discover new point mutations that delete the toxic activity of Doc. Furthermore, we have shown that the regulatory activity of a given mutant can be compared to that of the wild-type protein so that the affect of the mutation on auto-regulation of the system may be demonstrated.

We anticipate that the sequence data resulting from our work will show that toxicity is dependent upon a conserved and continuous region centered around the previously demonstrated residues necessary for post-segregational killing. This region should comprise some or all of the amino acids in the 60 to 80 position on the protein. Moreover, we anticipate that our data will help to identify the regions necessary for regulation as well as toxicity by revealing other mutations that affect the protein's regulatory properties as well as its ability to kill cells. These full results and sequence data will be published in a peer-reviewed journal at a later date.

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