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Undergraduate Honors Thesis

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Abstract: The wild-type *Deinococcus radiodurans* has the ability to repair extensive DNA damage after being exposed to doses of ionizing radiation of 5000 Gray. The resistance of mutant strain IRS41 (Ionizing-Radiation Sensitive) is restored by the cosmid pMM69 and pNS38 using dot transformation. The mutant strain IRS34 is not restored by either pMM69 or pNS38.

Introduction to *Deinococcus radiodurans*

D. radiodurans is a Gram-positive, nonsporeforming, nonmobile bacterium. It is spherical and convex in shape, pink to red in color, and approximately 1.5 to 3.5 micrometers in diameter. It was originally identified as a contaminant of irradiated canned meat in 1956 and it has also been isolated from other sources such as soil, animal feces and processed meats, all rich in organic nutrients. However, *D. radiodurans* has also been found in some nutrient-poor environments such as weathered granite, dry Antarctic valley, room dust, and irradiated medical instruments. This suggests "that the deinococci could have evolved, much as the spore-forming organisms did, to survive periods of prolonged environmental stress," (Battista 208).

White et al explains that *D. radiodurans* is highly resistant to many conditions that damage DNA such as UV radiation, hydrogen peroxide and, most importantly, ionizing radiation. The typical radiation survival curve shows complete resistance up to 5,000 Gray (one Gray is a unit of absorbed dose equal to 100 rads) with no loss of viability. In fact, exponential phase cultures routinely survive exposure to 15,000 Gray. For comparison, *E. coli* does not survive radiation above 1,000 Gray. *D. radiodurans* also exhibits resistance to desiccation. Battista describes a connection between desiccation resistance and ionizing radiation resistance. Forty-one ionizing radiation-sensitive strains of *D. radiodurans*, were also found to be sensitive to desiccation. Additionally, during dehydration DNA damage accumulates. "It appears that *D. radiodurans* is an organism that has adapted to dehydration and that its DNA repair capability is one manifestation of that evolutionary adaptation," (209).

The basis of research of *D. radiodurans* is its incredible tolerance to DNA damage. A dose of 3,000 Gray causes the chromosomes of all *D. radiodurans* cells to be cleaved in to multiple, subgenomic fragments (approximately 120 double stranded breaks per genome), which, for most species, is irreparable and lethal. *D. radiodurans* has the capacity to reform its chromosomes from these fragments in less than three hours without any loss of viability or evidence of mutation. Battista et al explains that *D. radiodurans* appears to contain most, if not all, of the typical complement of prokaryotic DNA-repair proteins. "This observation suggests two equally intriguing possibilities: (1) *D. radiodurans* uses the same DNA-repair strategies as other prokaryotes but does so in a manner that is somehow more effective than in other species or (2) *D. radiodurans* uses a DNA-repair system that has novel components," (362).

D. radiodurans is multigenomic and its stationary phase cells carry an estimated four genome equivalents. Each chromosome is a covalently closed circular molecule with a high GC content. It contains approximately 3×10^6 base pairs. Naturally occurring plasmids have been found in all species most of which are larger than 20 kb but their function is unknown. The function of the multigenomic characteristic is important in scrutinizing the ability of *D. radiodurans* to reassemble an intact chromosome after DNA damage from ionizing radiation. "Because strand breaks are generated randomly, the probability of losing genetic information at the same site on every chromosome is very low at sublethal doses of radiation," (Battista 216). This is assuming that the cell has the ability to mediate the reassembly of a chromosome from multiple fragments, which has yet to be proven.

There are many additional theories as to why *D. radiodurans* is so resistant to ionizing radiation. The limited information argues that *D. radiodurans* may have the ability to control replication of the chromosomes until the DNA is repaired. After cultures of the bacteria are irradiated, replication ceases. The movement of the DNA polymerase may be blocked by lesions or an undefined regulatory process may specifically prevent chromosome replication until the DNA is repaired. An additional theory is that *D. radiodurans* controls the exonuclease activity that degrades chromosomal DNA at the site of DNA strand breaks thus inhibiting significant DNA degradation and consequently providing protection against the lethal effects of ionizing radiation. Furthermore, the degradation of the chromosome is accompanied by the export of damaged and undamaged nucleotides from the cell. In *D. radiodurans*, the removal of damaged bases could be a way to avoid an increase in mutation frequency (2).

IRS41 and IRS34

Chemical mutagenesis produces mutant strains of *D. radiodurans* when treated with a simple alkylating agent such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). The cultures are treated with MNNG, diluted into fresh media, and allowed to grow before identification of the desired mutant phenotype is attempted. Recalling that *D. radiodurans* is multigenomic, the bacteria needs time to segregate and form cells homozygous for that mutation if the mutation is a recessive one (1).

IRS41 and IRS34 are two strains of the wild-type *D. radiodurans* that were created in this fashion, both of which are sensitive to ionizing radiation. IRS41 contains a mutation in the gene *irrI* and IRS34 contains a mutation in the gene *irrF*. The cosmid

library was previously screened to search for a cosmid that would restore the resistance to both IRS41 and IRS34. The hypothesis was that IRS41 could be restored with the cosmids pNS38 and pMM69 and IRS34 could be restored with only pNS38. IRS41 would be the control from which to compare IRS34. When pNS38 and pMM69 were sequenced and identified in the genome database, it was discovered that pMM69 was approximately 39kb long and pNS38 was approximately 40kb long and they significantly overlapped. Therefore, the area of interest was the approximate 1380bp of leftover sequence that restored resistance to IRS34 that was not contained in pMM69. Furthermore, the overlapping sequence contained a significant amount of the gene encoding for the chromosome-partitioning protein *parA* (Appendix A). The assumption was that the mutation of IRS34 was located within the *parA* gene.

Dot Transformation

Initially, a survival curve for IRS34 was conducted and compared to the survival curve of the wild-type R1. This confirmed the ionizing-radiation sensitivity phenotype of IRS34 (Appendix B).

Bacterial transformation is a process that involves the recombination of genetic material between bacteria as the result of transfer of extracellular pieces of DNA that are taken up by a living bacterium. Dot transformation simply involves directly applying a specific concentration of DNA onto a lawn of *D. radiodurans* and searching for successful transformation. *D. radiodurans* is fully competent and naturally transformable throughout exponential growth. The protocol for dot transformation is as follows:

1) Inoculate IRS34 and IRS41 frozen perm cells into 2ml TGY* broth.

2) Grow at 30 degrees C for 24 hours.

3) Transfer 100 microliters of the culture into 50ml TGY broth.

4) Grow with shaking at 30 degrees C for approximately 10 hours, to OD 600nm of 0.09-0.11 (approximately 8×10^6 to 1×10^7 CFU/ml)

5) Spin 25 ml of the cells at 10,000 rev/min for 10 minutes.

6) Resuspend in 2.5ml $MgSO_4$.

7) Plate 100 microliters onto TGY plates.

8) Grow in 30 degrees C for 2 hours.

9) Dot ~1 microgram of DNA of both the control and the sample onto the plates.

10) Grow in 30 degrees C for ~22 hours, until a very faint pink is seen.

11) Replica plate each plate.

12) Expose to 10,000 Gray of gamma radiation (235 minutes).

13) Grow at 30 degrees C for 1-2 days, checking often.

* TGY broth is made with tryptone, glucose, and yeast extract.

The result of dot transformation is a light lawn of cells, which is considered background with distinct areas of denser cell growth corresponding to the location of DNA addition. Background is due to the fact that the irradiation is coming in contact with a certain percentage of cells regardless of cell density. A certain number of the cells will never encounter the radiation and, therefore, isolated colonies will appear randomly around the plate.

The above protocol calls for pure DNA to dot onto the growing bacterial lawns. Since the cosmids are incorporated into *E. coli*, the cosmids that are to be used must be isolated from the cell. Cosmids are simply hybrid vectors constructed using parts of the lambda chromosome and plasmid DNA. It contains the *cos* sequence of phage lambda needed for packing phage DNA into the phage protein coats, plasmid sequences for replication, and an antibiotic resistance gene. After the cosmid enters the bacterial host cell, it replicates as a plasmid. The importance is that cosmids can carry DNA inserts that are 4 to 5 times larger than phage vectors and plasmids. The cosmids for *D. radiodurans* were created from randomly cutting the *D. radiodurans* wild-type genome (3.2 Mbp) into a myriad of pieces and cloning the overlapping pieces into a cosmid that also contains an ampicillin resistance gene. This genomic library, kept in a –80 degree C freezer, can be efficiently screened for clones that are capable of restoring the wild-type phenotype to mutant strains.

The kit used to isolate the cosmids is manufactured by QIAGEN Inc. and involves the QIAprep Spin Miniprep Kit Protocol (Appendix C). To begin, 5mls of LB broth containing ampicillin is inoculated with *E. coli* carrying the specific cosmid and is allowed to grow in a 36 degree C water bath with shaking for approximately 10 hours. Next, the cells are pelleted and subjected to a number of solutions and buffers.

According to the QIAGEN handbook, the components of the cell membrane are degraded leading to cell lysis and release of the cell contents. In an optimal lysis time, maximum release of plasmid DNA without release of chromosomal DNA is achieved while minimizing the exposure of the plasmid to denaturing conditions. A high salt concentration causes everything to precipitate except for the plasmid, which remains in

the solution. The solution is separated and then applied to a filter to ensure only plasmid DNA is left over and then it is washed from the filter with deionized water and can then be stored in the freezer. The absorbance of the DNA is measured using a spectrophotometer at the wavelengths of 280 and 260nm. The concentration is then calculated to determine the amount needed to dot onto the bacterial plates (about 1 microgram is desired).

Results

Dot transformation was conducted six times. The results of the trials indicated successful transformation with definite areas of denser cell growth relative to the background for IRS41. Both pMM69 and pNS38 showed positive results. With IRS34, however, all trials resulted with a relatively heavy amount of background growth with large colonies and no areas of successful restoration. When the dose of radiation was increased to 15,000 Gray, less background appeared but there was no increase in restoration.

Instead of isolating a small batch of cosmids each time, one large cell culture was grown and the cosmids were isolated in an attempt to increase DNA concentration. The idea was that higher DNA concentrations would lead to a better chance of restoration. Regular dot transformation was conducted with the same results as before. Positive results were identified for IRS41 and negative results for IRS34. Liquid transformation was also conducted three times in which the cell cultures were grown to an exponential phase and $CaCl_2$ was added to facilitate the transformation. The isolated DNA was then added to each tube and the culture was diluted out and irradiated at 10,000 Gray within

the tubes. The cultures were then dotted onto the plates directly and allowed to grow for 2-3 days. The results were inconclusive.

Conclusion

After several attempts with dot transformation and liquid transformation the results show that the ionizing radiation resistance of IRS41 can be restored using pMM69 and pNS38 and dot transformation. The ionizing radiation resistance of IRS34 cannot be restored by either pMM69 or pNS38. In order to restore the resistance of IRS34, other cosmids must be screened.

Works Cited

1) Battista, John R. (1997) *Annu. Rev. Microbiol.*, 203-24.

2) Battista, John R., Earl, Ashlee M. and Mie-Jung Park. (1999) *Trends in Microbiology* 9, 362-365.

3) Cummings, Michael R. and William S. Klug. Concepts of Genetics. 5th edition. New Jersey: Prentice Hall, Inc. 1997.

4) White, Owen et al. (1999) *Science* 286, 1571-1576.

Appendix A

Overlapping sequence of pNS38 (appr. 1380 bp)

GCTCATCGACGAGGACGGGCGGGTGGGCAGTTCGCTGCGCTGGGCGCGCCGCCGTGAGGAGGGCCTGGGCTTTCCGGTGCTGGA
CGCCGACGAGGTGAAGCCCAAGAAGCTGGCCGCGCTCGACGCCGTGCTCATCGACACCGAGGGCCGGCCCCGGCGCAAGGACCT
GCGGGCGCTGGCCGAGCGCGCCGACCTGATTCTGATTCCGAGCGGCCCGACCATGCTGGAACTGGAAGCCACCCGCGAGCTGCT
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AGCGCGCGAAGACCTGCGCGACGACGGCTGGACCGTATGCAACACGGCGCTGCGTTCGTACACCGTCTACCAGAAAGCCGCCGA
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CCACGCCTACCCCACGCACGTACAATGCCCGGCGATGACCGTCACGGGCCGCTTTGCTCCCAGTCCCACCGGGGCCATGCACCT
GGGCAACGCCCGCACCGCGCTGCTGGCGTGGCTGCACTCGCGCGCGCTCGGCGGGCGGCACCTGCTGCGTTTCGAAGACCTCGA
CACTGGCCGGGTGCGAGGCTGGGCCTACGACCTGACCCGGCGCGACCTGGAGTGGCTGGGGCTCGACTGGGACGAG

>ORF04056 chromosome partitioning protein, ParA family {Agrobacterium radiobacter}

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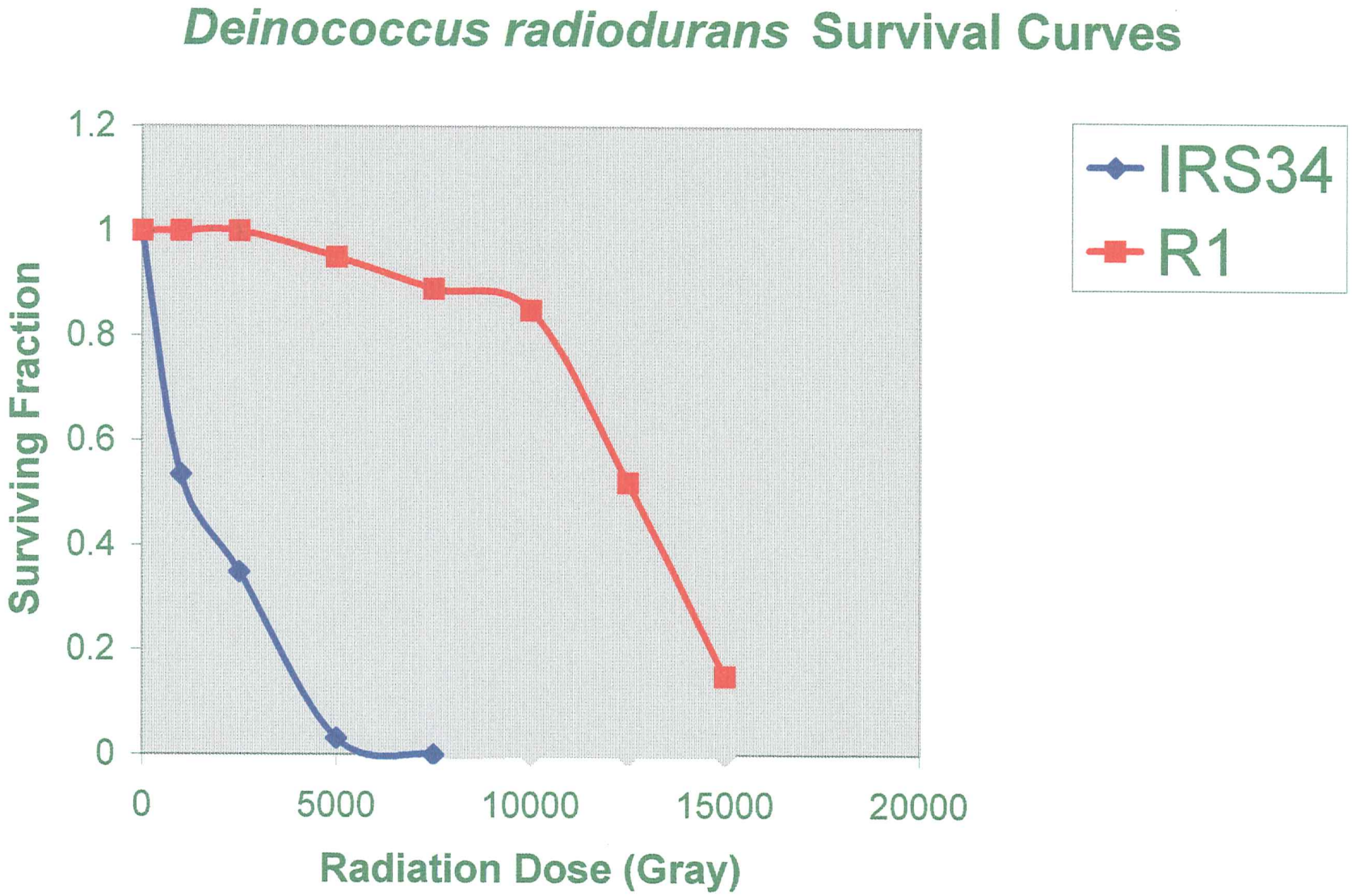
Deinococcus radiodurans Survival Curves
Surviving Fraction
1.2
1
0.8
0.6
0.4
0.2
0
0
5000
10000
15000
20000
Radiation Dose (Gray)
IRS34
R1

QIAprep Spin Miniprep Kit Protocol

using a microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5-ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 31.

! **Please read Important Notes for QIAprep Procedures on pages 14–15 before starting.**

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl of Buffer P1 and transfer to a microfuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

2. **Add 250 µl of Buffer P2 and gently invert the tube 4–6 times to mix.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

3. **Add 350 µl of Buffer N3 and invert the tube immediately but gently 4–6 times.**

   To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of Buffer N3. The solution should become cloudy.

4. **Centrifuge for 10 min.**

   A compact white pellet will form.

   During centrifugation, place a QIAprep spin column in a 2-ml collection tube.

5. **Apply the supernatants from step 4 to the QIAprep column by decanting or pipetting.**

6. **Centrifuge 30–60 sec. Discard the flow-through.**

7. **(Optional): Wash QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuging 30–60 sec. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.

8. **Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging 30–60 sec.**

9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   ! **IMPORTANT:** Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. **Place QIAprep column in a clean 1.5-ml microfuge tube. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of each QIAprep column, let stand for 1 min, and centrifuge for 1 min.**

QIAprep Spin Miniprep Kit Protocol

using 5-ml collection tubes

The QIAprep Spin Miniprep procedure can be performed using 5-ml centrifuge tubes (e.g., Greiner, Cat. No. 115101 or 115261) as collection tubes to decrease handling. The standard protocol on pages 18–19 should be followed with the following modifications:

Step 4: Place QIAprep spin column in a 5-ml centrifuge tube instead of a 2-ml collection tube.

Step 6: Centrifuge at 3000 x *g* for 1 min using a suitable rotor (e.g., Beckman® GS-6KR centrifuge at ~4000 rpm). (The flow-through does not need to be discarded).

Steps 7 & 8: For washing steps, centrifugation should be performed at 3000 x *g* for 1 min. (The flow-through does not need to be discarded).

Step 9: Transfer QIAprep column to a microfuge tube. Microcentrifuge at maximum speed for 1 min. Continue with step 10 of the protocol.