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Final Technical Report

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Abstract

Current forensic DNA genotyping technology requires 0.2-2.0 nanograms duplex DNA that is at least 100-500 base pair in length. However, many evidence samples fail to meet these minimal requirements, because the target DNA has been exposed to environments capable of extensively damaging DNA. This damage reduces the size of the target DNA, making it too small to be amplified and can block PCR amplification. Since DNA samples found at crime scenes exhibit varying degrees of damage, the effectiveness of DNA typing technology is limited by sample condition, and at present there are no methods of circumventing this problem. Our principle objective was to develop tools to facilitate STR DNA genotyping through a) improving the quality of the DNA found in degraded forensic samples, and b) enhancing the ability to retrieve amplifiable DNA from forensic samples. Our efforts were focused on two areas. First, we attempted repairing damaged DNA *in vitro* using cell extracts isolated from repair proficient microorganisms. Extracts were emphasized because there is no way of knowing *a priori* what type of DNA damage is present in biological evidence, and intact cells express an extensive array of DNA repair proteins capable of dealing with most DNA damage. These efforts met with minimal success; it appears we were able to repair DNA *in vitro*, but the technique was difficult to reproduce. The cell extracts inhibited the reaction used to monitor repair and prevent an accurate assessment of the extent of repair. Second, we have developed a method for retrieving selected DNA fragments from complex mixtures. DNA damage occurs randomly and so the double strand breaks resulting from that damage are also random. A sample – even a highly degraded sample – is expected to have a distribution of fragment sizes, and our efforts are aimed at isolating fragments that include the intact STR sequences from such mixtures. To that end we have developed an *in vitro* method using three purified proteins that permits selective capture of specific DNA sequences. This method relies on the use of a targeting DNA fragment, a sequence that is complementary to the sequence you wish to retrieve. Combining this fragment, the three proteins, and appropriate cofactors allows recovery of homologous DNA from solution with high efficiency.

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Executive Summary

Since its inception in the 1980s, DNA typing of biological evidence has taken on a central role in forensic science. This technology, in conjunction with the CODIS network and the implementation of national standards for convicted offender DNA databasing, provides law enforcement with a highly effective method for solving violent crimes nationwide. In addition, DNA evidence has led to the exoneration of a number of wrongly convicted persons. Current forensic DNA technology requires 0.2-2.0 nanograms duplex DNA that is at least 100-500 base pair in length. However, many evidence samples fail to meet these minimal requirements, because the target DNA has been exposed to environments capable of extensively damaging DNA. This damage can reduce the size of the target DNA, making it too small to be amplified by the polymerase chain reaction (PCR) during the typing protocol, and some forms of damage result in modification of nitrogenous bases that block PCR amplification. Since DNA samples typically found at crime scenes exhibit varying degrees of damage, the effectiveness of DNA typing technology is limited by sample condition, and at present there are no methods of circumventing this problem. If treatments can be found that improve the condition of degraded samples without affecting the genetic profile of DNA evidence, the use of DNA typing can be expanded to the benefit of law enforcement and society.

The long-term objective of this project is to develop a protocol for restoring heavily degraded DNA evidence to a condition suitable for STR DNA genotyping – a task that requires the capacity to increase the amount of available undamaged DNA fragments in the necessary 100-500bp range prior to amplification via the PCR process. With appropriate technical training, it is relatively straightforward to generate a genotype from an abundant pristine sample, such as that obtained from a cheek scraping. However, forensic scientists do not always have the luxury of abundant high quality DNA evidence to work with. Samples are affected by their history. DNA evidence is often exposed to the environment for varying lengths of time; in some situations, it could be decades between the time a crime is committed and the time the evidence is collected and analyzed. Sample DNA can be damaged in a myriad of natural and unnatural processes, and this degradation in sample quality makes obtaining a genotype difficult, if not impossible.

Two general types of degradation are particularly important when considering forensic genotyping. First, DNA damage can modify DNA bases, and these modifications can physically block movement of the DNA polymerase used in PCR amplification, reducing or eliminating the signals (derived from the STRs) used to create the genotype. Second, DNA damage causes DNA strand breaks, and as the number of strand breaks increases the probability of separating the STR from one of the primer sites needed for amplification increases. The number of strand breaks increases with time, creating smaller and smaller fragments. Eventually, the accumulation of strand breaks causes individual STR signals to drop out of the genotype, the larger STRs disappearing first.

The approach taken during this project toward improving DNA genotyping focused on 1) improving damaged sample DNA prior to attempts at PCR amplification, and 2)

retrieving those specific sequences needed for PCR amplification, thereby improving the chances for obtaining a genotype. The protocols described are not intended to supplant existing methodology. The effort was intended to provide a "pretreatment" that would help guarantee successful genotyping, augmenting existing protocols approved for forensic applications. These efforts were guided by the knowledge that DNA repair systems are present in every living cell – systems that have evolved to repair damaged DNA efficiently without introducing sequence changes. If these systems could be harnessed for use *in vitro* with degraded DNA samples, it may be possible to increase the efficiency with which forensic samples are processed.

During the funding period, two lines of investigation were pursued. First, extracts from *Deinococcus radiodurans* R1 were examined for their ability to repair DNA damage *in vitro*. *D. radiodurans* is one of the most DNA damage tolerant species known and that capability is the consequence of an unusually efficient DNA repair. Second, an *in vitro* system for repairing DNA double strand breaks was developed and used as a mechanism for capturing and concentrating any DNA sequence.

Repairing DNA *in vitro* using extracts of *D. radiodurans* R1

The experimental design for this study was straightforward. Samples of damaged DNA would be treated with extracts obtained from cultures of *D. radiodurans* exposed to 3000Gy dose of ionizing radiation. Earlier work had established that aged forensic samples exhibit a pattern of DNA damage similar to that generated when DNA is exposed to ionizing radiation. To follow DNA repair we used a bioassay in which viability was the end point. The bacterial strain *Acinteobacter baylyi* ADP6 carries a defect that prevents this strain from growing in the presence of 4-hydroxy-benzoate (POB). Transferring the *pcaEFDBCHG* operon into ADP6 can restore POB prototrophy. Since *A. baylyi* strains are easily and efficiently transformed with naked DNA, we designed a system in which we attempted to repair damaged plasmids carrying the *pcaEFDBCHG* operon with the extracts from *D. radiodurans*. Restoration of POB prototrophy in ADP6 transformed with a damaged plasmid would be taken as evidence of *in vitro* repair.

Initial trials suggested that extracts were repairing damaged DNA, but the controls indicated that undamaged DNA was adversely affected by the extract, lowering transformation efficiency. This effect of the extract made it impossible to accurately assess the extent of DNA repair *in vitro*. Attempts to determine the mechanism responsible for the inhibition by the extract were unsuccessful, and indicated that there could be more than one inhibitory factor present. After obtaining this result, no further studies of the use cell extracts for *in vitro* DNA repair were pursued.

In vitro DNA double strand break repair and selective capture

A procedure for the joining together two DNA molecules that share an 18- 55 nucleotide region of homology was developed during the funding period. The process, referred to as

in vitro double-strand break repair reaction, requires only three proteins: RecA protein, single strand binding (SSB) protein and a DNA polymerase. To date, all three proteins are obtained from *Escherichia coli*. RecA and SSB are needed for identifying the region of homology and promoting invasion by the 3' end of single strand DNA molecule recombining with duplex DNA. The DNA polymerase extends the 3' ends, sealing the spliced fragments formed by the action of RecA and SSB.

The purpose of this reaction is twofold. First, it can be used to repair double strand breaks found between the STR and the primer sites for STR amplification. If the single strand fragment used to initiate *in vitro* double strand break repair (referred to as a targeting fragment) bridges the strand break the resulting union eliminates that break. Second, if the targeting fragment includes at its 5' end a feature that allows efficient retrieval such as biotin that can be harvested with streptavidin-coated magnetic beads, recombined fragments can be isolated and concentrated for further manipulation (e.g. PCR amplification).

To establish that *in vitro* double strand break repair was possible, an assay was developed that utilized the plasmid pUC19 cleaved by a pair of restriction enzymes: AatII and PstI. The plasmid pUC19 DNA digested with AatII shares homology with the ends of the PstI digested pUC19 DNA. The ends overlap by 504 nucleotides and 2182 nucleotides. Strand pairing, strand exchange, and extension of the 3' end of the exchanged strand can theoretically produce two products: one of 3190 nucleotides and another of 4868 nucleotides. When attempted, the reaction generated all of the expected products with unprecedented efficiency. It is estimated that as much as 40% of the starting material recombined. Additional investigation established that the following components were necessary for the reaction: RecA (in the presence of ATP), SSB, DNA Pol I, and all four dNTPs; omit any one of these components and the products are not formed

To test the feasibility of using synthetic oligonucleotides in the double strand break reaction, biotinylated capture fragments of 50, 100, 150 and 200 nucleotides in length were examined for the ability to initiate the reaction. Each fragment was complementary to DNA sequence upstream of the *gltS* gene of *Escherichia coli*, which simulated a target sequence. An attempt was made to recover this sequence from purified *E. coli* genomic DNA. The double strand break reaction was conducted as described above. The 100, 150, and 200 base oligonucleotides initiate the double strand break reaction and allow a discrete set of fragments that are larger than 1500 base pairs to be retrieved.

It appears that as long as complementary DNA sequence is available, targeting DNA fragments can be designed that will promote *in vitro* DNA double strand break repair. In theory, this protocol can be used to selectively capture any DNA sequence. In addition to autosomal STRs, Y chromosome STRs, and mitochondrial DNA sequences could be retrieved by this procedure. This procedure has three properties that make it potentially useful for forensic applications: i) it does not require knowledge of DNA sequence on both ends of a DNA segment to be captured, as in PCR, ii) it does not introduce any enzymes that could damage or alter a target DNA sample, and iii) it should allow the retrieval of desired DNA segments even from samples that are highly contaminated with

DNA from other species.

The capacity to selectively target DNA sequences within a complex mixture is one of the most useful features of the method, allowing one to retrieve the proverbial "needle from the haystack" because the method relies on a RecA-facilitated search for complementarity between a targeting DNA fragment and the genomic DNA sequence with a sequence of interest. The mechanism of a RecA-mediated search for homology is not well understood, but *in vitro* evidence (derived from the literature) indicates that RecA can identify its target within an excess of 200,000-fold heterologous substrate within 15 minutes – far faster than if the same DNA fragment were added to this mixture in the absence of RecA.

It is expected that selective capture through *in vitro* double strand break repair will enhance the ability to recover the DNA fragments from low abundance samples, degraded samples, and samples that are contaminated with mixtures of DNA from other species. The incredible specificity of RecA-mediated reactions, all but excludes cross-reaction.   The ability to selective capture a sequence of interest and recover it with magnetic beads simplifies subsequent steps in genotyping and will remove ambiguity from those analyses generated from samples obtained under less than ideal circumstances.

Final Technical Report

I.   Introduction

A. Statement of the problem

This project sought to answer a single question. Can DNA evidence that has been deemed quantitatively and qualitatively inadequate for PCR-based genotyping be restored to a usable condition if pre-treated with mixtures of proteins that either repair existing damage or allow efficient recovery of DNA sequence suitable for amplification? During the funding period, we have worked toward developing methods for repairing damaged DNA *in vitro* and for retrieving DNA fragments of interest to forensic scientists from evidence samples.

The long-term objective of this project is development of protocols for facilitating STR DNA genotyping from heavily degraded forensic DNA evidence; a task that requires the capacity to increase the amount of available undamaged DNA fragments in the necessary 100-500bp range prior to amplification via the PCR process. Two approaches have been taken: 1) We have attempted to add extracts from highly repair proficient bacteria to extensively-degraded biological evidence in the belief that we can improve the quality of a large enough fraction of the target DNA in that sample to support STR genotyping, and 2) we have developed a method that allows us to capture and concentrate the targets of STR typing.

B. Literature citations and review

1. STR typing

DNA genotyping based on the PCR amplification and electrophoretic analysis of Short Tandem Repeats (STRs) plays a prominent role in forensic science (Butler, 2006). A STR is a polymorphism found in mammalian DNA, a sequence of nucleotides (ranging between 2-10 bases) that is tandemly repeated at a locus. By examining several STR loci one can establish the unique genetic profile of an individual, linking biological evidence from a crime to the perpetrator or to other crimes by the same person.

Tetranucleotide repeats are the mainstay of forensic DNA typing and criminal offender databasing (Butler, 2006). There are only 33 possible tetranucleotide motifs (Jin *et al*., 1997), and the consensus motif sequences, mostly AGAT and GATA, are ubiquitous in the human genome. The number of repeat units at these loci varies from as few as four to as many as 50. In 1997 the forensic community in the United States chose thirteen STR loci to form the essential core of its Combined DNA Index System (CODIS) casework and offender databases. These loci are: D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, CSF1PO, FGA, Th01, TPOX, and vWA. There are enough different alleles at these STR loci in any major population or subpopulation to ensure that individuals will be heterozygous at most loci, enabling unambiguous identification (Butler, 2006; Jin *et al*., 1997).

2. DNA Degradation and STR Typing

Under conditions where biological samples are well preserved, genotyping with STRs is a robust technology that can be applied with confidence. However, forensic scientists are often confronted with biological evidence in which the DNA is present in a degraded form that interferes with PCR amplification, limiting the effectiveness of this technology (Hochmeister, 1998; Hoff-Olsen *et al*., 2001; Pfeiffer *et al*., 1999). In these samples the DNA is highly fragmented and contains a large number of modified nucleotides. STR analysis generates PCR fragments of between 100 – 500 base pairs; if the fragments of target DNA are on average smaller than this, effective PCR amplification will not be obtained. Although many other types of DNA damage can interfere with PCR amplification, double strand breaks are the major impediment to successful DNA genotyping in degraded DNA samples. Any double strand break in the region between the primers used to amplify the DNA at a particular locus will prevent amplification of that segment.

3. DNA Repair

Given the fundamental role of DNA in the storage and transmission of genetic information, it is somewhat surprising that this molecule is relatively unstable *in vivo* and *in vitro* (Lindahl, 1993). DNA is susceptible to spontaneous decomposition and it can be damaged by a myriad of physical and chemical agents derived from endogenous and exogenous sources (Friedberg *et al*., 2005). The effect that DNA damage has on a cell is

in large part determined by the interaction between the lesion formed and the replicative DNA polymerase (Wellinger and Thoma, 1996). Lesions frequently inhibit this polymerase, blocking DNA replication, and unless the blockage is removed the cell will die. As a consequence, all characterized species express proteins that either detect and repair DNA damage or allow the offending lesion to be bypassed. The primary function of most DNA repair proteins *in vivo* is to correct DNA damage before the polymerase reaches the lesion, thus avoiding the problem. An impressive arsenal of proteins is dedicated to this function and repair is accomplished by direct reversal of base damage, or by excising damage and using the undamaged complementary strand to restore the original sequence (Friedberg *et al*., 2005). Mechanisms for reversing a lesion are not as common as that involving excision repair. Direct reversion is of necessity lesion specific and known mechanisms affect only pyrimidine dimers and certain methylated bases. In contrast many lesions are targeted by excision repair systems. There are two types of excision repair: nucleotide excision repair (NER) and base excision repair (BER). During NER, the lesion is recognized and the repair complex nicks the DNA backbone 5' and 3' of the lesion. When the lesion is removed a 12 or 13 base gap is generated in the strand. A DNA polymerase restoring the original DNA sequence fills this gap. The proteins that catalyze NER recognize unnatural bends that are introduced by the damaged base into DNA; they do not identify specific lesions. As a consequence NER can remove many types of DNA damage and, in general, a single species encodes only one NER complex. Base excision repair utilizes a similar strategy, but there are many types of BER proteins, as they tend to be specific in their substrates, each protein recognizing a limited number of lesions. The damaged base is removed during BER, leaving an apurinic/apyrimidinic (AP) site in the DNA. The sugar remaining in the AP site is then removed by the action of an AP endonuclease and deoxyribophosphodiesterase, resulting in a one base gap in the DNA that is filled in by a DNA polymerase. If the lesion cannot be removed by one of these processes and the polymerase is stopped, there are back up systems that allow the cell either to repair the stalled replication fork and correct the damage found there, or to bypass the damage with a special class of DNA polymerases capable of translesion DNA synthesis (tls) (Battista and Earl, 2004; Friedberg *et al*., 2005). The repair of stalled replication forks employs genetic recombination, utilizing the redundant genetic information found in sister duplexes to replace the damaged DNA and permit accurate replication restart (Cox *et al*., 2000). The tls DNA polymerases are not affected by blocking lesions, and insert a nucleotide opposite the lesion to extend the growing DNA chain and effect bypass (Lopes *et al*., 2006; Rattray and Strathern, 2003; Woodgate, 1999). Thus, DNA repair processes limit the lethal and mutagenic effects of DNA damage as it arises *in vivo*, and so maintain viability and preserve the fidelity of DNA replication. For the purposes of this proposal, it is instructive to recognize that the problems DNA damage causes *in vivo* are not unlike those that occur during attempts to amplify degraded DNA evidence during STR genotyping. In both situations damaged DNA bases block the movement of a DNA polymerase. By removing the offending base prior to the polymerase reaching the site of damage *in vivo*, the cell avoids a potentially lethal circumstance. If damage to DNA evidence can be repaired *in vitro* prior to PCR, it should be possible to successfully genotype that sample. It was our contention that proteins utilized *in vivo* by all species to repair DNA damage have potential use as

reagents to facilitate the forensic typing of heavily damaged biological samples.

4. DNA double strand break repair

All species repair DNA double strand breaks by a process involving the action of RecA-class recombinases, helicases, nucleases, DNA polymerases, and DNA ligases (Friedberg *et al*., 2005). Briefly, nucleases and helicases are used to unwind the DNA at the broken end and degrade the 5'-ending strand. The region of single-stranded DNA (with a terminal 3' end) thus created is bound by a recombinase. The recombinase promotes a DNA strand invasion, to create a D-loop. DNA polymerase can extend the 3' end of the invading DNA strand. If the invading strand is then separated from the invaded DNA, it can be joined to its cognate broken end via strand annealing. Replication and DNA ligation completes the repair process.

5. *Deinococcus radiodurans* R1

There are a small number of bacterial species that have the capacity to survive massive amounts of DNA damage. During the funding period, we explored the potential of proteins isolated from *Deinococcus radiodurans* R1 (Battista *et al*., 1999). The *Deinococcaceae* are distinguished by their extraordinary ability to tolerate the lethal effects of DNA damaging agents, particularly those of ionizing radiation. Although the physiological basis of the deinococci's extreme radiotolerance has never been adequately explained, it is clear that irradiated cells are not passively protected from the damaging effects of the incident radiation. Instead, evidence argues that the deinococci suffer massive DNA damage following irradiation, and that extensive DNA repair is necessary if these cells are to survive such exposures.

The D37 dose (the dose that on average is required to inactivate a single cell) for *D. radiodurans* R1 is approximately 6500Gy, at least 200 fold higher than the D37 dose of *E. coli* cultures irradiated under the same conditions (Cox and Battista, 2005). The energy deposited by 6500Gy γ radiation should introduce approximately 200 DNA double strand breaks, over 3000 single strand breaks, and greater than 1000 sites of base damage per *D. radiodurans* genome (Cox and Battista, 2005). Despite this overwhelming genetic insult, *D. radiodurans* can reconstitute its genome in a manner that maintains the linear continuity of the parent organism, accomplishing this feat through an error-free process. The types of DNA damage generated following the irradiation of *D. radiodurans* are similar in type and quantity to what is found in ancient DNA and presumably similar to that observed as mammalian DNA is degraded after cell death.

C. Statement of hypothesis or rationale for the research

1. Forensic DNA samples that are damaged and degraded can be restored for use in STR genotyping by treatment with DNA repair proficient extracts from prokaryotic cells.

2. Intact short tandem repeats suitable for forensic analysis can be recovered with high efficiency from extensively degraded DNA samples.

II. Methods

During the three years of this projects existence, we have pursued two lines of investigation. For the first one and one half years, we attempted to use cell extracts derived from *D. radiodurans* R1 as a means of "repairing" extensively damaged sample DNA *in vitro*. This approach resulted in the creation a convenient assay for *in vitro* repair, followed by iterative exploration of the best conditions for isolating and adding extracts to the samples of interest. The last one and one half years of the project focused on the development of a technique we refer to as *in vitro* DNA double strand break repair. This method allows us to selectively capture DNA containing STR sequences, concentrating the sequences of interest for genotyping, and making those sequences available for further repair, if necessary. Each sub-project will be dealt with in separate sections.

A. The use of cell extracts to repair damaged DNA

1. Background

At the outset it was critical to establish a set of assays to facilitate the screening of cell extracts and sets of conditions for repair activities relevant to STR typing. We believed that the initial assays should be simple, rapid, cost-effective, and sensitive enough to detect weak signals that can later be enhanced by optimizing reagents and conditions. Initially, assays involving non-human DNA were used to provide a proof of concept and help identify the most successful strategies. Assays employing human DNA samples (derived from cultured cells) and the more expensive National DNA Index System (NDIS)-approved STR typing kits were planned for later stages in the investigations provided the assays worked in the simpler system.

The assay designed relied on properties of the bacterium *Acinetobacter baylyi* ADP1 (Vaneechoutte *et al.*, 2006). ADP1 will efficiently take up exogenous DNA when added to the growth medium, and if that DNA shares homology with sequences in the *A. baylyi* genome, the exogenous DNA is incorporated into the *A. baylyi* chromosome with observed transformation frequencies ranging up to 10%.   Wild type strains of ADP1 can grow on media in which 4-hydroxy benzoate (POB) is the sole carbon source (Young *et al.*, 2005). Growth on POB requires a number of catabolic enzymes found in a large operonic cluster (*pcaEFDBCHG*) in the ADP1 genome. The pathway (known as the beta-ketoadipate pathway) converts POB or benzoic acid to succinyl-CoA and acetyl-CoA is diagrammed in Figure 1.

There are derivatives of ADP1 (for example ADP6) that cannot grow on POB due to alterations in the *pcaEFDBCHG* operon (Young *et al.*, 2005). The plasmid pZR1 expresses the cloned operon and can complement the ADP6 genotype; growth on POB is, therefore, dependent on successful transformation with the appropriate plasmid. Since

introducing DNA damage to this plasmid dramatically reduces the efficiency of transformation to POB prototrophy with pZR1, the plan was to assess the function of extracts or individual DNA repair proteins on *in vitro* repair of pZR1.   Since we can control the type and extent of damage introduced into the transforming DNA, we felt this system could be used to quickly screen extracts for sub-fractions (or repair proteins) capable of restoring DNA *in vitro*.   If the efficiency of transformation in incubations containing a repair protein or extract is better than that of the damaged plasmid alone, we could infer that the repair protein or extract is repairing the damaged DNA.   A biological assay of this type has the potential to detect very small numbers of successful DNA repair events, and was expected to be especially useful in the early stages of our studies.   After incubation with extract, each damaged pZR1 DNA sample added to an ADP6 culture, spotted onto plates containing only POB as a carbon source, and then scored the next day for colonies containing repaired DNA. Controls to determine the number of colonies produced with the same DNA, incubated without extract, were included in each study. Increases in colony number were assumed to signal successful repair.

2.   Inactivation of pZR1 by gamma irradiation

The plasmid pZR1 was created by cloning the *pcaEFDBCHG* operon of *Acinetobacter baylyi* ADP1 (Doten *et al*., 1987) into the plasmid pUC18, allowing easy propagation of the plasmid in the *E. coli* strain JM109. JM109/pZR1 was grown in LB broth containing 100μg/ml ampicillin. Large-scale isolation of the plasmid pZR1 was accomplished using the Qiagen Maxiprep Protocol (QIAGEN Inc., Valencia, CA). The purified plasmid was placed in sterile eppendorf tubes in a volume equivalent to 5μg of plasmid DNA. The plasmid samples were then dried in desiccators maintained at 5% relative humidity. Drying and rehydrating did not affect the plasmids ability to transform ADP6 and provided a convenient method of storing the plasmid without need for refrigeration.

We artificially degraded pZR1 DNA by exposing the hydrated plasmid to gamma radiation. Gamma radiation "ages" the DNA, introducing the same pattern of damage found in forensics samples that have been exposed to the environment for prolonged periods of time (Paabo, 1989).   Five micrograms of pZR1 was rehydrated in 15μl of water and exposed in Model 484R ^{60}Co irradiator (J. L. Shepherd & Associates, San Fernando, CA) at a dose rate of 16Gy/min. Plasmids were removed after exposure to 10,000, 20,000, 30,000, 40,000, and 50,000 Gray (Gy). (One Gray is an absorbed dose of 100 rad.)

3.   Transformation of *Acinetobacter baylyi* ADP6

Transformation of ADP6 was carried out by adding one microgram of linearized plasmid DNA to 0.1 ml of an exponential phase culture of ADP6 (between 1.5 x 10^6 and 1x 10^{7}cfu/ml) and incubating for 30 min at 37°C before plating on M9 minimal media plates containing 5mM POB.   An EcoR1 digest was used to linearize pZR1. This step is necessary to ensure that the product of recombination is stable, arising through a double crossover event. After incubation with transforming DNA, cells were diluted to 1ml with 0.9% saline. 0.1 ml aliquots were plated, and plates stored at 37°C for 24 hours to permit

colony formation.   Transformation efficiency was determined by dividing the number of cells transformed to POB prototrophy by the number of cells to which transforming DNA was added. Titers of the number of cells to which transforming DNA was added were determined by serial dilution on plates of M9 minimal medium containing 10mM succinate. Three independent trials were conducted for each transformation with three replicates plated for each trial.

4. Generating extracts from *D. radiodurans* R1

Previous experiments indicated that *D. radiodurans* cells were able to fully recover growth within three hours after γ-irradiation at 3,000Gy under optimal growth conditions in TGY medium (Battista, 1997), suggesting that cells efficiently repaired massive DNA damages following irradiation. Subsequent studies of gene expression indicated that the proteins most necessary for conferring radioresistance of this species were synthesized one hour post-irradiation (Tanaka *et al*., 2004).   Cells extracts used in all studies were obtained from cultures of *D. radiodurans* one hour post-irradiation after exposure to 3000Gy γ-radiation.

TGY broth (200 ml) was inoculated with a 2 ml culture (2×10^8cfu/ml) of *D. radiodurans*, and allowed to grow to approximately 1×10^7cfu/ml at 30^oC. Cultures were irradiated and harvested by centrifugation at 4^oC at 6,000 x g for 15 min. Pellets were re-suspended in 20 ml 95% ethanol and held at room temperature for 10 minutes to remove *D. radiodurans*' outer membrane. The ethanol-stripped cells were collected by centrifugation at 4^oC at 6000-x g for 15 min and the resulting pellet gently re-suspended in 1 ml of 2 mg/ml lysozyme (Sigma Chemical, St. Louis, MO) in TE buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0). This mixture was incubated at 37^oC for 30 min and centrifuged at 4^oC at 6000 x g for 15 min to remove debris and unbroken cells. This extract was frozen and stored at -80^oC in 0.1ml aliquots.   To examine the *in vitro* DNA repair capability of the extracts, the frozen aliquots were thawed on ice and varying amounts added to damaged and undamaged plasmid pZR1 prior to attempts to transform that plasmid into ADP6.

B. *In vitro* DNA double strand break repair

1. Background

We have developed a procedure for the joining together two DNA molecules that share a significant region of homology, using a simple but robust *in vitro* double-strand break repair reaction. The process requires only three proteins: RecA protein, single strand binding (SSB) protein and a DNA polymerase. To date, all three proteins are obtained from *Escherichia coli*.

Figure 2 illustrates the mechanism of the method developed. Once a sequence to be retrieved is decided upon, a single stranded DNA targeting fragment (which is complementary to a region adjacent to the sequence of interest) is designed. The targeting fragment typically shares at least 18- 55 nucleotides of homology with this

DNA sequence, and must be single-stranded to initiate the reaction.

The single-stranded DNA targeting fragment is then bound by RecA protein or a RecA homologue and SSB, and used to promote a strand invasion reaction into the duplex genomic DNA sequence.

A DNA polymerase, displacing one strand of the invaded duplex DNA in the process, then extends the 3' end of the invading targeting strand. DNA polymerase also extends the 3' end of the invaded duplex. The result is a lengthened DNA that combines sequences from both DNA fragments.

To exploit the specificity of this reaction, the targeting DNA fragment includes at its 5' end a feature that allows efficient retrieval such as biotin that can be harvested with streptavidin-coated magnetic beads.   Retrieving the targeting fragment and the target in this manner allows concentration of the target sequence and further manipulation of the target (e.g. PCR amplification) without the potential interference of biological and chemical contaminants found in the mixture from which the target was obtained.

2. Protocol: *in vitro* DNA double strand break repair

a. Proteins, DNA, and Reagents

Escherichia coli proteins RecA and SSB were purified natively as described previously (Lohman *et al*., 1986; Shan *et al*., 1996). DNA substrates were derived from pUC19 DNA that was purified using $CsCl_2$ banding. DNA was digested with either AatII or PstI (NEB (Ipswich, MA)). The reaction was then subjected to phenol/chloroform/isoamyl alcohol (25:25:1) extraction. Finally, the DNA was ethanol precipitated and re-suspended in water. DNA polymerase I was purchased from NEB (Ipswich, MA). The dNTPs were purchased from Promega (Madison, WI). ATP, creatine phosphokinase, phosphocreatine, formamide, phenymethanesulfonyl fluoride and DTT were purchased from Sigma (St. Louis, MO).   SYBR Green I dye was purchased from Invitrogen (Carlsbad, CA).

b. Double-strand break repair

The AatII digested pUC19 DNA was heated in water to 100^oC for 10 min in a thermocycler, then quick-chilled in an ice-water bath for 10 min. To this DNA (1.6μM) was added a reaction mix containing the following (concentrations reported as final): RecA buffer (25mM Tris-Acetate, 5% glycerol, 3mM potassium glutamate, 10mM magnesium acetate, pH 7.5), ATP (2mM), dithiotheritol (DTT) (1mM), additional glycerol (7.5%), and an ATP-regeneration system of creatine phosphokinase (10U) and phosphocreatine (12mM). RecA protein (3μM) was added and this reaction was incubated at 37^oC for 3 minutes. SSB protein (0.8μM) was added and allowed to incubate at 37^oC for 3 minutes. The PstI digested pUC19 DNA was then added and allowed to incubate at 37^oC for 10 minutes. Finally, DNA polymerase I (1 unit) was

added with all four dNTPs (2mM), and allowed to incubate at 37°C for at least 30
minutes. Proteins were digested with 1µL of proteinase K undiluted from manufacturer
[NEB (Ipswich, MA), 20mg/ml], and incubated at 37°C for 30 minutes. Loading buffer
(20mM EDTA, 8.3% glycerol, 0.07% bromophenol blue, in water) was added to the
reaction and the entire aliquot was loaded onto a 1% agarose for electrophoresis.
Following electrophoresis, the gel was stained with SYBR Green I dye (Invitrogen
(Carlsbad, CA)), and the DNA was visualized using an Amersham Typhoon Imager
model 9410.

c.   The use of synthetic oligonucleotides in selective capture

A specific single-strand DNA capture fragment can be chemically synthesized using
available genome sequence data to guide that synthesis. The process is inexpensive and
synthetic oligonucleotides as large as 200 nucleotides may be purchased commercially.
The protocol for selective capture is simplified if biotinylated oligonucleotides specific
for the target sequences of interest can be prepared on demand.

To test the feasibility of using synthetic oligonucleotides in the double strand break
reaction, we designed biotinylated capture fragments of 50, 100, 150 and 200 nucleotides
in length. Each fragment was complementary to DNA sequence upstream of the *gltS*
gene of *Escherichia coli*, which simulated a target sequence. A double strand break
reaction was conducted as described above.

One nanomole of the biotinylated oligonucleotide is added to the reaction mixture
composed of 1mM DTT, 2mM ATP, 7.5% glycerol, 12mM phosphocreatine, and 10U
creatine phosphokinase.   RecA from *E. coli* is then added to a final concentration of
2µM, and incubated for three minutes at 37°C. Single-stranded binding protein from *E.
coli* is added to the reaction mixture to a final concentration of 0.8µM, and incubated an
additional three minutes at 37°C. 250ng of target DNA is added to the reaction mixture
and incubated for 30 minutes at 37°C before the addition of one unit of DNA polymerase
I from *E. coli* and deoxynucleotide triphosphates (2mM final concentration).   This final
reaction mixture is incubated for 30 minutes at 37°C.   The reaction is stopped by adding
Proteinase K (1mg/ml final concentration) for one half hour at 37°C, followed by the
addition of phenymethanesulfonyl fluoride (5µM final concentration).
Phenymethanesulfonyl fluoride was left in the reaction mixture for one hour.

The streptavidin-coated magnetic beads (Dynal M-280) used to recover the capture
fragment and bound target sequence from each reaction is washed twice in phosphate
buffered saline prior to use. Fifty microliters the washed bead suspension is combined
with the reaction mixture and incubated for three hours at room temperature with
agitation. The beads are separated from reaction mixture with a magnet, and washed two
times with phosphate buffered saline (37 mM NaCl, 2.7 mM KCl, 10 mM sodium
phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) and once with
deionized water. The magnet is removed; 95% formamide in 10mM EDTA is added to
the beads and the mixture heated for 5 minutes at 65°C.   A magnet is used to separate the
free beads; the supernatant is collected to retrieve the captured target DNA.

III. Results

A. Statement of Results

1. The use of cell extracts to repair damaged DNA

a. **Application of ionizing radiation reduces the transformation of ADP6 to POB prototrophy.** Table 1 illustrates that increasing doses of ionizing radiation result in a progressive decrease in transformation efficiency. A dose of 30kGy was chosen for attempting *in vitro* repair as it was felt that this dose would provide the clearest indication of an incremental increase in transformation efficiency.

b. **Addition of cell extract appeared to increase the transformation efficiency of irradiated plasmid DNA.** The assay we are using is straightforward; extracts of *D. radiodurans* R1 are added to the damaged plasmid (pZR1*) and if repair is taking place, the efficiency of plasmid transformation increases. If increases in transformation efficiency take place, it is direct evidence of *in vitro* DNA repair. Table 2 indicates the effect of *D. radiodurans* cell extracts on transformation efficiency in the three trials described. The extract increases the transformation efficiency of the irradiated plasmid 8.8 fold. However, the extract is also inhibiting transformation with the undamaged plasmid, suggesting that extract contains components that severely limit our ability to judge the effectiveness of *in vitro* DNA repair and so our ability to use the extract to improve the condition of evidence samples. Since this assay depends on the ability to a) reproducibly generate an active extract and b) specifically repair the plasmid, our efforts on this project shifted toward trying to determine what in the extract was limiting transformation efficiency of undamaged pZR1. This effort has been unsuccessful to date. We are convinced that there is at least one nuclease activity present that destroys the plasmid, but we have not been able to identify it.

2. *In vitro* DNA double strand break repair

a. *In vitro* **double strand break repair occurs *in vitro*.** To establish that *in vitro* double strand break repair was possible an assay was developed the utilized the plasmid pUC19 cleaved by a pair of restriction enzymes: AatII and PstI (Fig. 2). The plasmid pUC19 DNA digested with AatII shares homology with the ends of the PstI digested pUC19 DNA. The ends overlap by 504 nucleotides and 2182 nucleotides. Strand pairing, strand exchange, and extension of the 3' end of the exchanged strand can theoretically produce two products: one of 3190 nucleotides and another of 4868 nucleotides. So for example, invasion of the PstI digested pUC19 DNA with a single strand of AatII digested pUC19 DNA produces a joint molecule with two 3' ends that can be extended by DNA polymerase I (Pol I) to

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produce a fully double-stranded molecule of the above mentioned lengths. The results we obtained show the appearance of two products of almost equal intensity, of approximately 3.2kb and 4.8kb (Fig 3). The appearance of these two higher molecular weight bands is dependent on the presence of the two DNAs (AatII digested pUC19 that has been rendered single-stranded, and the PstI digested pUC19, which is double-stranded), RecA (in the presence of ATP), SSB, DNA PolI, and all four dNTPs. Omit any one of these components and the products are not formed (Fig. 4). Thus, we have shown that these components (a ssDNA and a dsDNA sharing a region of homology, RecA (in the presence of ATP), SSB, DNA PolI, and all four dNTPs) are both necessary and sufficient for product formation.

b. **A biotinylated oligonucleotide can be used in an *in vitro* double strand break repair reaction to retrieve a specific DNA sequence from purified DNA in solution.** As illustrated in Figure 6, a 200 base oligonucleotide initiates the double strand break reaction and allows us to retrieve a discrete set of fragments that are larger than 1500 base pairs (Lane 8, boxed in the figure). The protocol for retrieval and release was successful, but only when 95% formamide and heat treatment (65°C for five minutes) was used to separate streptavidin and biotin. Double strand break repair was accomplished when using oligonucleotides of 150 and 100 bases in length were used as well, but the double strand break reaction was not initiated with a 50 base oligonucleotide.

3. Tables

Table 1. The inactivation of plasmid pZR1 by gamma radiation. Increasing radiation dose results in a reduction in the efficiency of pZR1 to transform ADP6 to POB prototrophy, presumably by damaging DNA in a manner that prevents recombination of the wild type *pcaEFDBCHG* sequence with the ADP6 genome. Efficiencies are the mean of values obtained from three independent experiments with three replicates per experiment.

Dose (kGy)	Transformation Efficiency (%)
0	4.6 ± 0.03
5	4.2 ± 0.05
10	0.4 ± 0.01
20	0.04 ± 0.01
30	0.002 ± 0.0004
40	--[a]
50	--

[a] **Transformation not detected**

Table 2. Attempt to increase the efficiency of transformation to heavily irradiated pZR1. 50μl of undiluted extract from cultures of *D. radiodurans* R1 irradiated exposed to 3kGy γ-radiation was added to 5 μg of pZR1 that had been exposed to 30kGy γ-radiation. Irradiated pZR1 is denoted as pZR1*. Efficiencies are the mean of values obtained from three independent experiments with three replicates per experiment.

Transformation Efficiency				
Trial	pZR1	pZR1*	Extract + pZR1	Extract + pZR1*
1	8.6± 0.03	0.0008± 0.0005	0.4 ± 0.01	0.0074± 0.003
2	11± 0.01	0.002± 0.001	0.7 ± 0.09	0.016± 0.008
3	9.2± 0.05	0.002± 0.0009	1.4 ± 0.13	0.017± 0.006

4. Figures

Figure 1. The β-ketoadipate pathway of *Acinetobacter baylyi*. This pathway comprises two parallel metabolic branches. One branch, mediated by six enzymes encoded by the cat genes, converts catechol to succinate and acetyl coenzyme A (acetyl-CoA); the other branch, catalyzed by products of the *pca* genes, converts protocatechuate to succinate and acetyl-CoA by six metabolic reactions analogous or identical to those of the catechol sequence.
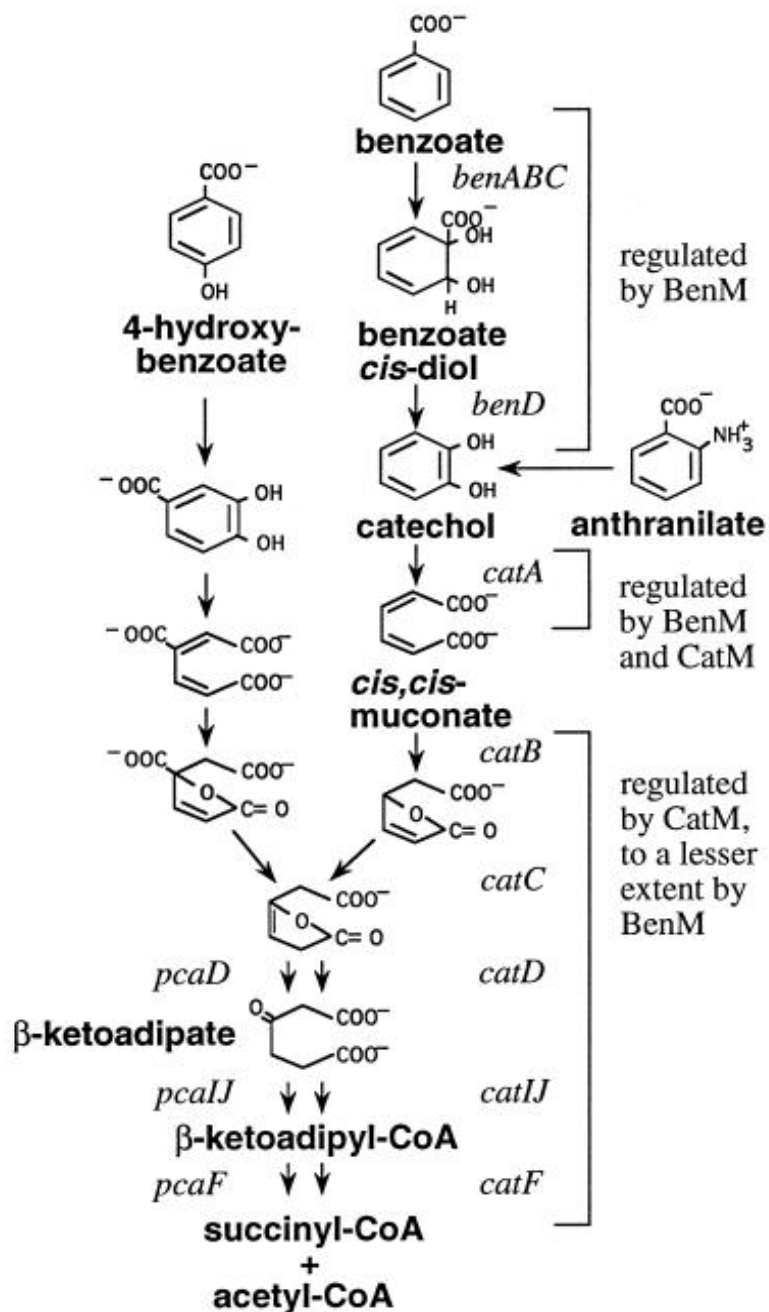

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Figure 2. A double-strand break repair reaction *in vitro*. The reaction shown is the splicing together of two DNA segments with an overlapping region of sequence, shown in red. One of the segments is rendered single-stranded, preferably by heat denaturation, nuclease/helicase action, or asymmetric PCR (PCR amplification using only one PCR primer). The 3'-ending strand is bound by RecA and SSB, and is used to invade the other duplex DNA. DNA polymerase I extends both available 3' ends and displaces one strand of DNA segment 2. The result is a new segment in which the sequences of segments 1 and 2 are combined.
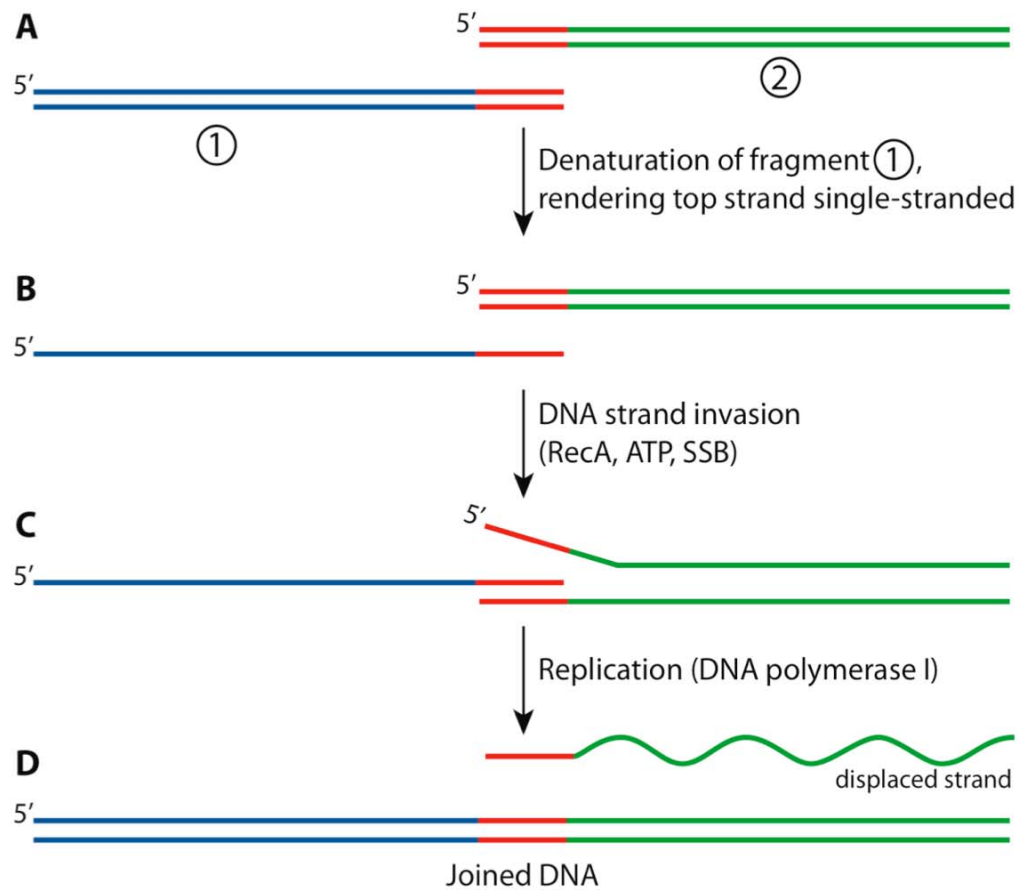
Figure 3. In the test reaction a single plasmid DNA is cut in two different ways to generate two different full-length linear segments. These two segments have all the same nucleotide sequences, but the sequences at the ends are different, and the two segments can overlap in two different ways. This situation leads to two different double-strand break repair reactions and two possible products.
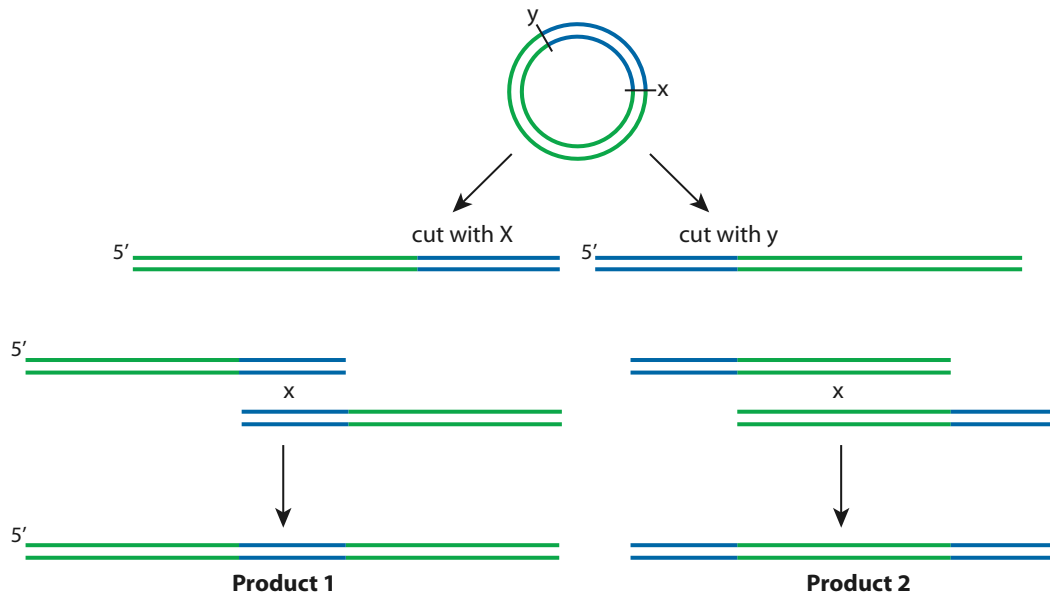
Figure 4. The plasmid used in this experiment is pUC19 (2686 bp), cleaved with either AatII or PstI. The first lane after the marker ladder shows heat-denatured AatII cleaved pUC19 (single-stranded DNA or ssDNA). Lane 2 shows PstI cleaved pUC19 (linear duplex DNA or dsDNA). Lane 3 shows both of these DNA substrates together. Lane 4 shows the ssDNA after 3 min of incubation with RecA protein (some has reannealed, some is in the well). Lane 5 shows the DNA after addition of RecA and SSB. Lane 6 shows the DNA after the addition of the dsDNA, just prior to the addition of DNA polymerase I. The final three lanes show the generation of products at 10, 20, and 30 min after the addition of DNA polymerase I. S: substrate DNA; P: expected products; I: reaction intermediates.
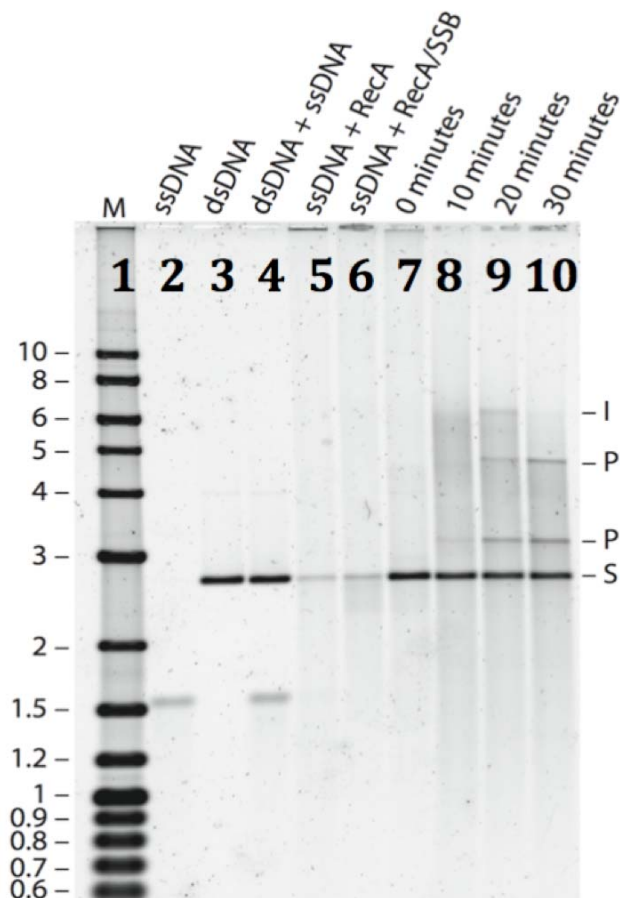
Figure 5. Requirements for double-strand break repair *in vitro*. The same marker ladder is used in lane 1, and lanes 2-4 are again simply the denatured and intact DNA segments, as in panel B. The next three lanes (5-7) show the effects of incubating the indicated components with denatured DNA (ssDNA) at 37 ºC for 46 min. Lanes 8-10 contain the same components incubated in the same way with intact dsDNA. Lanes 11-16 illustrate reactions containing both single stand and double strand DNA substrates. In lanes 11-15, one or more components were omitted. Lane 16 shows the complete reaction. Wherever DNA polymerase I was present, dNTPs were also added. S: substrate DNA; P: expected products; I: reaction intermediates.
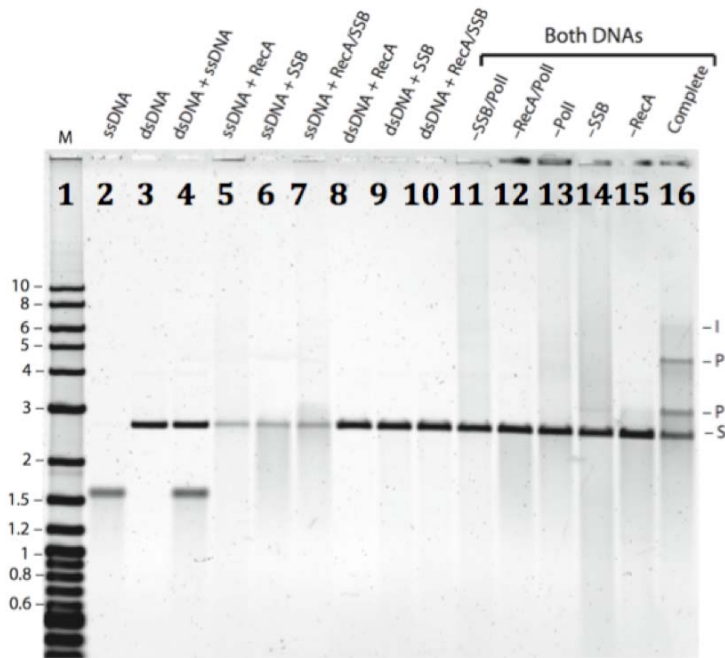
Figure 6. Retrieving target DNA using a biotinylated 200 base oligonucleotide as the capture fragment. One nanomole of a biotinylated 200 base oligonucleotide was used to target the region upstream of the *gltS* gene from genomic DNA isolated from *E. coli* MG1655. Following the double strand break reaction, streptavidin-coated magnetic beads (Dynal M-280) were used to recover captured DNA. Fragments were freed from the magnetic beads by heating them to 65°C in the presence of 95% formamide. Freed fragments are separated on 1% agarose gel and visualized with SYBR-Gold. Lanes 1 and 9 contain the TriDye -2 log ladder. Lane 2 is 1nmol of the 200 nucleotide capture fragment. Lane 3 is 150ng of *E. coli* MG1655 genomic DNA. Lane 4 combines the 200 base capture fragment and the *E. coli* genomic DNA. Lane 6 is material released from magnetic beads loaded with captured DNA when treated with water. Lane 7 is material released from magnetic beads loaded with captured DNA when treated with 95% formamide for five minutes at 25°C. Lane 8 is material released from magnetic beads loaded with captured DNA when treated with 95% formamide for five minutes at 65°C. Lane 5 is empty.
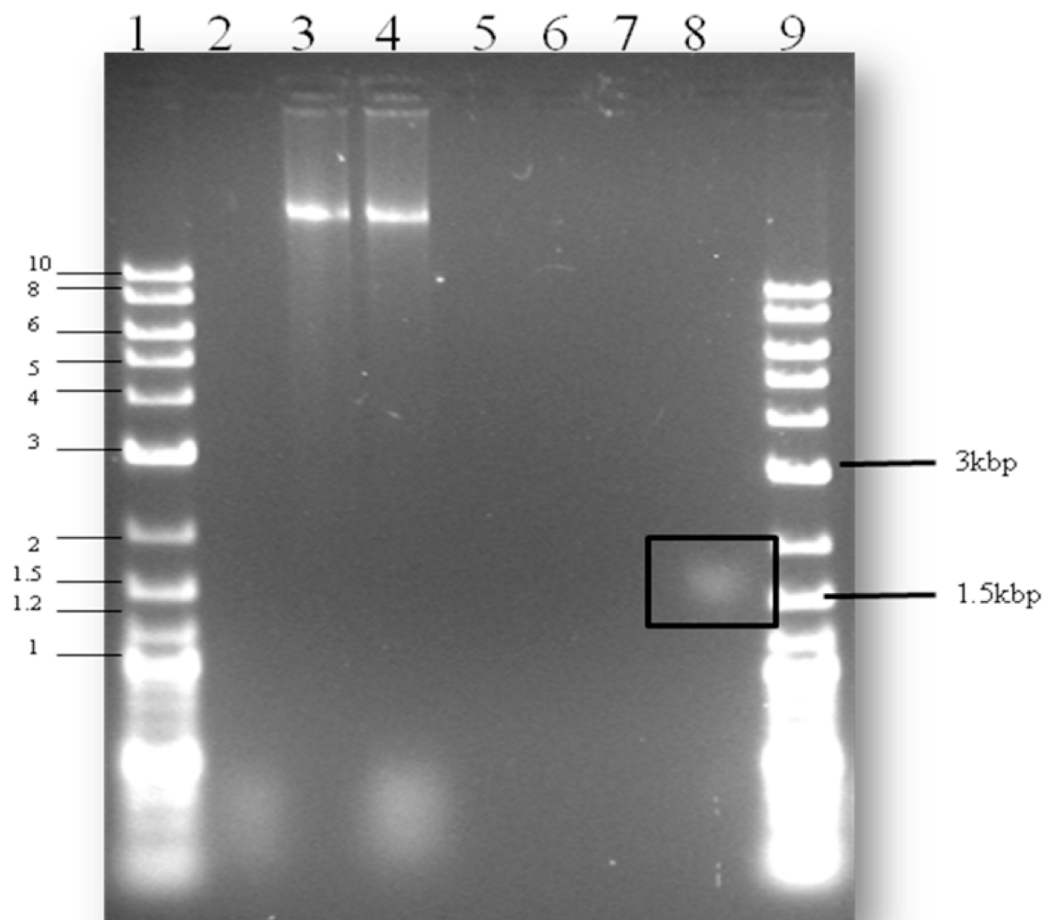
IV. Conclusions

1. Discussion of findings

a. Our efforts to develop a method for repairing damaged DNA using extracts from DNA repair proficient bacteria were unsuccessful. We expected that the assay system (conversion of ADP6 to POB prototrophy following transformation with plasmid DNA) would be sensitive enough to detect small increases in repair and that ultimately we could use these results to isolate the proteins responsible for those repairs. While we were able to see a nine fold increase in transformation efficiency in trials described in Table 2, we did not expect the extracts to reduce the transformation efficiency observed when undamaged DNA was used in the assay. Clearly this inhibitory activity limits the utility of the assay, preventing us from assessing the true extent of repair. Our attempts to isolate the inhibitor by fractionating the extract were not successful; several different protein fractions exhibited inhibitory activity. The simplest explanation assumes that there is more than one inhibitory agent. Rather than be bogged down in investigating further, we changed strategy and focused on developing a method of *in vitro* double strand break repair.

b. We have developed a procedure for the joining together of two DNA molecules that share a significant region of homology, using a simple but robust *in vitro* double-strand break repair reaction. The process requires only three proteins: RecA protein, SSB and a DNA polymerase. All three proteins are obtained from bacterial sources. As long as complementary DNA sequence is available, *in vitro* DNA double strand break repair can be used to selectively capture any DNA sequence. In addition to autosomal STRs, Y chromosome STRs, and mitochondrial DNA sequences could be retrieved by this procedure. To exploit the specificity of this reaction, the targeting DNA fragment may include at its 5' end a feature that allows efficient retrieval of that fragment and any DNA with which it has recombined. Non-limiting examples of this feature include a ligand such as biotin that can be harvested with streptavidin-coated magnetic beads, or a DNA sequence that binds tightly to a specific protein affinity column. Retrieving the targeting fragment and the target in this manner allows concentration of the target sequence and further manipulation of the target (e.g. PCR amplification) without the potential interference of biological and chemical contaminants found in the mixture from which the target was obtained.

c. This procedure has three properties that make it potentially useful for forensic applications: i) it does not require knowledge of DNA sequence on both ends of a DNA segment to be captured, as in PCR, ii) it does not introduce any enzymes that could damage or alter a target DNA sample, and iii) it should allow the retrieval of desired DNA segments even from samples that are highly contaminated with DNA from other species.

d. The capacity to selectively target DNA sequences within a complex mixture is one of the most useful features of the method, allowing one to retrieve the proverbial "needle from the haystack" because the method relies on a RecA-facilitated search for complementarity between a targeting DNA fragment and the genomic DNA sequence with a sequence of interest. The mechanism of a RecA-mediated search for homology is not well understood, but *in vitro* evidence indicates that RecA can identify its target within an excess of 200,000-fold heterologous substrate within 15 minutes – far faster than if the same DNA fragment were added to this mixture in the absence of RecA (Bazemore *et al.*, 1997; Rao and Radding, 1994)

e. As DNA fragments, it becomes more and more difficult to amplify the larger STRs. Selective capture targeting conserved genome sequence immediately upstream or downstream of an STR should permit retrieval and concentration of any intact STR remaining in that sample. This property should allow a greater number of degraded samples to be successfully analyzed.

f. Variants of RecA protein, SSB and/or DNA polymerase can presumably substitute for the *E. coli* proteins used in the initial trials. Such variants would carry conservative amino acid substitutions, but maintain equivalent functional activity. We envision that RecA mutants that are more effective than the wild type RecA protein can be created and substitute the RecA protein in the method described herein. For example, we suspect that the RecA protein mutant called E38K will enhance the reaction. This mutant is much more effective at binding to short regions of homology. Other recombinases that can substitute are (a) RecA proteins from other bacterial species (all such recombinases from bacteria are called RecA; recombinases in the same family, but from different classes of organisms, have some different names, as follows), (b) RecA family recombinases from bacteriophages, such as the UvsX protein of bacteriophage T4, (c) the eukaryotic Rad51 and Dmc1 proteins, and (d) the RadA proteins of Achaeans. Similarly, other DNA polymerases or SSBs could be used in the reactions.

2. Implications for policy and practice

The failure to type DNA evidence has significant consequences: criminal cases go unsolved with un-apprehended perpetrators continuing to inflict injury and damage on the community, investigations are extended, trials that DNA evidence might have obviated or at least shortened are extended, and wrongly-convicted persons remain incarcerated or are even put to death – all at enormous costs to the victims, the criminal justice system, and cumulatively to society.

The method described above can be used to selectively capture specific DNA sequences in forensic DNA samples and, thus, potentially recover DNA evidence samples that are quantitatively and qualitatively adequate for PCR-based genotyping.   If successful this

process will: (a) permit accurate amplification of STR loci in degraded DNA samples; (b) prevent alteration of STR repeat length in the DNA sample to be analyzed; (c) prevent any additional degradation of or damages to the DNA sample to be analyzed.   The availability of this technology should expand the capability of the forensic scientist, improving their ability to accurately genotype samples compromised by DNA degradation.

3.   Implications for further research

The utility of selective capture through *in vitro* double strand break repair has yet to be proven, and there are three major questions concerning this protocol that must be answered as soon as possible.

> **A. How efficient is the protocol at retrieving sequences of interest from degraded samples?** At present, the protocol recovers about thirty percent of the molecules targeted, but these are from pristine samples. To answer this question, we have cloned the human DNA sequences immediately upstream and downstream of the 13 STRs specified by CODIS, and which must be included in any forensic DNA typing kit used in the United States. These have been cloned into plasmids, and set up so that they can be cleaved at the STR-proximal end. We have set up conditions for asymmetric PCR so that we are getting good yields of single- stranded targeting fragments derived from these constructs (step 1 in Figure 2). We are working out conditions for the separation of these single strand-targeting fragments from the template they are derived from. This work will create a set of reagents that will allow us to construct targeting fragments that can retrieve these STRs from complex mixtures. The 3' end of each targeting fragment will be identical to the sequence of the DNA flanking one side of the STR, from a point beginning a few base pairs from one end of the STR to a point beyond the sequence to which PCR primers are targeted to amplify the STR sequence in current forensic DNA typing protocols.   We have initiated field trials with the help of the Orange County Crime Laboratory, Orange County, CA. We are working with Jeanne Putinier and Russell Baldwin, forensic scientists at that lab. We are sending them reagents to test on forensic samples, and are also designing protocols for them to use. A refinement of that effort is underway in which we will use a sample of white blood cells from a human donor, splitting them into 6 aliquots. One will be un-irradiated. The others will be irradiated to 2, 4, 6, 8, and 10kGy. Irradiation artificially ages DNA introducing the same damage encountered when evidentiary DNA is degraded in the environment. We plan to dilute these samples to levels specified by the Orange County Crime lab, soak squares of cloth in them, dry the cloth squares, and finally cut each impregnated cloth into small uniformly sized pieces. These pieces will be used to test repair protocols in our lab, and we will then send the treated samples to the crime lab where they can simply run the same analysis that they usually run. We anticipate this approach will speed up the field trial process.

B. Will the double strand break reaction be affected by the chemistry of the medium in which the forensic sample is suspended? We have not yet tested to see how robust the proteins needed for this reaction are under conditions that simulate what might be found in a forensic sample. Trials of the model reactions in which the matrix containing the sample is changed need to be conducted.

C. Are the proteins we are using for this analysis optimal? As developed initially, this protocol used the RecA, SSB, and DNA polymerase I proteins of *E. coli* exclusively. We have initiated studies to refine the double strand break repair reaction, using these proteins and the STR-derived DNA substrates. We are taking one targeting fragment, and using it to target its cognate STR region (also a cloned DNA substrate), varying RecA, SSB, and Polymerase I concentrations and other parameters. In addition, we are also doing some experimentation with other proteins including DNA polymerase V and mutants of RecA. This polymerase lacks a proofreading subunit and more easily bypasses lesions that arise in the DNA as a result of degradation. Our goal is to optimize the protocol while we complete construction of the clones for generating the oligonucleotides that will target the human STR sequences.

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