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A New Selectable Marker System for Genetic Studies of Bacteria: Final Report

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A New Selectable Marker System for Genetic Studies of Bacteria

Final Report

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Abstract:
Genetic manipulations in bacteria currently rely on the introduction of antibiotic resistance genes into a bacterial strain; for those organisms that will be used for commercial or industrial applications, the genetic cassette encoding the antibiotic resistance is sometimes removed after selection. It is clear that if alternative technologies could obviate the need to introduce antibiotic resistance into bacteria, they would most certainly become a standard tool in molecular microbiology for commercial, industrial as well as research applications. Here, we present the development of a novel genetic engineering technology based on toxin-antitoxin systems to modify bacterial genomes without the use of antibiotic resistance in the mutagenesis process.

Introduction
We are working to develop a novel genetic system that will allow scientists to manipulate bacterial genomes completely without the use of antibiotics. Currently, antibiotic resistance cassettes are mandatory for replacing or disrupting genes in bacteria. These cassettes confer resistance to a particular antibiotic, which is used to easily select those bacteria that have the correct mutation. All current methods of gene disruption use antibiotics at some point during the process, even if the final product contains no antibiotic resistance.

Our method employs a naturally occurring bacterial system encoding a toxin and antitoxin that take the place of the antibiotic and antibiotic resistance cassette respectively. This toxin/antitoxin system is harmless to all organisms except for bacteria and provides a powerful means of selection that can replace antibiotics. We have adapted this system to be a controlled inducable system that will allow for the selection of genes that have been disrupted. In our system the bacterial gene to be disrupted is replaced by the antitoxin gene on the chromosome. Then a plasmid containing the toxin is introduced into this bacterial strain. The toxin will kill any bacteria that do not contain the antitoxin leaving only those bacteria that have a disrupted gene.

Toxin-antitoxin systems, naturally encoded on bacterial plasmids and chromosomes, can be used as a replacement for antibiotic-based selection of genetically modified bacteria. The advantage of this method over other non-antibiotic genetic engineering strategies (e.g. auxotrophy complementation or heavy metal resistance) is that the toxin, in many respects, acts similarly to many antibiotics but is not used in clinical applications, therefore will not contribute to increasing antibiotic resistance and or compete with therapeutic countermeasures. In addition, prior modification of the target cells, or prior cloning and characterization of bacterial target genes does not need to be repeated for each target organism.

We have developed a delivery system whereby the toxin (or a modified version of the natural toxin) and antitoxin are encoded on separate plasmids under tight regulation so that they can be introduced when selection becomes necessary. This system can be adapted for maintenance of plasmids, gene deletion, production of antibiotic free products and other molecular manipulations where antibiotics are detrimental to the process or disallowed by NIH biosafety guidelines. This system acts as a “poison/antidote” system whereby bacteria need to produce

antitoxin while toxin is present to survive. In this sense the toxin acts as the antibiotic and the antitoxin acts as the antibiotic resistance gene.

As an example, we have adapted the zeta/epsilon toxin-antitoxin system for use as an alternative to antibiotic selection. Here, the structural gene coding for zeta toxin or a modification of the zeta toxin is placed under the control of tightly regulated promoter in a plasmid that does not have an antibiotic resistance gene for selecting its presence in cells, but it carries a gene coding for a fluorescent protein. This plasmid is introduced by transformation into the strain to be mutated (Fig. 1 a-c). We have previously demonstrated that a colony carrying the fluorescence producing gene, i.e. the plasmid, can be selected without the use of an antibiotic. A DNA fragment including the epsilon antitoxin gene (constitutive) flanked by fragments of the gene to be mutated is generated by PCR or other methods, or a suicide plasmid including a fragment with these characteristics is generated and introduced into the strain to be mutated by chemical transformation or electroporation (Fig. 1 step 2). Of those cells that have received the transforming DNA a percentage will undergo a double crossover by homologous recombination generating a mutation in the gene of interest, which will be interrupted by the antitoxin gene (Fig. 1e). At this stage the there will be three main cell populations: a) cells that did not receive any transforming DNA, b) cells that received transforming DNA but will lose it before homologous recombination took place, these cells will be rapidly converted to cells identical to those "a", and c) cells in which a double crossover leads to loss of the intact gene and acquisition of a mutated gene interrupted by the epsilon antitoxin gene (Fig. 1e). To select the desired cells (cells in which a double crossover leads to loss of the intact gene and acquisition of a mutated gene interrupted by the epsilon antitoxin gene), the zeta toxin gene is turned on by addition of the inducer (Fig. 1 step 3). This procedure will induce death of all those cells that do not carry a copy of the epsilon antitoxin gene (Fig. 1f). This will provide a guaranteed elimination of all bacteria that are not successfully mutated with the antitoxin. This system is highly adaptable and can be used in many types of bacteria.

Antibiotic-free selection of genetically modified select agents

Our primary goal is to develop antibiotic-free selection for genetically altered select agent pathogens. We are adapting the toxin-antitoxin system to enable gene replacement in select agent pathogens since the NIH restrictions introducing antibiotic resistance into select agent pathogens have hindered research with select agent pathogens. Below are examples of efforts to adapt the toxin/antitoxin system for use in *Yersinia pestis*.

Main approach

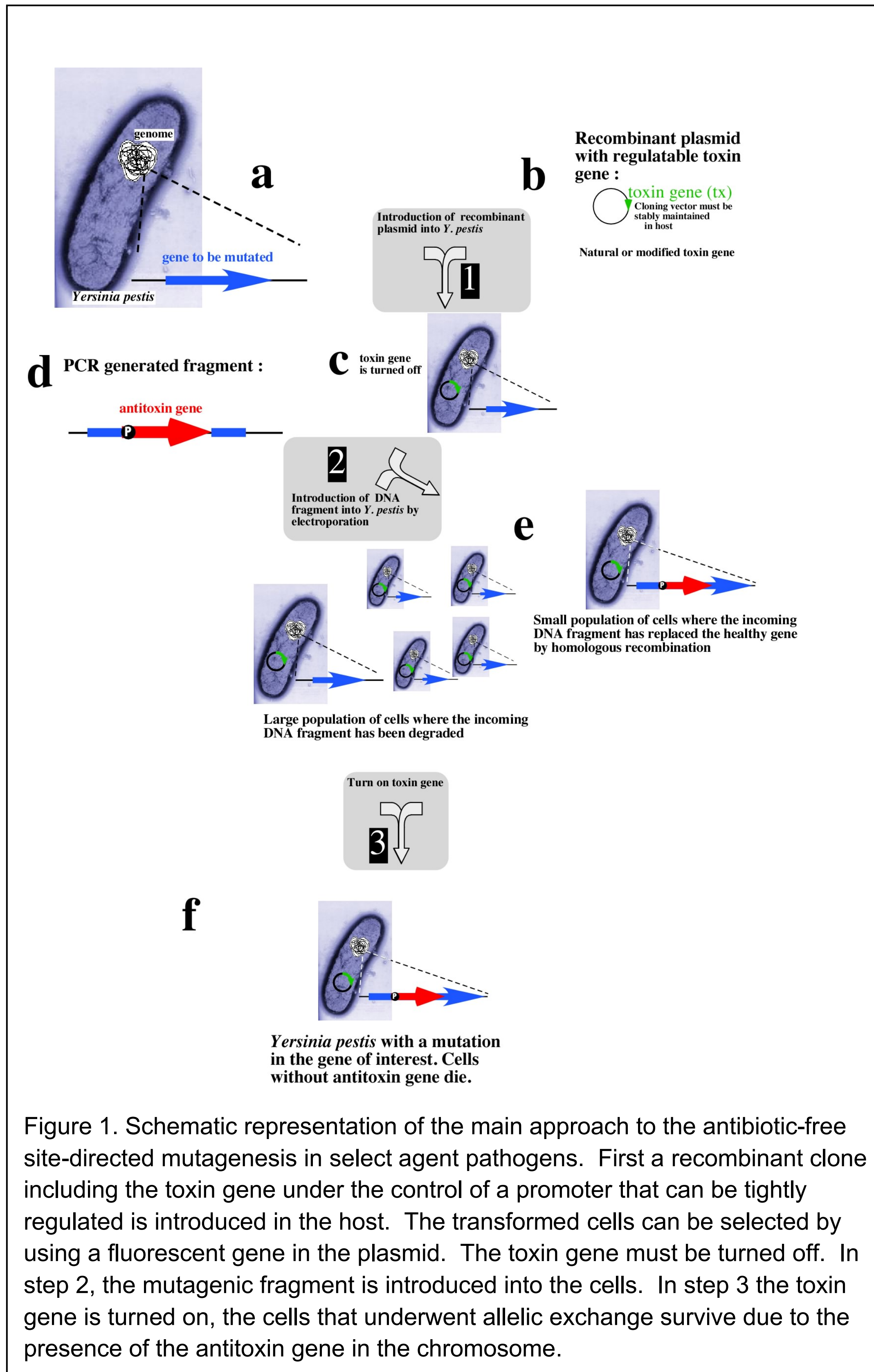

Figure 1. Schematic representation of the main approach to the antibiotic-free site-directed mutagenesis in select agent pathogens. First a recombinant clone including the toxin gene under the control of a promoter that can be tightly regulated is introduced in the host. The transformed cells can be selected by using a fluorescent gene in the plasmid. The toxin gene must be turned off. In step 2, the mutagenic fragment is introduced into the cells. In step 3 the toxin gene is turned on, the cells that underwent allelic exchange survive due to the presence of the antitoxin gene in the chromosome.

i. *Generation of a strain harboring a plasmid including a regulated gene coding for the zeta toxin or a derivative of this toxin.*

A plasmid including a fluorescent gene for selection and the toxin gene under the control of a tightly regulated promoter is introduced into *Y. pestis* by chemical transformation of electroporation (Fig. 1). Simple visual examination of the colonies provided selection of the transformed cells based on fluorescence.

ii. *Generation of a DNA region consisting of a functional antitoxin gene flanked by sequences of the gene to be mutated.*

We have targeted several *Y. pestis* genes with known and assayable phenotypes for initial proof-of-principle antibiotic-free gene deletion studies (Fig 1a). These include the periplasmic nitrate reductase gene napA, which can be assayed colorimetrically based on methyl viologen reduction, and the hmsS gene encoded in yersiniabactin iron uptake locus, which can be assasyed colorimetrically based on uptake of a red dye. A linear DNA fragment consisting of the antitoxin gene flanked by fragments of the target gene are generated by PCR using standard molecular methods (Fig. 1d). This DNA fragment is introduced into *Y. pestis* that carry the zeta toxin encoding plasmid by chemical transformation or electroporation, a routine transformation procedure to permit uptake of foreign DNA and allelic replacement in the chromosome based on homologous recombination (Fig. 1, step 2). As a consequence two populations of *Y. pestis* are generated: a) a large majority of the cells are identical to the original strain transformed by the toxin gene-containing plasmid because the incoming linear DNA fragment (or the suicide plasmid carrying the fragment) is degraded before recombination has taken place or no DNA was taken up during the electroporation (Fig. 1e), and b) a small minority of the cells will have undergone allelic exchange by homologous recombination before the incoming DNA was degraded. These latter cells (targeted knockout strains) will have a mutation in the gene of interest (Fig. 1e) while the wild-type gene will be lost to degradation. Because of the design of our linear DNA fragment, these cells also incorporated a copy of the functional antitoxin gene (see Fig. 1e).

iii. *Elimination of cells that did not undergo allelic exchange via homologous recombination (ie. selection for engineered recombinant clones).*

To select the *Y. pestis* cells harboring the mutated target gene and functional antitoxin gene, the parental wild type cells must be eliminated from the mix (Fig. 1 step 3). This is achieved by inducing expression of the toxin gene by adding the appropriate inducer to the culture medium. Only those cells that harbor a functional antitoxin gene will survive the action of the toxin. All those cells where the mutagenic DNA fragment was lost will not survive (Fig. 1f).

Alternate strategies

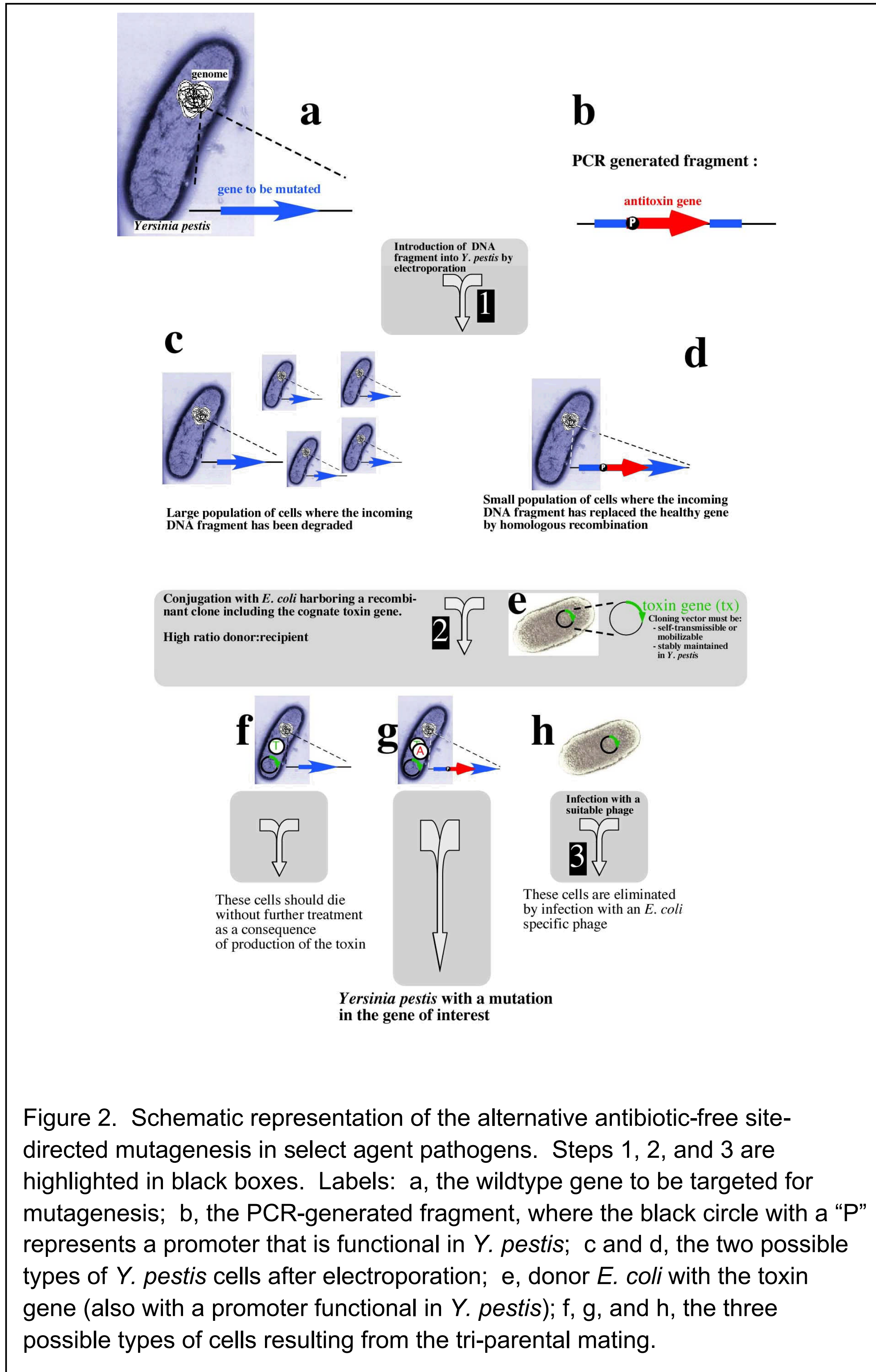

Figure 2. Schematic representation of the alternative antibiotic-free site-directed mutagenesis in select agent pathogens. Steps 1, 2, and 3 are highlighted in black boxes. Labels: a, the wildtype gene to be targeted for mutagenesis; b, the PCR-generated fragment, where the black circle with a “P” represents a promoter that is functional in *Y. pestis*; c and d, the two possible types of *Y. pestis* cells after electroporation; e, donor *E. coli* with the toxin gene (also with a promoter functional in *Y. pestis*); f, g, and h, the three possible types of cells resulting from the tri-parental mating.

An alternative method consists of introducing the mutagenic DNA before introducing the toxin gene.

i. *Generation of a DNA region consisting of a functional antitoxin gene flanked by sequences of the gene to be mutated.*

A linear DNA fragment consisting of the antitoxin gene flanked by fragments of the target gene is generated by PCR using standard molecular methods (Fig. 2). This DNA fragment is introduced into *Y. pestis* by electroporation to permit uptake of foreign DNA and allelic replacement in the chromosome based on homologous recombination (Fig. 2, step 1). As a consequence two populations of *Y. pestis* will are generated: a) a large majority of the cells will be identical to the original strain because the incoming linear DNA fragment is degraded before recombination has taken place or no DNA was taken up during the electroporation (Fig. 2c, and b) a small minority of the cells will have undergone allelic exchange by homologous recombination before the incoming DNA is degraded. These latter cells (targeted knockout strains) have a mutation in the gene of interest (Fig. 2d) while the wild-type gene is lost to degradation. Because of the design of our linear DNA fragment, these cells have also incorporated a copy of the functional antitoxin gene (see Fig. 2).

ii. *Elimination of cells that did not undergo allelic exchange via homologous recombination (ie. selection for engineered recombinant clones).*

To select the *Y. pestis* cells harboring the mutated target gene and functional antitoxin gene, the parental wild type cells must be eliminated from the mix. This will be achieved using a recombinant plasmid that includes a functional origin of replication together with a gene encoding the functional cognate toxin. This recombinant plasmid is introduced into *Y. pestis* cells by conjugation with a suitable *Escherichia coli* donor (Fig. 2e). Tri-parental matings is used to mobilize recombinant plasmids into a variety of recipient bacteria. We use RK2 vector derivatives such as pVK102 that have a wide host range and are easily mobilizable with helper plasmids like pRK2073. The ratio of donor to recipient cell must be empirically determined to ensure the highest possible efficiency of transfer of the plasmid into target bacterial cells (Fig. 2, step 2); this generally approaches 100% transfer into recipient cells. After conjugation takes place, three populations are expected in the mix: a) wild type *Y. pestis* carrying the plasmid (Fig. 2f), b) mutant *Y. pestis* carrying the plasmid (Fig. 2g), and c) donor *E. coli* (Fig. 2h) carrying the plasmid. Recipient *Y. pestis* cells that did not engage in the conjugation, though unlikely, may remain in the mix. Wild type *Y. pestis* carrying the plasmid die as a consequence of the production of the plasmid-encoded toxin. Donor *E. coli* cells are eliminated from the mix by either utilizing a strain of *E. coli* that requires a specified amino acid or vitamin, by infecting the mix with an *E. coli*-specific bacteriophage (Fig. 2, step 3), or by plating the mating mix in the presence of an antibiotic to which *Y. pestis* has higher intrinsic resistance.

Simultaneous introduction of antitoxin with inducible toxin (third approach)

Another permutation of our system is to introduce a mutation in the genome containing by allelic replacement with the antitoxin. This strain contains an inducible toxin on a plasmid maintained without the use of antibiotics that controls the expression of the zeta toxin with the pBAD

promoter. Once the gene replacement has taken place, toxin production is induced with the addition of arabinose and selection for the antitoxin gene replacement occurs. Cells that did not recombine the antitoxin are eliminated, leaving only those that have undergone successful recombination (Fig. 2).

Results

First proof of principle experiments using zeta-toxin/antitoxin for selection in *E. coli* and attenuated *Y. pestis*

We took a multipronged approach, starting with proof-of-principle experiments introducing zeta-toxin/antitoxin into *E. coli* and attenulated strains of *Y. pestis*. We first cloned the zeta-toxin gene into an expression plasmid with an IPTG inducible promoter and a kanamycin resistance selection maker. We cloned the anti-toxin into a compatible expression plasmid with an arabinose promoter and a kanamycin resistance selection marker. Bacteria were transformed with both plasmids and transformed bacteria were selected with kanamycin. Zeta-toxin induction of with IPTG resulted in zero colonies on agar, kanamycin, IPTG plates. Zeta-toxin induction in combination with anti-toxin induction with arabinose resulted in a somewhat reduced colony count compared to controls (control plates are kanamycin in agar without IPTG or arabinose). These experiments showed that zeta-toxin kills *E. coli* and attenuated *Y. pestis* and that antitoxin rescues the bacteria from the toxin.

Proof of principle experiments to obtain precursor strains transformed with zeta-toxin expression plasmid

Having demonstrated the first proof of principle that zeta-toxin kills *E. coli* and attenuated *Y. pestis* and that antitoxin rescues the bacteria from the toxin, we attempted the first steps in our main approach to antibiotic-free selection of genetically modified select agent pathogens. We first tried to transform *E. coli* and attenuated *Y. pestis* with an expression plasmid encoding the zeta-toxin under the control of an IPTG promoter. We were unable to obtain any transformants, either because the plasmid was not stable or the promoter was leaky. We then transformed *E. coli* with a plasmid encoding an ampicillin selection marker and zeta-toxin under the control of an arabinose promoter, which is supposed to be a more tightly controlled promoter. Even under ampicillin selection and no induction no transformants were obtained—suggesting leaky expression of zeta-toxin, even under the tight control of the arabinose promoter, was killing the cells. The unanticipated potency of the zeta-toxin, along with the leaky expression, leading to cell death in the absence of anti-toxin caused us to pursue our alternative strategy.

Proof of principle experiments to obtain precursor strains transformed with antitoxin and recombined into the genome

As a first proof of principle that we could disrupt a chromosomal segment of the bacterial genome with anti-toxin from linear DNA we constructed a plasmid that contained a contiguous stretch of DNA encoding an arabinose promoter, a kanamycin resistance gene, red fluorescent protein, and anti-toxin. This contiguous stretch of DNA was flanked on both sides by sequence that flanks the chromosomal region to be disrupted in the bacterial genome. The flanking sequence enables homologous recombination such that the kanamycin resistance, red fluorescent protein, anti-toxin cassette replaces the genomic segment between the flanking

sequences. Having constructed the plasmid we amplified a linear segment of DNA with the flanking sequences and the cassette using PCR. The PCR product, a linear piece of DNA, was then transformed into *E. coli*. We were able to select for viable colonies using kanamycin selection—suggesting that recombination had been achieved and that kanamycin resistance had been introduced into the bacterial genome. Replating the viable mutant strains in the absence of kanamycin resulted in a large number of viable colonies, but only a few were red—suggesting that few of the viable kanamycin resistant strains inherited the intact cassette, but a some did. Colony PCR studies on the strains expressing red fluorescent protein confirmed that a subset of the strains encoding red fluorescent protein had inserts in their genomes of the expected size and therefore likely had the anti-toxin encoded in the genome as well. These studies showed that a linear piece of DNA could be targeted to the bacterial genome, but also showed that the cassette was not entirely stable and only a small subset of transformants contained the expected genetic alteration. Despite extensive effort we were not able to obtain similar results in attenuated *Y. pestis*, suggesting that the transformed cassette was even less stable in *Y. pestis,* perhaps because the *Y. pestis* genome itself undergoes rapid rearrangement.

Attempts to select antitoxin containing strains by introduction of zeta-toxin expression plasmid

Having obtained genetically altered strains of *E. coli* that encode an antitoxin on the chromosome, we next attempted to transform these *E. coli* strains with expression plasmid encoding the IPTG controlled zeta-toxin. The expectation was that we would be able to obtain strains of *E. coli* with chromosomally encoded anti-toxin and plasmid encoded toxin and that we would be able to induce cell death by inducing the expression of the toxin or prevent cell death by inducing the expression of anti-toxin. We were not able to obtain any viable transformants with the toxin encoding plasmid—suggesting that there was leaky expression of the zeta-toxin and that the low levels of zeta-toxin, even uninduced, overwhelmed the anti-toxin. We hypothesize that the copy number of toxin molecules expressed, even uninduced, exceeds the copy number of anti-toxin molecules produced, even induced, and therefore the toxin overwhelms the anti-toxin and lead to cytotoxicity.

Use of attenuated toxin

Given the lack of success transforming bacteria with zeta-toxin encoding plasmid, except in combination with plasmid encoded anti-toxin, we developed expression plasmids encoding a recently reported attenuated zeta-toxin. We obtained a plasmid encoding the attenuated toxin and from a collaborator and subcloned the attenuated toxin into an expression plasmid with an arabinose promoter and a kanamycin resistance cassette. We again created strains with two plasmids, one encoding the anti-toxin and one encoding the attenuated toxin. We were further able to show that induction of the attenuated toxin absent the antitoxin resulted in cell death and that co-induction of antitoxin and attenuated toxin lead to viable colonies. We were also able to transform bacteria with the plasmid encoding attenuated toxin alone and obtain viable colonies without induction of the attenuated toxin.

Attempts to select antitoxin containing strains by introduction of attenuated zeta-toxin expression plasmid

Having obtained a plasmid that was lethal to bacteria only upon induction we transformed previously developed stains that putatively contained chromosomally encoded anti-toxin with the attenuated zeta-toxin encoding plasmid. Ultimately we were not able to select for a strain that contained both chromosomally encoded antitoxin and plasmid encoded attenuated toxin—presumably due to genetic instability and plasmid instability.

Future needs

In order to achieve the intended goals of this project, it is apparent now that a range of toxin-antitoxin systems should be tried and all derived strains should be sequenced, at least in proximity to any chromosomal alterations and around expressed gene sequences in plasmids. Other promoter systems might be tried or other selective growth media that might provide tighter control of toxin expression. Glucose free minimal defined media might be used for example to better control the activation of the IPTG promoter. Other approaches to homologous recombination might also be tried, such as the use of a suicide plasmid instead of linear DNA. Helper recombinases, such as red recombinase, might also be useful.