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THE CREATION OF A KNOCK-OUT MUTATION OF A GAMMA AND BETA CARBONIC ANHYDRASE IN DEINOCOCCUS RADIODURANS AND THE CONSTRUCTION OF EXPRESSION PLASMIDS

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THE CREATION OF A KNOCK-OUT MUTATION OF A GAMMA AND BETA CARBONIC ANHYDRASE IN *DEINOCOCCUS RADIODURANS* AND THE CONSTRUCTION OF EXPRESSION PLASMIDS

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Abstract

Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyzes the reaction that interconverts carbon dioxide into bicarbonate. There are three major classes: α-, β-, and γ-CAs. It was once thought that different organisms only had one form of the enzyme or another: animals only had α-CAs, plants only had β-CAs, and bacteria could have either β- or γ-class enzymes. But this system is slowly breaking down as some CAs have been found in organisms that should not have had that class of CA. For example, the higher plant *Arabidopsis thaliana* contains three γ-CA genes as well as several α- and β-CA genes. Several prokaryotes also contain members of each CA class, and many contain at least two of them.

In this experiment, two carbonic anhydrases were found in *Deinococcus radiodurans*. Examination of the *D. radiodurans* DNA database revealed two potential CA-encoding genes, one of the β class, the other, the γ class. At least one, and possibly both, of these CAs is required for growth.

In order to determine which *D. radiodurans* CA is required for growth, a plasmid was designed to knock-out each CA gene. Two plasmids, one for each CA gene, were constructed from TufA promoter and the chloramphenicol-resistance gene of pGTC101 (Earl *et al.*, 2002), pBluescriptII KS, and two pieces of the gene. The 5' and 3' halves of each gene were amplified separately and then cloned into either side of the TufA promoter and the chloramphenicol-resistance gene that had been cloned into pBluescriptII KS. Each plasmid was linearized and transformed into *D. radiodurans*. These were then used to knock-out the corresponding CA genes in the *D. radiodurans* genome.

Literature Review

Carbonic Anhydrase

Carbonic anhydrase (CA), which catalyzes the reversible hydration of CO_2, is found in nearly every type of organism. There are three classes of CA, designated α, β, and γ. Although all carbonic anhydrases catalyze the same reaction, the structures and sequences are very different for each class.

Originally it was thought that the α-class of carbonic anhydrase was found in animals and some alga, the β-CAs were found in plants and eubacteria, and the γ-CAs in archaebacteria and eubacteria. The enzymes were classified according to their structures and what organisms they are found in. However, the older system of classification of the CAs is starting to break down. Classes of CA that would normally be found only in one type of organism are now being found in other organisms.

The first CA, an α-CA, was found in human erythrocytes about 70 years ago, and CA activity was discovered in plants shortly thereafter. The first γ-CA was found less than 10 years ago in the archaebacterium *Methanosarcina thermophila*. The β-class, the most abundant CA, is found in photosynthetic plants. Recently, however, the classes of CA that would not normally have been found in particular organisms are now being found in those organisms. All three classes have been found in *Arabidopsis thaliana* and an α-CA has been found in *Neisseria gonorrhoeae* (Björn *et al.*, 2000). Although all three classes have been found in prokaryotes, the most predominant classes are still the β and γ (Smith *et al.*, 1999)

In addition to catalyzing the interconversion of CO_2 and HCO_3^-, the CAs have other activity, such as ion transport and pH regulation. The γ-CA from the archaebacterium *M.*

thermophila converts "the methyl group of acetate, methanol, or methylated amines to methane" but has no acetlyase activity (Smith and Ferry, 1999). This CA may be a secretory protein found in the periplasmic space of *M. thermophila* as it contains what looks to be a signal peptide.

The structure of these enzymes, the β-class structure most recently discovered, are different for each class, and their sequences have little, if any, homology. The α-class are generally monomers, the β-class can be dimers, tetramers, hexamers, or octamers, showing that a dimer is probably a structural unit, and the γ-class are all trimers. The structural and sequence differences indicate that these enzymes evolved independently. The γ-class is thought to be the oldest at 3.0 and 4.5 billion years ago (Tripp *et al.*, 2001), and the β-class probably the next youngest (Smith *et al.*, 1999). The γ class is most interesting not only for its age, but also for its structure, since it contains the novel structure of a β-helix. The α- and β-classes have various arrangements of α-helices and β-strands (Liljas and Laurberg, 2000), the more common protein structures.

The α-class is mostly populated by mammalian CAs, while prokaryotes are not as widely represented yet. Crystal structures of some of the isozymes of the α-class have been done, showing the orientation of the important His residue (either "in," or towards the zinc molecule, or "out," or away from the zinc molecule). The wild-type protein His residue is usually in the "in" position (Tripp *et al.*, 2001).

The β-class, although not the oldest class evolutionarily, is the most diverse. They have now been found in eubacteria, archaebacteria, and yeast as well as plants, and might be used for CO_2 fixation when it is scarce. The enzymes in this class have been separated into two subclasses, the plant type and the Cab type. The plant subclass consists of two clades, one for

dicotyledonous plants and one for monocotyledonous plants. The Cab subclass contains five clades, all related to the Cab enzyme from *Methanobacterium thermoautotrophicum* (Tripp *et al.*, 2001).

The γ-class is composed of three identical monomers. It also contains the novel left-handed β-helix structure of three parallel sheets. The first γ-class enzyme was found in *M. thermophila* and is called Cam. When expressed in *E. coli*, the enzyme from *M. thermophila* contains zinc. However, other forms of the enzyme containing iron and cobalt show a greater CO_2 hydration rate than the zinc-containing enzyme (Tripp *et al.*, 2001).

Though their overall structure is diverse, the active sites of each class of enzymes are remarkably similar. The active site of the β-class enzyme is the mirror image of the α-class active site (Kimber and Pai, 2000), and the active site of the γ-class enzyme is virtually the same as the α-class active site. All of the enzymes contain at least one zinc atom at the active site, though the atom can be ligated to three histidines (α- and γ-class) or two cysteines and a histidine (β-class). As indicated above, it is possible that the γ-class enzyme Cam from *M. thermophila* can use a transition metal other than zinc (Tripp *et al.*, 2001).

Interestingly, a δ-class of carbonic anhydrase has been proposed. A potential monomeric CA has been found that has no sequence homology to the other three classes of CA, though its active site resembles those of α- and γ-CAs. This enzyme, called TWCA1 from *Thalassiosira weissflogii* (a marine diatom), could be a totally new class of CA enzyme (Tripp *et al.*, 2001), though it is possible that it could belong in the established classes, or not even be a CA at all. The information about the δ-class is still controversial.

The activity of carbonic anhydrase can be inhibited by nitrate, azide, and iodide (Smith and

Ferry, 1999), as well as sulfide and cyanide (Pocker and Sarkanen, 1978). These bind the zinc atom in the active site and prevent the action of the enzyme. Although these are inhibitors of all the classes of CA, the concentrations needed to inhibit vary with each class (Smith and Ferry, 1999). Because these inhibitors could all inhibit the activity of other enzymes in addition to CA, two specific inhibitors, 6-Ethoxyzolamide (EZ) and acetazolamide (AZ), were used in the following experiments. EZ is membrane permeable, while AZ is not. Therefore use of these inhibitors can indicate whether a CA is outside or inside the plasma membrane.

Knock-out Mutation

Knock-out mutation is the replacement of a normal gene with a mutated gene (or the complete removal of the normal gene). By knocking out a normal gene with a mutated gene, the *in vivo* function of a protein or other important aspects of the protein can be learned. For a knock-out mutation to work, a mutated DNA sequence must be made and then inserted into the organism's genome. This occurs by the hybridization of the mutated gene with the homologous normal genomic DNA. If the mutated gene contains a selectable marker, cells harboring the mutated gene can be selected. Because *D. radiodurans* has more than one chromosome, the mutants will have to be screened to make sure that they are homozygous for the mutation. Then by studying the effects of the mutated protein, its function can be ascertained (Lodish *et al.*, 2000).

Deinococcus radiodurans

D. radiodurans cells are strictly aerobic, spherical or rod-shaped bacteria that form

reddish colonies. The cells are easily transformable without prior treatment except the addition of calcium chloride to exponentially growing cells. DNA can then be added directly to the liquid culture, and the calcium chloride-treated cells take up the DNA. If the DNA is linear, it will then recombine with the homologous DNA on one or more of the chromosomes.

Materials and Methods

I. Inhibition of CA by EZ and AZ

In order to test if EZ or AZ will inhibit the CAs in *Deinococcus radiodurans*, whether the CAs are internal or external, and if the CAs are required for growth of *D. radiodurans*, an experiment was set up as follows. Six tubes were used: three tubes for *E. coli*, three for *D. radiodurans*. For each of the bacteria, one tube contained just TGY media, one contained TGY media plus 200μM AZ, and one contained TGY media plus 200μM EZ. The bacteria were then inoculated into the correct tubes and incubated for 48 hours at 30°C.

II. Construction of the plasmids pBGGCA and pBGBCA

1. Creation of a truncated pGTC101

All procedures were done according to the standard methods (Sambrook *et al.*, 1989). All restriction enzymes were purchased from New England Biolabs. Dr. Battista's lab furnished the purified plasmid pGTC101 (Earl *et al.*, 2002). Examination of the sequence of pGTC101 revealed a StuI site in the TufA promoter about 200bp upstream of the start codon for the chloramphenicol-resistance gene. Since the vector contained a StuI site upstream of the TufA promoter, we proposed to shorten the TufA promoter by about 1.8kb by digestion with StuI and then religating the plasmid.

The pGTC101 plasmid was transformed by electroporation into competent DH5α cells, and the bacteria were then spread on LB agar plates containing the antibiotic ampicillin. Individual colonies were selected off of the plates and inoculated into 3mL LB overnight tubes. The DNA was then harvested by the alkaline lysis method and digested with StuI. However, digestion with StuI only linearized the vector. After reexamining the sequence of pGTC101, we

found that one site was potentially methylated and could not be digested with StuI. Because of this, the plasmid was then transformed into the *dam⁻dcm⁻ E. coli* strain, GM2163. The cells were grown on ampicillin plates, colonies were picked, and the DNA harvested, as before. The plasmid was then digested with the StuI enzyme, truncating the promoter of the *tufI* gene. The digested DNA was run on a 1.2% low-melting agarose gel, the bands excised, and the ends of the plasmid ligated back together. This DNA was then transformed into DH5α and the cells grown on ampicillin plates. The DNA harvested from these cells was then digested with BglII and HindIII and subjected to electrophoresis to ensure that the StuI fragment was eliminated.

2. Creation of PCR inserts

Dr. Battista's lab supplied the *Deinococcus radiodurans* R1 genomic DNA for this experiment. All procedures were done according to standard methods (Sambrook *et al.*, 1989). PCR primers were manufactured by Operon Technologies. Eight primers were designed so that for each gene, there was a 5' end with an EcoRI site attached to the 5' end of the primer, a 3' middle (that is, the middle of the gene) with a PstI site attached to the 5' end of the primer, a 5' middle with a NotI site at the 5' end of the primer, and a 3' end with a SacI site at the 5' end of the primer. The primers were also designed to have a melting temperature of 64°C, not counting the restriction sites. In effect, these primers would amplify four DNA fragments; two for each of the two CA genes to be studied. Two pieces, one for the gamma-CA, one for the beta-CA, will have an EcoRI site at the 5' end and a PstI site at the 3' end. These pieces are the 5' halves of each CA gene. The second pieces have a NotI site at the 5' end and a SacI site at the 3' end. These pieces are the 3' halves of each CA gene. The 5' end primer for the gamma-CA is termed DRGCAEco (for *Deinococcus radiodurans* gamma-CA with the EcoRI site)

(GAATTCAGTAGGCGATGCCGCGCGA), the 3' middle primer is DRGCAPst (CTGCAGAGCCGGGGTCGGCGTGCA), the 5' middle primer is DRGCANot (GCGGCCGCCCTGCACGCTGCACGAGGA), and the 3' end primer is DRGCASac (GAGCTCAAGTTGTGGTCAGCCGCGCA). The beta-CAs have a similar naming scheme, DRBCAEco (GAATTCGTCATGGGGCGCAGGCTAG), DRBCAPst (CTGCAGCGTTGCCCGCCACCCGCA), DRBCANot (GCGGCCGCTGGTGGGCGAAGCGGCCT), and DRBCASac (GAGCTCCCCTTAGAGTGCGAGGTCGTC). The primers were paired, and the resulting gene fragments given the names DRGCAEco/Pst, DRGCANot/Sac, DRBCAEco/Pst, and DRBCANot/Sac. For clarification of the PCR procedure, see Figure 2.

Betaine and DMSO were used in the PCR reactions since *Deinococcus* DNA is G+C rich (Earl *et al*, 2002). For the PCR reaction, 1X PCR buffer, 1μL of genomic DNA, 100ng of each primer, 200μM dNTP mix, 13 μL of Betaine, 0.65 μL of DMSO, and 2.5 units of the YieldAce DNA polymerase (Stratagene) were used in a total reaction volume of 50μL. The parameters used for PCR were: 92°C for 2 minutes, 30 cycles of 95°C for 20 seconds, 64°C for 20 seconds, and 72°C for 1 minute, then 72°C for 7 minutes. This reaction amplified the DRGCANot/Sac, DRBCAEco/Pst, and DRBCANot/Sac fragments, but not the DRGCAEco/Pst fragment. Another program was used to obtain that gene fragment. The reaction components remained the same. However, the parameters used for PCR were 92°C for 2 minutes, 30 cycles of 95°C for 20 seconds, 62°C for 20 seconds, and 72°C for 1 minute, then 72°C for 7 minutes.

3. Ligation of PCR products into pGEM-T Easy

Electrophoresis of the products of the PCR yielded four ~400bp bands. These bands were

then cut out of a 1.2% low-melting agarose gel and ligated into pGEM-T Easy (Promega) following the manufacturer's instructions. The ligation was diluted and transformed into DH5α. The cells were grown on ampicillin plates with X-gal. White colonies were picked and inoculated into 3mL of LB media and grown overnight. The DNA harvested from these cells was digested with EcoRI and was subjected to electrophoresis on an agarose gel to confirm that the ligation and transformation had been successful. These products were saved for later use.

4. Construction of the pBluescriptII KS + pBC chloramphenicol cassette (pBlueBC)

Since pBC (from the truncated pGTC101 and containing a chloramphenicol cassette) needed to be digested with StuI again, it was transformed into the *dam*$^-$*dcm*$^-$ *E. coli* strain GM2163. The DNA was then digested with StuI and NotI, and the ~900bp insert was then ligated into pBluescriptII KS. This was then transformed into DH5α and the cells grown on LB agar plates. Colonies were picked and inoculated into 3mL of LB and grown overnight. The DNA was harvested and digested with PstI and NotI. The digested DNA was then subjected to electrophoresis on an agarose gel to confirm that the ligation had been successful. Two bands were found, one was about 900bp (the *tufI* promoter/chloramphenicol cassette from the modified pBC) and the other was around 2.9kb (the pBluescriptII KS vector). This plasmid will now be called pBlueBC.

5. Construction of the plasmids pBBGCA and pBBBCA

The DRGCAEco/Pst and DRBCAEco/Pst DNA fragments (the PCR fragments from above) were then excised out of pGEM-T Easy using EcoRI and PstI. The pBlueBC vector was also digested with these enzymes. All products were run on a 1.2% low-melting agarose gel and the bands excised out of the gel. Each piece, DRGCAEco/Pst and DRBCAEco/Pst, were then

ligated separately into the vector. These ligations were transformed into DH5α, and the cells grown on agar plates. Colonies were picked and inoculated into 3mL LB tubes to grow overnight. The DNA was harvested from these cells, and the plasmid DNA was digested with EcoRI and PstI. The digested DNA was subjected to electrophoresis on an agarose gel to confirm the ligation was successful.

These constructions, now called pBBGCAEco/Pst (for the pBlueBC vector with DRGCAEco/Pst fragment) and pBBBCAEco/Pst (for the pBlueBC vector with DRBCAEco/Pst fragment), were then used as the vector for adding DRGCANot/Sac and DRBCANot/Sac. The vectors, pBBGCAEco/Pst and pBBBCAEco/Pst, and the pGEM-T Easy vectors containing the DR(G/B)CANot/Sac fragments were digested with NotI and SacI. These were all then run on a 1.2% low-melting agarose gel, and the bands were excised from the gel. The fragments were ligated into the pBBGCAEco/Pst and pBBBCAEco/Pst vectors. These were then transformed into DH5α, and the colonies were picked and inoculated into 3mL of LB to grow overnight. The DNA was harvested and digested with NotI and SacI. The DNA was then subjected to electrophoresis on an agarose gel to check that the transformation and ligation had been successful.

These new plasmids will be designated pBBGCA for the DRGCAEco/Pst and DRGCANot/Sac fragments in the pBlueBC vector and pBBBCA for the DRBCAEco/Pst and DRBCANot/Sac fragments in the pBlueBC vector.

6. Transformation of *Deinococcus radiodurans* with pBBGCA and pBBBCA

Linear DNA is required for homologous recombination into the genome of *D. radiodurans*. Because of this, the plasmids pBBGCA and pBBBCA were digested with the

restriction enzyme HindIII to linearize the plasmid. The digested DNA was phenol:chloroform extracted, purified, and used to transform *D. radiodurans*. This was done because proteins, such as the restriction enzyme HindIII, can be transformed into *D. radiodurans* along with the DNA. If an active restriction enzyme were to be transformed, digestion of the genome could occur.

Purification of the phenol:chloroform extracted DNA was completed by adding ½ volume of 7.5M ammonium acetate, then 2 volumes of 100% ethanol. This was placed on ice for 10 minutes, then centrifuged at 14000rpm for 10 minutes. The liquid was removed. The product was washed with 70% ethanol, centrifuged for 10 minutes at 14000rpm, and the liquid was removed. The DNA was then resuspended in 40μL of TE buffer.

To transform wild type *D. radiodurans*, cells were grown in 50mL of TGY media at 30°C to an OD_{600} of 0.1 to 0.2 (exponential growth). To this culture, 1.5mL of 1M $CaCl_2$ that had been filter sterilized was added and this was incubated for 80 minutes. The purified DNA was added to 1mL of this culture and allowed to incubate at 30°C for 8 hours. This was then spread on TGY plates containing 3μg/mL of chloramphenicol.

The two plasmids were transformed separately. Only the *D. radiodurans* that contained the plasmid pBBBCA produced reddish colonies on the chloramphenicol-containing plates. The colonies were visible approximately 120 hours after the cells were plated.

7. Genomic DNA extraction from *Deinococcus radiodurans*

Colonies were picked from the plate containing the pBBBCA plasmid. The colonies were inoculated into 3mL of GYT media containing 3μg/mL of chloramphenicol. Once the cultures had grown to saturation, 1.5mL of media was microcentrifuged for two minutes. The resulting pellet was resuspended in 567μL of TE buffer (pH=8.0). To this, 30μL of 10% SDS and 3μL of

20mg/mL proteinase K was added, mixed, and incubated at 37°C for one hour.

Then 100μL of 5M NaCl was added, followed by 80μL of CTAB/NaCl solution. This was then incubated at 65°C for 10 minutes. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed, and then microcentrifuged for five minutes. The aqueous layer was then transferred to a new tube, and an equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed, and centrifuged for five minutes. Again, the aqueous layer was collected into a new tube.

To the new tube, 0.6 volume of isopropanol was added and mixed, then placed at -80°C for 15 minutes, allowing the DNA to precipitate. This was microcentrifuged for 10 minutes and most of the liquid removed (about 100μL was left behind). To this, 1mL of 70% ethanol was added to wash the pellet. This was microcentrifuged for five minutes, the liquid discarded, and the DNA pellet completely dried in a 37°C incubator. The pellet was resuspended in 30μL TE buffer.

III. Construction of the plasmids pDRGCA and pDRBCA

1. PCR and purification of the whole genes

Two of the primers used in the PCR reactions to amplify the fragments were also used to amplify the whole genes. The 5' end primers (DRGCANco: CTTCCATGGCGCGCGACTTCCGGCCTT and DRBCANco: CTTCCATGGGGCGCAGGCTAGCG), however, were redesigned to include an NcoI site instead of the EcoRI site. The SacI site at the 3' end remained the same. These primers will amplify each whole gene, adding an NcoI site at the 5' end, and a SacI site at the 3' end.

For the PCR reaction, 1X Herculase reaction buffer, 200μM of dNTP mix, 1μL of genomic DNA, 100ng of each primer, 13μL of Betaine, 0.65μL of DMSO, and 2.5 units of the Herculase DNA polymerase (Stratagene) was used. The Herculase was used instead of the YieldAce since the Herculase has a better proofreading ability, and also works well for G+C rich templates. The protocol used for this PCR was: 98°C for 3 minutes, then 10 cycles of 98°C for 40 seconds, 64°C for 30 seconds, 72°C for 60 seconds, then 25 cycles of 98°C for 40 seconds, 64°C for 30 seconds, 72°C for 70 seconds, then 72°C for 10 minutes.

All procedures were done according to standard methods (Sambrook *et al.*, 1989). The PCR products, named simply DRGCA for the gamma-CA and DRBCA for the beta-CA, were then purified and A-tailed. A shorter procedure was used for purification to minimize handling of such small amounts of DNA that can easily be lost. The procedure used is as follows: to the PCR product, add ½ volume of 7.5M ammonium acetate, then 2 volumes of 100% ethanol. This was placed on ice for 10 minutes, then centrifuged at 14000rpm for 10 minutes. The liquid was removed. The product was washed with 70% ethanol, centrifuged for 10 minutes at 14000rpm, and the liquid was removed. The product was then resuspended in water, and 1X reaction buffer, 25mM $MgCl_2$, 10 mM dATP, and *Taq* DNA polymerase (Promega) was added. This was placed in a 70°C water bath for 30 minutes, run on a low-melt 1.2 % agarose gel, and the bands cut out of the gel. Each band was about 800bp.

2. Ligation of PCR products into pGEM-T Easy

The A-tailed DNA fragments were then ligated into pGEM-T Easy and transformed into DH5α. The cells were spread onto agar plates containing ampicillin and X-gal. White colonies were picked and inoculated into 3mL LB tubes to grow overnight. The plasmid DNA was

harvested and digested with EcoRI. The digested DNA was then subjected to electrophoresis to check that the ligation had been successful.

3. Checking the orientation of DRGCA and DRBCA in pGEM-T Easy

The orientation of the DRGCA and DRBCA products in pGEM-T Easy was determined as follows. The DRGCA plasmid DNA was digested with Eco0109I and PstI and subjected to electrophoresis to determine the size, and therefore the orientation, of the digested DNA fragments. The backward orientation would yield a 580bp band, while the forward orientation would yield a 200bp band. The DRBCA plasmid DNA was digested with NheI and PstI and subjected to electrophoresis to determine the size of the digested DNA. The forward orientation would yield a fragment too small to see on a gel, and no band will be formed. The backward orientation would yield a 800bp band.

The DRGCA in pGEM-T Easy will now be designated pDRGCA, and the DRBCA in pGEM-T Easy will now be designated pDRBCA.

4. Ligation into pET28-b(+) and transformation into DH5α

After the orientation assay, both pDRGCA and pDRBCA were digested with NcoI and SacI. The vector pET28-b(+) (Novagen) was also digested with these enzymes. The products were subjected to electrophoresis on a low-melting agarose gel and then excised from the gel. The DRGCA and DRBCA were ligated separately into the pET28-b(+) vector overnight.

The ligations were then diluted and used to transform DH5α cells by electroporation. The cells were grown on kanamycin LB plates plus 200μM EZ. Colonies were picked and miniprepared to extract the plasmid DNA. The DNA obtained was then digested with NcoI and SacI. The DNA was subjected to electrophoresis an agarose gel to verify that the ligation and

transformation had been successful.

5. Transformation into BL21(DE_3), induction, sonication, and SDS-PAGE

The DRGCA in pET28-b(+) and DRBCA in pET28-b(+) DNA was diluted 1:100 and then transformed into the *E. coli* strain BL21(DE_3) by electroporation. The cells were then spread on LB agar plates containing 200μM EZ and kanamycin. Colonies were picked and miniprepped to extract the DNA. The DNA was then digested with NcoI and SacI and subjected to electrophoresis on an agarose gel to ensure that the transformation had been successful.

The cells were then reinoculated into 3mLs of LB broth and allowed to grow overnight. Then, 100μL of the overnight cells were inoculated into 3mL of LB broth and allowed to grow to an OD_{600} of 0.6 (about 4 hours). To these tubes was added 3μL of 1M IPTG to induce production of the proteins. This was incubated at 37°C for 3 hours.

After induction, the cells were spun down and resuspended in 100μL of 0.1M Na_2CO_3 and 0.1M DTT. The cells were then sonicated in three sets of 10 second cycles, allowing cooling time in between sets. These were then microcentrifuged for 10 minutes at 15000rpm. The supernatant was saved, and to 20μL of this, ½ volume of loading dye was added. The pellet was then resuspended in 100μL of Na_2CO_3/DTT, then 2μL of 1M DTT and 1μL of loading dye was added to 20μL of the resuspended pellet solution.

The resulting samples were then boiled for five minutes and were loaded onto a Laemmli SDS-PAGE. After electrophoresis for 1 hour, the gel was then stained and destained to show the bands.

Results and Discussion

I. Inhibition of CA by EZ and AZ

In previous studies we had observed that neither AZ nor EZ is toxic to *E. coli*. And, as expected, all of the *E. coli* tubes grew. The *D. radiodurans* that did not contain any EZ or AZ grew. However, the tube that contained EZ did not grow, while the one that contained AZ did grow. This shows that at least one of the two CAs in *D. radiodurans* is required for growth and is located inside the plasma membrane

II. Construction of the plasmids pBGGCA and pBGBCA

1. Creation of PCR inserts

The original PCR protocol did not yield the DRGCAEco/Pst fragment, although it worked well for the other three fragments (Figure 3). However, lowering the extension temperature from 64°C to 62°C resulted in amplification of the DRGCAEco/Pst fragment (Figure 4).

2. Ligation of PCR products into pGEM-T Easy

The DNA fragments amplified in the PCR reaction were excised from the low-melt agarose gel and ligated into pGEM-T Easy. The plasmid DNA harvested from the DH5α cells was digested with EcoRI and yielded the same fragments of the appropriate size (Figures 5a and 5b). Only certain ones were actually correct, and the rest were thrown out.

3. Construction of the plasmids pBBGCA and pBBBCA

The 5' fragment of each gene (that is, DRGCAEco/Pst and DRBCAEco/Pst) was first inserted into the pBlueBC vector. A vector control was used for the ligation and transformation, and the resulting DNA was digested with EcoRI and PstI to check for the fragment in the pBlueBC vector. Only a few of the colonies picked were actually correct and were used for the

next step (Figure 6).

The new vectors, pBBGCAEco/Pst and pBBBCAEco/Pst were then digested with NotI and SacI so that the DRGCANot/Sac and DRBCANot/Sac fragments can be added to their respective vectors as the first fragments were added to pBlueBC (Figure 7a and 7b).

4. Transformation of *Deinococcus radiodurans* with pBBGCA and pBBBCA

Only the pBBBCA-containing *D. radiodurans* produced colonies on the TGY plates. Because the pBBGCA-containing *D. radiodurans* plate did not produce colonies, there may have been a problem with the transformation, and this will have to be redone. It is unlikely that the knock-out mutation of the γ-CA was lethal, since all of the chromosomes would need to be mutated in order to have a homozygous knock-out. This is probably not possible in only one round of chloramphenicol growth. Therefore, from the initial growth on the plates, it is still unclear whether the mutations are lethal.

The PCR results of the β-CA, which did grow, will show whether or not the mutation is homozygous. If it is not, then the cells will be grown continuously in chloramphenicol-containing media. If no more growth occurs after several rounds of reinoculation into chloramphenicol media, the mutation may be lethal. However, if the PCR results show that the β-CA mutation is homozygous, then it can be assumed that the mutation of the β-CA is not lethal.

Problems with the genomic DNA harvesting prevented final results of the knock-out to be seen. Because of this, the cells were reinoculated into TGY broth, and the DNA was then harvested. The first PCR gave no results, but subsequent PCR may reveal results.

III. Creation of the plasmids pDRGCA and pDRBCA

1. PCR results

When the PCR product was run out on a gel to check if the PCR had worked, the lane containing the DRGCA gene had two extra bands. It is unclear what these bands were, but as the correct, ~800bp band was present, the other two bands were disregarded. The band for the DRBCA gene was correct at around 800bp (Figure 8).

2. Checking the orientation of DRBCA and DRGCA in pGEM-T Easy

After digestion with Eco0109I and PstI, it was found that DRGCA in pGEM-T Easy was in the backwards orientation. The electrophoresis gel revealed a ~600bp band, which shows that the orientation is backwards (Figure 9).

After digestion with NheI and PstI, it was found that the DRBCA in pGEM-T Easy was also in the backwards orientation. The electrophoresis gel revealed a ~800bp band, which shows that the orientation is backwards (Figure 10).

The backwards orientation of both of these inserts suggests that the expression of the insert is probably toxic to *E. coli*. Therefore, EZ was used in all the procedures following the orientation assay.

3. Transformation into BL21(DE_3), induction, sonication, and SDS-PAGE

Transformation into BL21(DE_3) was successful and induction was performed. The SDS-PAGE results were not very good. Some of the reagents in used may have been too old. However, results indicated that DRBCA was expressed, and that some of it was soluble. No unique polypeptides could be identified in the extracts of the DRGCA.

Conclusions and Future Direction

In the studies described here, I created plasmids to disrupt the β- and γ-CA genes in *D. radiodurans*. Some colonies appeared on selection plates containing *D. radiodurans* cells transformed with the β-CA mutation. However, I have not yet obtained a PCR product from the genomic DNA isolated from these cells. No chloramphenicol-resistant colonies were obtained with the γ-CA mutation. At this point, it is not clear why this experiment failed.

In this study, I also constructed plasmids for expression in *E. coli* of the β- and γ-CA genes from *D. radiodurans*. The β-CA was expressed and some was found in the soluble fraction. When this experiment is repeated, the soluble fraction can be assayed for CA activity. The γ-CA did not appear to be expressed under the same conditions. Others in the laboratory have attempted to express a γ-CA from *A. thaliana* without success. At present, it is not clear why the γ-CAs are so difficult to express.

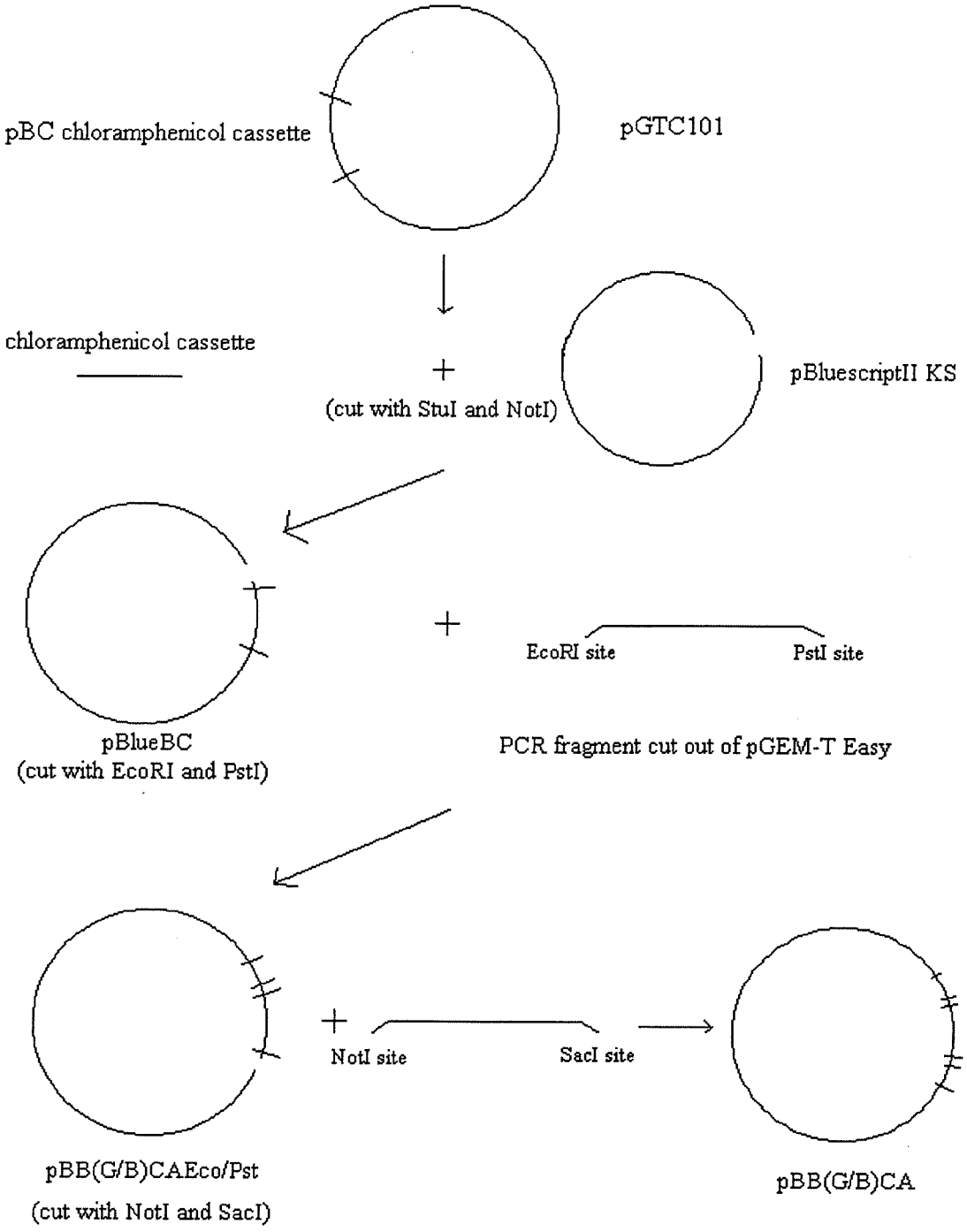


Figure 1: Overview of construction of pBBGCA and pBBBCA

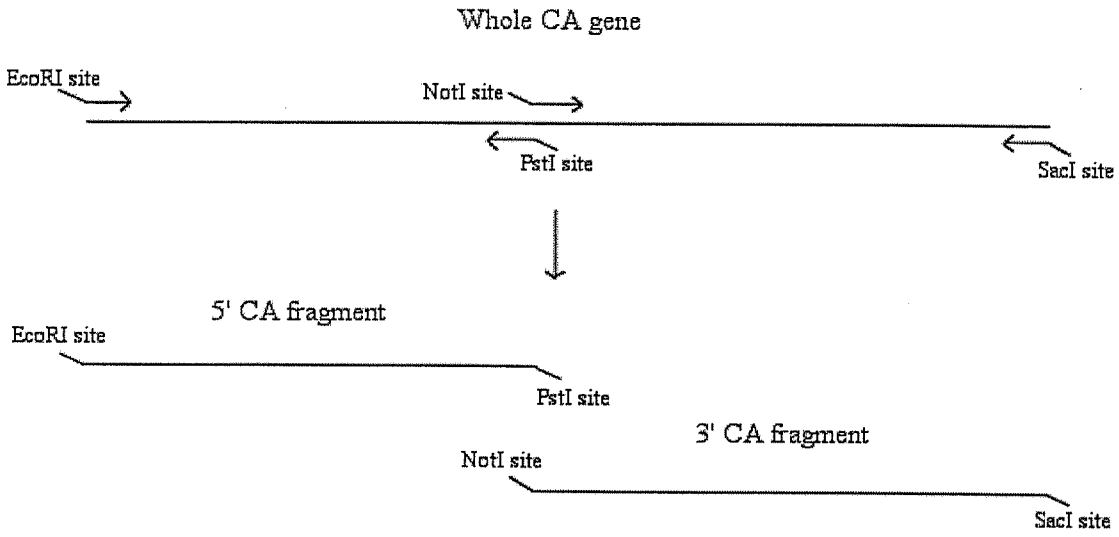
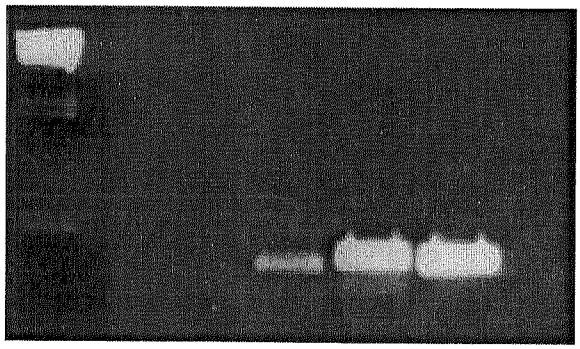
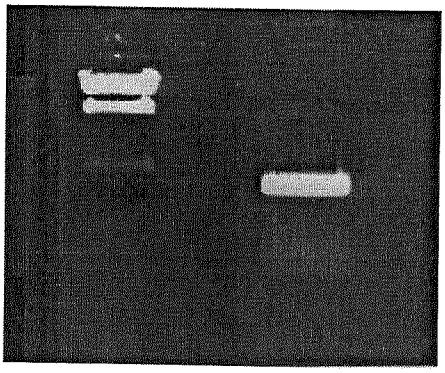


Figure 2: PCR reactions



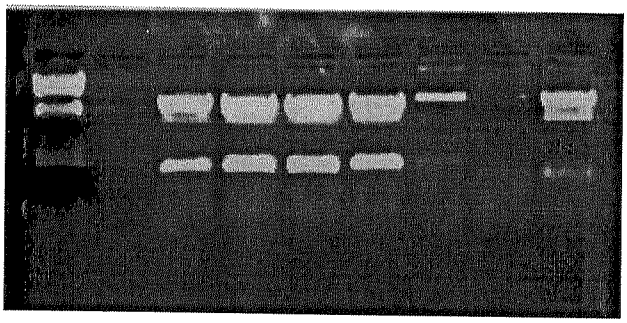
1 2 3 4 5

Figure 3: PCR gel results
Lanes: 1: Lambda HindIII marker, 2: missing DRGCAEco/Pst band,
3: DRGCANot/Sac, 4: DRBCAEco/Pst, 5: DRBCANot/Sac



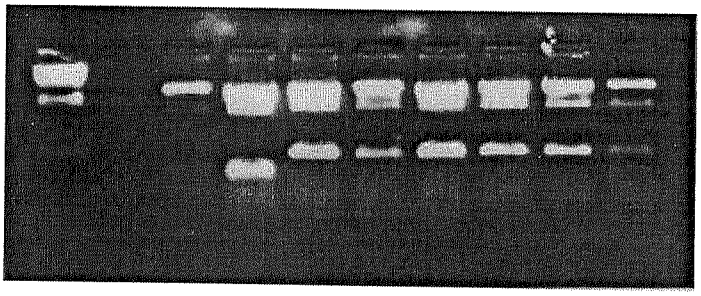
1 2

Figure 4: PCR results for DRGCAEco/Pst fragment
Lanes: 1: Lambda HindIII marker, 2: DRGCAEco/Pst fragment



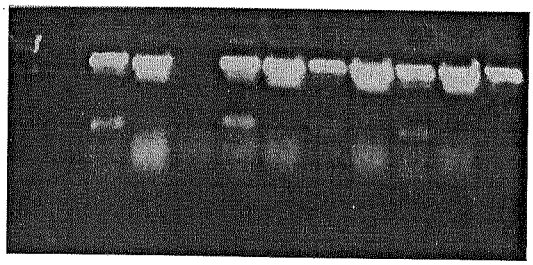
1 2 3 4 5 6 7 8 9

Figure 5a: Miniprep results after transformation into pGEM-T Easy, cut with EcoRI
Lanes: 1: Lambda HindIII marker, 2-4: DRGCAEco/Pst, 5-9: DRGCANot/Sac



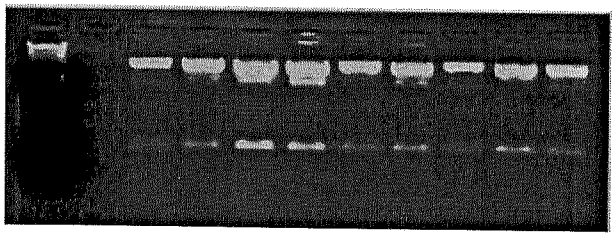
1 2 3 4 5 6 7 8 9

Figure 5b: Miniprep results after transformation into pGEM-T Easy, cut with EcoRI
Lanes: 1: Lambda HindIII marker, 2: DRGCANot/Sac, 3-5: DRBCAEco/Pst, 6-9: DRBCANot/Sac



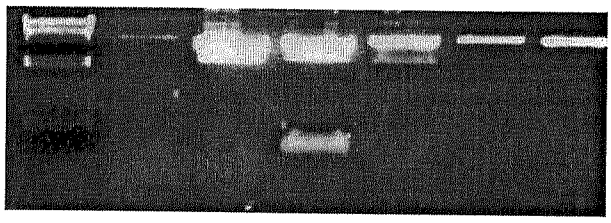
1 2 3 4 5 6 7 8 9 10 11

Figure 6: Miniprep results after transformation into pBlueBC, cut with EcoRI and PstI
Lanes: 1: Lambda HindIII marker, 2-5: pBBGCAEco/Pst (only lanes 2 and 5 are correct),
6-11: pBBBCAEco/Pst (only lanes 7 and 9 are correct)



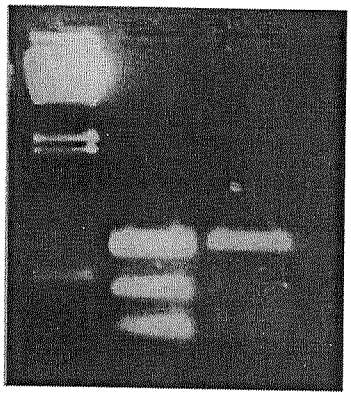
1 2 3 4 5 6 7 8 9 10

Figure 7a: Miniprep results after transformation into pBBBCAEco/Pst
Lanes: 1: Lambda HindIII marker, 2-10: pBBBCA



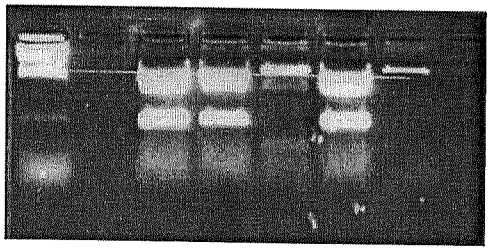
1 2 3 4 5 6 7

Figure 7b: Miniprep results after transformation into pBBGCAEco/Pst
Lanes: 1: Lambda HindIII marker, 2-7: pBBGCA (only lane 4 is correct)



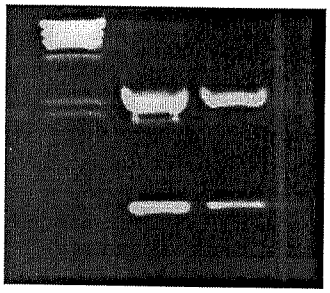
1 2 3

Figure 8: PCR results for DRGCA and DRBCA
Lanes: 1: Lambda HindIII marker, 2: DRGCA (with the two extra bands below the correct one), 3: DRBCA



1 2 3 4 5 6

Figure 9: Checking orientation of DRGCA in pGEM-T Easy
Lanes: 1: Lambda HindIII marker, 2-6: DRGCA in pGEM-T Easy (lanes 4 and 6 are incorrect)



1 2 3

Figure 10: Checking the orientation of DRBCA in pGEM-T Easy
Lanes: 1: Lambda HindIII marker, 2-3: DRBCA in pGEM-T Easy

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