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A Genetic Analysis of the Influence of *Escherichia coli* Host Factors on Tn5 Transposition and Excision.

by

Trudy Fuchs

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

A Genetic Analysis of the Influence of Escherichia coli Host Factors on Tn5 Transposition and Excision

by

Trudy Fuchs

Adviser: Dr. Sharon D. Cosloy

The role of Escherichia coli host factors in Tn5 transposition and excision was studied. In insertion studies, the role of E. coli general recombination pathways and their components were examined for any influence on transposition. Strains were infected with a lysogeny defective derivative of λ, which harbors Tn5, and transposition of Tn5 was measured by selection with kanamycin. Insertion frequencies were statistically analysed to determine whether differences existed among the means derived from replicate experiments. Both recombination pathway and gene effects were observed. A RecF strain was observed to have 10-36 times higher insertion frequencies than strains utilizing other general homologous recombination pathways. The effects of ExoV and ExoVIII were equivalent with respect to their individual impact on insertion, however a strain with both ExoV and ExoVIII (RecBC/E pathway), had significantly higher insertion frequencies than a strain in which both were absent, (Rec-). The frequency of insertion in a Rec- strain was recF dependent. Although ExoV does not seem to be involved in insertion because RecBC and Rec- strains had equivalent insertion frequencies, the presence of ExoV influenced insertion frequencies in recF mutants. Thus, transposition is influenced by host general recombination enzymes and pathways.

Precise excision was studied in strains harboring Tn5 in the lacZ gene on an F'lac plasmid. Precise excision frequencies were calculated in strains that were isogenic except with respect to recombination pathways. In addition, thyA and polA mutants were constructed to harbor the same F'lacZ::Tn5 plasmid. A precise excision was signalled by the formation of a red pigmented papilla on a colony grown on MacConkey agar. Papillae appeared only after 48 hours of incubation. Among the host factors found to affect excision were the RecF recombination pathway, DNA Polymerase I and the concentration of deoxythymidylate. A RecF strain was reduced in precise excision by two orders of magnitude and precise excision was found to be inhibited by five orders in thyA mutants. Excision was also reduced in polA mutants, and the degree of inhibition depended on the particular mutant allele. Excision frequencies were depressed at 30^0 as compared to 37^0 and 42^0. This was due to the fact that at 30^0 the cells reached maturity at a later time than at other temperatures. We conclude that precise excisions occur in mature colonies during repair synthesis mediated by DNA Polymerase I. Several aspects of current models of excision, including a requirement for DNA synthesis and formation and filling of gaps are strengthened by these findings.

When transposons are used as tools for mutagenesis, excision can result in reversion of the mutation. These results provide a way to stabilize such insertional mutations by using strains harboring a thyA mutant allele or utilizing growth medium supplemented with fluorouracil.

Author's Note:

The format of this dissertation is in accordance with the guidelines set forth by the thesis advisory committee of this doctoral candidate and the new guidelines of the C.U.N.Y. Graduate Center. It is as follows. There is a general abstract followed by a general introduction and bibliography. These are followed by the three chapters of the thesis. Each of the chapters is a complete manuscript written for submission to The Journal of Bacteriology. The appendices in Chapters 2 and 3 are for clarification purposes for the thesis only and will not be submitted for publication.

This work is dedicated to my father, William Fuchs, who sparked my interest in biology when I was a child, and whose memory is my inspiration.

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I would like to express deep gratitude to my mentor, Dr. Sharon Cosloy. Without her capable guidance, I would never have accomplished my goal. The knowledge and skills she has imparted to me, have helped me become a scientist. I am grateful for the energy and effort she has invested in me. I share the pride I have in my work with her and want to thank her for showing me the way.

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Introduction

Recombination in E. coli

Recombination can be defined as a series of reactions catalyzed by enzymes which process donor and recipient DNAs, resulting in a new molecule with novel DNA-DNA junctions, having portions contributed by both parent molecules. Escherichia coli carries out three kinds of genetic recombination. (For a review see Kopecko, 1980). The first, homologous recombination occurs at regions of physical homolgy between host and donor DNAs. There is a requirement for a sequence of at least 20 base pairs of homolgy between donor and recipient in order for homologous recombination to occur (Shen and Huang, 1986). The second type, site specific recombination, is illustrated by the lysogenization of phage lambda, when it integrates into a specific site on its host's chromosome called the "att" site (for reviews see Nash, 1981; Gottesman, 1981). The third type of recombination is transposition.

Transposition has been studied extensively within the last decade, but the phenomenon of mobile genetic elements was brought to the attention of the scientific community by McClintock (1951), in cytogenetic studies using maize. Transposition involves small segements of DNA ubiquitous in nature, called insertion sequences and transposons. They are found residing in the genomes of many classes of organisms including bacteria, yeast (for reviews see Fink, et. al., 1980; Greer, et. al., 1980), plants, (Saedler and Nevers, 1985); nematodes (Emmons, et. al., 1983), fruit flies (for reviews see Green, 1980; Engels, 1983) and man (Paulson,

et. al., 1985). The subject of this thesis is the bacterial transposon Tn5.

Transposition is an addition event because the whole element inserts itself into the host DNA. This can occur at many sites on the recipient's chromosome. Large regions of homology between recipient and donor are not required, nor is there any limited number of specific sites of insertion in the host genome as there is with λ, although there are preferred sites for some elements (Shaw and Berg, 1979). As a result, this type of recombination cannot be classified as homologous.

Upon insertion, transposons can act as null mutations in the gene in which they have inserted since they disrupt the gene. They can also behave as polar mutations when inserted into bacterial operons (Berg, et. al., 1980a). Genomic rearrangements can occur including duplications, inversions, and deletions of parts of the host's genome (for a review see Kleckner, 1977). Duplications of 5-12 base sequences at the target site occur upon insertion. The number of sequences duplicated is element specific. Transposons can insert at the same site in the chromosomes of cells in opposite orientations. This can cause different genetic effects at the same site in different cells (Rubens, et. al., 1976).

Non-Homologous Recombination: Transposition

At the molecular level, transposition can be defined as the precise and symmetrical joining of particular nucleotides at the ends of the transposon to a target DNA molecule in which both strands have been nicked in a staggered fashion. Transposition, a non-homologous recombination event was thought not to be dependent on the recA gene product and therefore was believed not to depend on host recombination

systems (Hirschel, et. al., 1982a). However, the true role of RecA in transposition is not clear (Ahmed, 1986). Most transposons code for their own recombination enzymes termed transposases. However one, Tn903, does depend on host general homologous recombination systems (Young and Smith Grillo, 1980).

Transposable elements have historically been classified into 3 categories (for a review see Kleckner, 1981), although very recent evidence (Ahmed 1986), may make reclassification necessary. Class I elements include insertion sequences and the compound elements derived from them such as Tn5, Tn9 and Tn10. Class II elements are those like Tn3 and $\gamma\delta$, which do not have mobil insertion sequences at their ends. Class III elements are large (greater than 30kb) transposing bacteriophages such as Mu. The rest of this discussion will be restricted to Class I and II elements. Class II elements encode site specific recombination systems for resolving cointegrates although the mechanism of cointegrate formation is not a site specific recombination event. Cointegrates are fused replicons with two directly repeating copies of the transposon at the fusion junction. They may arise as intermediate structures during the process of transposition as a result of transposon replication. The site specific recombination system includes a "res" site within the element, as well as a resolvase protein encoded by the element which acts at the "res" site. (Krasnow and Cozzarelli, 1983; Grindley et. al., 1982; for a review see Grindley, 1983). This system is capable of resolving cointegrates into two separate molecules each containing the transposon. Class I elements on the other hand do not seem to contain such a system, and it has been suggested that Tn5 and Tn10 transpose via an entirely different mechanism (Berg, 1983; Berg et. al., 1984; Bender and Kleckner, 1986; for

a review see Berg and Berg, 1983). However, Tn5 can form cointegrates. The formation and or resolution of which may be dependent on the host's recombinational machinery (Hirschel et. al., 1982a, Hirschel et. al., 1982b). This is feasible since the cointegrate contains two copies of the element directly repeated, and in the case of Class I elements neither a resolvase nor a res site has been identified. Formation of Tn5 cointegrates is indeed recA dependent (Hirschel, et. al., 1982; Ahmed, 1986). Evidence to the contrary however, shows that transposase of Tn5, can catalyse homologous recombination between the direct repeats of IS50 at Tn5's two ends (Zupancic et. al., 1983). In another study, (Phadnis and Berg, 1985), evidence was presented to refute the claim that recombination between direct repeats of IS50 was transposase mediated.

Other variables used to classify elements are size, complexity, the absence or presence of antibiotic resistance determinants, the number of host sequences duplicated at the target site, and the proposed mechanism of transposition insertion. One factor common to all transposable elements is the presence of either direct or inverted repeated base sequences flanking a central segment of DNA. It is these flanking sequences that confer mobility to the element. Within these flanking sequences are the genes necessary for transposition functions. Those elements whose central DNA (within the flanking ends) does not code for any extraneous proteins, are the smaller "insertion sequences" (for a review see Starlinger and Saedler, 1976). The insertion sequences are less than 200 base pairs. Transposable elements whose central portion of DNA often codes for one or more antibiotic resistance determinants are the larger more complex "transposons", whose flanking regions are sometimes discrete recognizable insertion elements that can transpose independently

from the rest of the transposon (Berg, et. al., 1982). In this case the transposon is called composite or compound.

This research thesis will employ the use of Tn5 which is a 5800 base pair compound transposon with inverted repeating flanking sequences called IS50L and IS50R. Each repeat is 1534 base pairs long, and they differ from each other by only one base pair (Rothstein and Reznikoff, 1981). The functional transposase enzyme is entirely coded for by the right flanking sequence. IS50R also codes for a repressor polypeptide which regulates transposition frequencies (Biek and Roth, 1980; Isberg et. al., 1982; Johnson, et. al., 1982; Lowe and Berg, 1983). There is evidence that it may act in concert with host factors to regulate copy number (Johnson and Reznikoff, 1984a; Johnson and Reznikoff, 1984b). These two polypeptides share a portion of the same coding sequence but differ in their N termini (Rothstein and Reznikoff, 1981). The single base pair difference between IS50L and IS50R results in an ochre termination codon in IS50L and therefore production of truncated versions of these two polypeptides from this region. In addition it provides a promoter sequence necessary for the regulation of the kanamycin- neomycin resistance determinant in the unique central portion of the element (Rothstein and Reznikoff, 1981). The inverted repeats are themselves insertion sequences. The central portion of Tn5 (approximately 2700 bases), which in addition to coding for resistance to kanamycin and neomycin also contains two other genes encoding resistance to bleomycin (Mazodier, et. al., 1985), and streptomycin in hosts other than E. coli (O'Neill, et. al., 1984; De Vos et. al., 1984). These three determinants operate from 3 different open reading frames and may be regulated in operon fashion

(Mazodier, et. al., 1985).

Tn5 bears no sequence homology to the E. coli chromosome (Berg and Drummond, 1978). It exhibits little overall sequence specificity for selection of insertion sites but there are some preferred hotspots, (Shaw and Berg, 1979; Bossi and Ciampi, 1980). Upon insertion, Tn5 duplicates 9 base pairs of the host's target sequence, resulting is short directly repeated target sequences flanking the element.

The controversy surrounding the mechanism(s) of transposition has recently become polarized. Studies on transposition of the composite elements such as Tn5, Tn9, and Tn10 have failed to elucidate any one mechanism and depending upon the researcher, similar results are interpreted in different ways. In addition, different studies can provide conflicting answers to the same questions. Two basic models currently exist to explain transposition and each has variations with respect to the symmetry of the initial cuts made in both the target and the ends of the transposon, the order in which the events occur and the possible pathways and products that arise (for a review see Grindley and Reed, 1985). One is a replicative model in which the transposon is replicated during a transposition event forming the intermediate structure, a cointegrate. The cointegrate as described previously consists of two fused replicons with two copies of the transposon at their junctures. The cointegrate is later resolved in some cases by an element encoded resolvase, part of the site specific recombination system encoded by what are defined at this time as the Class II elements. The products of this resolution event are two molecules both containing the transposon. The second model is a conservative model which proposes a cut and paste mechanism to explain transposition. Until recently the larger composite elements in the Class I

category such as Tn5 were believed to have transposed via a conservative pathway based on experimental evidence provided by Berg, 1977; Berg, 1983, Bender and Kleckner, 1986. (For a review see Berg et. al., 1984). In contrast, Class II elements are believed to transpose via a replicative mechanism based on models by Grindley and Sherratt (1979), Arthur and Sherratt (1979), Galas and Chandler (1982), Shapiro (1979), Galas and Chandler (1981), and Ahmed (1986). However, it appeared that Tn5 was capable of forming cointegrate structures, the intermediates in the replicative pathway (Hirschel, et. al., 1982a, 1982b). Ahmed (1986), utilizing a galactose sensitivity plating assay on MacConkey agar, was able to progressively follow and phenotypically select Tn5 mediated events such as deletions, cointegrates and transpositions. He has been able to show the processive steps in the transposition of Tn5. He demonstrated that transposition is lower in recA strains, and that cointegrates form prior to transpositions. This led him to conclude that the conservative model of transposition might not be applicable to Tn5 and that replicative models of Shapiro (1979) and Arthur and Sherrat (1979) are accurate. The evidence he presented suggests that elements such as Tn5, Tn9 and Tn10 utilize the host encoded RecA protein to either form or resolve cointegrates. Neither a resolvase function nor a res site (the location of the site specific recombination in the resolution of cointegrates) has been identified for these elements. It would therefore seem logical that the recA function can substitute. Together with the emerging importance of RecA in transposition and the ambiguity with respect to models of transposition applicable to Tn5, it was of interest to investigate the effects of general homologous recombination on transposition of Tn5. Such studies might shed some light on the possible mechanism of Tn5

transposition and the influence of host recombination factors on this process.

Aside from recA, there are many host genes which influence the transposition frequencies of various elements, such as polA (Clements and Syvanen, 1981; Sasakawa, et. al., 1981); top (Sternglanz, et. al., 1981); gyr (Isberg and Syvanen, 1982); and tnm whose function is not well defined (Smirnov, et. al., 1981; Ilyina et. al., 1981). It is clear that although the element carries the essential machinery required for its mobility, there is a delicate interaction between it and the host's environment, particularly the condition of the DNA. This is true for the process of excision as well. Since to this date, there is little information available about the effect on transposition of the the recombinogenic state of the cell, this study is of value in contributing clues to the mechanisms involved in transposition.

Excision of Transposable Elements

Transposable elements can excise from the site into which they have inserted. As is the case with Tn5, the excision frequency, although site dependent, varies from 10^{-6} to 10^{-5} depending upon whether the excision is precise or imprecise (Egner and Berg, 1981). Excision events can lead to the restoration of a functional gene as in the case of a precise excision, where the entire element is lost along with one set of the duplicated host target DNA sequences (Foster, et. al, 1981). More frequently nearly precise or imprecise excision events occur, during which part of the element is left behind at the target site, thus not restoring function to the affected gene. Three types of Tn10 excision events have

been identified. Precise, nearly or imprecise, and precise excision of an imprecise excision remnant (Foster, et. al., 1981).

Excision of transposable elements appears to be independent of insertion because the transposase is not required for an excision event (Egner and Berg, 1981), and excisions are more rare than transpositions. Excision is not necessarily correlated with movement to a new site (Berg, et. al., 1980b), although we found (unpublished results) that in cells that had undergone precise excision, the kanamycin resistance determinant was detected, usually on the chromsome. This indicated that the element hopped to another location, but it is not known when the hopping had occurred. Thefore it cannot be determined whether the hopping was linked to the detected excision event or whether the transposition was replicative and independent of the excision. Excision is recA independent (Egner and Berg, 1981). Efficient excision does require the presence of inverted repeats which are present at the ends of most transposable elements. When the inverted repeats are converted to direct repeats excision is drastically reduced (Egner and Berg, 1981). Excision frequency is site dependent (Egner and Berg, 1981; DasGupta, et. al., 1987), as well as dependent on the length of the palindromes (the inverted repeats) within the segment to be deleted (DasGupta, et. al., 1987).

It has become increasingly clear in the last few years that there is a relationship between the host environment and the transposable element with respect to excision. Among these are genes involved in the repair of DNA (Lundblad and Kleckner, 1984; this thesis), DNA methylation, (Lundblad and Kleckner, 1984); various alleles of the recBrecC genes (Lundblad, et. al., 1984), and the RecF pathway of recombination, (Hickson and Emmerson, 1983; Collins, et. al., 1982; this thesis). These facts are

compatable with the fact that the element-encoded transposase does not participate in Tn5 excision. Deletions in the transposase gene do not affect excision frequency (Egner and Berg, 1981).

Current models of excision are adapted from models used explain spontaneous deletion formation in DNA similar to those observed in the lacI gene (Farabaugh, et. al., 1978). Spontaneous deletions can occur during DNA synthesis, due to copy errors in segments with inverted repeats or palindromes (Egner and Berg, 1981; Collins, 1980; Collins et. al.,1982), as well as with direct repeats (Albertini, et. al., 1982; Streisinger, et. al., 1966; Hasson, et. al., 1984)). It has been found that palindromic DNA sequences were not stable during attempts at cloning these kinds of sequences in E. coli (Collins, 1980). There are two models that currently describe the possible sequence of events that can account for transposon excision. Both involve inverted repeats within the segment to be deleted, direct repeats flanking the segment to be deleted and DNA synthesis and or repair. The two models are based on experimental evidence proposed by Egner and Berg, (1981), and DasGupta, et. al., (1987).

In the first model, the substrate containing the transposon is single stranded. Single stranded substrates can arise at a replication fork, or as a result of exonucleolytic degradation. Intramolecular base pairing at the inverted repeats of the element can give rise to a hairpin structure where the stem contains the inverted repeats and the loop contains the unique 2700 base pairs of the transposon. The secondary structure thus formed brings the direct repeats of the nine base pairs of the target DNA into close proximity forming the juncture of the stem of the hairpin. The secondary structure impedes the synthesis from the template at the juncture of the hairpin and a slipped mispairing (Streisinger, et. al., 1966)

could occur. The nascent DNA chain can slip across the junction of the hairpin from the template in the first direct repeat and reassociate within the second repeated sequence. In doing so, the nascent chain hydrogen bonds within the complementary bases of the second repeated sequence with a possible perfect restoration of the nine base pairs in the original target molecule, although an imprecise event is also possible. DNA synthesis resumes, the hairpin structure is cleaved endonucleolytically and repair synthesis restores the bases in the gap created by cleavage of the hairpin. In this way, a precise excision will have occurred when the entire element along with one of the direct repeats of target DNA has been excised.

In a second but similar model extensive DNA synthesis is not required and the substrate is double stranded DNA. In double stranded DNA inverted repeats or palindromic symmetry can give rise to a cruciform. Endonucleases cleave the cruciform leaving gaps at the regions of the direct repeats which would not have been included in the cruciform, but which would have been brought into proximity of each other because of it. Exonucleolytic degradation in a 3' to 5' direction and resectioning and reannealing in the regions of the direct repeats occur. A precise excision would arise following gap closing as a result of repair synthesis.

It is not known which polymerase may be responsible for the events described in these proposals. The data presented here will address this question. Although the excision event is recA independent, it is of interest to learn whether host recombination pathways and the enzymes involved play a role in excision.

Gene Products and Pathways that Mediate E. coli Recombination

In E. coli there are a number of gene products that govern the general homologous recombination process. The most important of these is the recA gene product. Mutations in recA eliminate the ability of the cell to carry out homologous recombination (for a review see Clark, 1973). The recA gene product is a DNA-dependent ATPase which rapidly promotes the synapsis of duplex DNA with homologous single stranded DNA, and a more slow strand exchange creating a heteroduplex molecule (Gonda and Radding, 1983). However, the recA gene is highly pleiotropic and exerts influence in other aspects of DNA metabolism including repair, mutagenesis and λ functions (for reviews see Eisenstark, 1977 and Radding, 1982). RecA promotes homologous pairing in three phases. In the presynaptic phase, single stranded DNA stoichiometrically binds with the recA protein (Flory and Radding, 1982). RecA binds to duplex DNA as well (McEntee, et. al., 1979). During the synaptic phase duplex DNA is bound to the complex and may be partially unwound by the recA protein, thus forming a D-loop structure (Cunningham, et. al., 1979), however this ternary complex is not necessarily in homologous register. The mechanism for "homology search" (Gonda and Radding, 1983) is not well understood. After homologous pairing has occurred, (synapsis), strand exchange follows. RecA protein promotes strand exchange in a unidirectional fashion displacing the 5' end of the + strand of the duplex molecule (Gonda and Radding 1983; Wu, et. al., 1982), replacing it with the new strand to produce heteroduplex DNA.

In addition to recA, a number of other genes play a decisive role in E. coli recombination pathways such as recB/recC, recE, recF, sbcA, and

sbcB, among others. In E. coli the recB/recC genes code for the synthesis of an ATP-dependent DNase called ExonucleaseV (ExoV). Recently, a third subunit of this enzyme, originally predicted by Lieberman and Oishi (1974), has been identified and designated recD (Amundsen, 1986). Strains harboring recD mutations lack ExoV nuclease activity but are recombination proficient. Mutations in either recB or recC reduce recombination proficiency to 1% of the wild-type level (Clark, 1974). It is believed that the single stranded regions necessary for recombination are provided by the RecBC enzyme (for a review see Radding, 1982). In the presence of double stranded DNA, ATP, and single stranded binding proteins, (possibly recA), the exonucleolytic functions of ExoV are suppressed and it acts to unwind duplex DNA, in the process exposing single stranded regions. These regions may eventually serve as the donor material (Telander Muskavitch and Linn, 1982).

In addition to its ATP-dependent exonuclease activity, (Oishi, 1969), it possess an ATP-stimulated endonuclease activity on single stranded DNA (Gillen and Clark 1974), it is a DNA-dependent ATPase (Oishi, 1969), and as mentioned above a DNA helicase. It has therefore been referred to as the 'RecBC enzyme' as opposed to ExoV (Telander Muskavitch and Linn, 1982), which simply implies exonucleolytic function. ExoV is inactive on closed or nicked double stranded circular molecules (Gillen and Clark, 1974), but does degrade gapped double stranded circular DNA (Karu, et. al., 1973).

Mutations in the recB/recC alleles eliminate the wild type pathway of recombination (RecBC). There are however other Rec pathways available to the cell, which restore recombination proficiency to wild-type levels. The two suppressor mutations sbcA and sbcB open the RecE and RecF

pathways, respectively, (Kushner, et. al., 1971; Barbor, et. al., 1970). Recently, a third suppressor designated sbcC (Lloyd and Buckman, 1985), has been shown to exist in commonly used RecF strains. Although the function of this suppressor gene is unknown it is responsible for the Mitomycin C and UV resistant phenotype of RecF strains.

Thus when a cell lacks a functional ExoV, two independent mutations can restore recombination proficiency. On one hand, a mutation in sbcB, the structural gene for ExoI causes a loss of that enzyme. ExoI is an ATP-independent exonuclease which acts strictly on single stranded DNA (Kushner, et. al., 1971). It has been shown in vitro that the enzyme degrades from the 3' hydroxylated end. Restoration of recombination in recB/recC strains by loss of ExoI led to speculation that the RecF pathway requires single stranded intermediates (Kushner, et. al., 1971; Barbor, et. al., 1970). It is thought to mediate the integration, of single stranded DNA into heteroduplex DNA (Lloyd and Thomas, 1983). The RecF pathway is considered a repair recombination pathway (Lovett and Clark, 1983, 1984), that depends on several other gene products, among them recF, recA, recJ, and recN. The recF gene product plays a role in recombination (Horii and Clark, 1973), repair (Wang and Smith, 1983; Thomas and Lloyd, 1983) and transposition (this thesis). The enzymology of RecF in these processes is not completely understood. The RecF protein appears to posses endonucleolytic activity, illustrated by the fact that multimeric plasmids, (circular oligomers) are linearized in its presence (Krivonogov, 1984). One possible role of recF in both recombination and repair is that it might modulate RecA in these processes. Suppression of recF mutants is accomplished by mutations designated srf and one has been located in the recA gene now designated recA441 (Volkert and Hartke, 1984; Volkert, et.

al., 1984), formerly designated tif-1 (Thomas and Lloyd, 1983).

A mutation in sbcA which opens the RecE pathway of recombination, causes a new nuclease to appear; ExoVIII, an ATP-independent DNase, whose structural gene is recE. Recent work (Fouts, et. al., 1983; Willis, et. al., 1983), has demonstrated that sbcA is a transcriptional regulatory locus such as a promoter or a translational regulatory locus instead of a previously believed repressor protein. Mutation in sbcA may create a new promoter or complete an already existing partial promoter upstream from the recE gene, however this proposal is still under investigation (Willis, et. al., 1985). Both sbcA and recE are located on the cryptic Rac prophage inserted in many E. coli strains (Gillen, et. al., 1981). ExoVIII is believed to be an isozyme of the λ red recombination function (Gillen and Clark, 1974). ExoVIII differs from ExoV in that it prefers double stranded DNA as substrate over heat denatured DNA, which is suitable substrate for ExoV (Kushner, et. al., 1974). In addition, ExoVIII is an ATP-independent exonuclease (Kushner, et. al., 1974).

Presumably, the particular pathway that is used, is determined by the combination of enzymes present or absent in the cell at any one time. The different pathways appear to process the donor DNA differently (Cosloy, 1982), however each pathway enables them to recombine to form a functional recombinant molecule.

We wish to investigate the frequency of Tn5 insertion and precise excision in strains that utilize different pathways of recombination as well as strains with other chromosomal mutations. In this way we can elucidate the mechanisms of transposition and excision and the influence of the host's recombinational machinery on these processes.

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Chapter 1.

Title:
Genetic Analysis of the Influence of Escherichia coli Homologous Recombination on Tn5 Transposition

Running Title:
Influence of Recombination on Transposition

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Abstract

The mechanism of transposition and the role of host general recombination in this process is not completely defined, however several host functions have been shown to influence the ability to transpose. We studied the possible involvement of Escherichia coli general recombination functions in transposition of Tn5 by analysing transposition in strains which are isogenic to each other except with respect to alleles that define particular Rec pathways. Insertion frequencies were determined after infection of these strains with a lysogeny defective derivative of λ which harbored Tn5. Statistical analysis applied to the means derived from replicate experiments revealed that recombination pathway and individual genes influenced Tn5 transposition. A strain which utilizes the RecF pathway of recombination exhibited elevated transposition frequencies when compared to isogenic Rec- and wild type (RecBC) strains. When a recF mutant allele was introduced into the RecF strain it lost this enhancement. A strain which utilizes a combination of the RecBC and RecF pathways demonstrated higher transposition frequencies than its isogenic Rec- counterpart. Transposition in Rec- strains is dependent upon a functional recF product because there was no significant difference in insertion frequencies between wild type and Rec- strains unless the Rec- strain harbored a recF allele, in which case transposition was significantly lower. This suggests that transposition may utilize ExoV when recF is not functional.

Introduction

Two basic models currently exist to explain transposition, (for a review see (16)). One is replicative (2,12,13,14, 27), in which the transposon is replicated during transposition forming the intermediate structure, a cointegrate. The cointegrate is resolved in some cases by an element encoded resolvase, part of the site specific recombination system encoded by Class II elements such as Tn3 (15). The second model is a conservative model which proposes a 'cut and paste' mechanism to explain transposition (7). Until recently the larger composite elements such as Tn5 were believed to have transposed via a conservative pathway (5,6,7,9).

When Tn5 cointegrates were identified, (18,19), it was not known whether they were obligate intermediates in Tn5 transposition. The formation and or resolution of Class I cointegrates may depend on the host's recombinational machinery (1). In the case of Class I elements such as Tn5, neither a resolvase nor a res site has been identified. Formation of Tn5 cointegrates is recA dependent (1,18). Ahmed , demonstrated that Tn5 transposition is lower in recA strains, and that cointegrates form prior to transpositions (1). This led him to conclude that the conservative model of transposition might not be applicable to Tn5 and that replicative models of Shapiro (27) and Arthur and Sherrat (2), implicating the formation of cointegrates as obligate intermediates, are accurate. The evidence he presents suggests that elements such as Tn5, Tn9 and Tn10 utilize the host encoded RecA protein to either form or resolve cointegrates.

With the emerging importance of RecA and the identification of cointegrates during Tn5 transposition, it is necessary to investigate the role of general homologous recombination functions in transposition other than RecA. We determined the insertion frequency of Tn5 in sets of strains that are isogenic to each other, but differ with respect to recombination pathways utilized, and have found that transposition is not equally efficient in these strains. Further, since the pathways utilize different exonucleases to process DNA, the impact that these enzymes have on transposition may be different.

Materials And Methods

Strains and phage. The strains used in this study are listed in Tables I and 1a. There are two sets. Each set consists of several strains derived from the same parent. Within sets strains differ from each other only with respect to structural and regulatory alleles involved in E. coli general homologous recombination. Enzyme assays were conducted to determine if the strains had the correct nucleases involved in the recombination pathways of E. coli. The method of Oishi (24), was used, to assay for ExoV, and the method of Weiss and Milcarek (32) was used to assay ExoI.

Tn5 was introduced into the cells by λ::Tn5 obtained from D. Berg (8). This phage has a temperature sensitive repressor and lacks the att site and int and xis genes, and therefore the ability to lysogenize. Phage stocks yielding titers of 2.0×10^9-5.0×10^{10} were grown on strain C600 in TB broth (10g of tryptone, 5g NaCl per liter of water) at 37^0. At a cell density of approximately 3.0×10^8/ml, 10mM $MgSO_4$ was added to the cells along with λ::Tn5 at an moi of 0.5-1.0. The lysate was allowed to incubate overnight.

Plating efficiencies. λ::Tn5 plating efficiencies were determined for each strain by standard phage titer assays in soft TB top agar overlays (0.75% agar) with 10mM $MgSO_4$ added to the overlay. Overnights of each strain were grown in TB broth containing 20μg/ml thymine, 10mmM $MgSO_4$ and 0.2% maltose.

Tn5 insertion assay. Transposition insertions were performed using the method of Shaw and Berg, (28). After infection the cells were diltued by a factor of ten with fresh TB broth and incubated for one hour at 30^0 with aeration, allowing for phenotypic expression of the kanamycin resistance marker. After phenotypic expression, the cells were diluted and plated on complex kanamycin medium (28), containing 30µg/ml kanamycin, and on Antibiotic Medium 3 (Difco) lacking kanamycin in order to determine the number of viable cells at plating time. In addition, viable counts of the original overnight cultures were determined. The plates were incubated for 24-48 hours at 30^0. Insertion frequencies were determined by dividing the number of kan^r colonies by the number of viable cells at plating time. Insertion frequency means derived from several replicate experiments were compared by an Analysis of Variance (29) and then a Student-Newman-Keuls Analysis (29) to determine whether there were any significant differences among them.

Results

Plating efficiencies. Escherichia coli utilizes several alternate pathways for recombination involving homologous segments of DNA. This investigation addresses the question of whether these pathways and the enzymes involved have an impact on the frequency of insertion of Tn5 into strains which utilize them. Two different sets of strains were used, (Tables1 and 1a). Within sets, strains were isogenic to each other except with respect to the pathway of recombination utilized. Since these two sets are derived from different parents and strain differences have been shown to affect transposition (17), only data within sets was compared.

The vehicle used to introduce Tn5 into the strains was a mutant derivative of λ. It has the cI857 temperature sensitive represssor (see Materials and Methods). To determine whether all strains supported the growth of the phage equally, plating efficiencies for each were tested by counting the number of plaques that were produced after their infection with λ::Tn5 in top agar overlays.

As can be seen in Table 2, the plating efficiencies ranged from 0.80-2.4x10^8/ml for strains JC5176, SDB1311, JC4584, and HF4733, and 2.9-4.7x10^8/ml for strains AB1157, JC7623 and JC5519. Thus it can be concluded that all strains support the growth of lambda with equivalent efficiency and any differences seen in insertion frequencies among the strains is due to recombination pathway effects.

Viable counts for the saturated overnight cultures ranged from 0.63-2.2 x 10^8/ml. Viable counts of the overnights did not vary from experiment to experiment. However the ability of the strains to grow after the infection and phenotypic expression incubation period varied. There

was no pattern observed for the percentage of cells surviving the procedure, either within one strain or between strains from experiment to experiment. The percent survival of Set I (Table 1) was 8-39% over 12 experiments and for Set II (Table 1a) about 30-100% over 6 experiments. The reason for the unpredictable pattern of recovery after the phenotypic expression is not known but it can be attributed to neither variation in the viability of the overnight cultures nor differential phage propagation. Variability in survival of the strains did not influence results because insertion frequencies were normalized by the number of viable cells at plating time.

Effects of recombination pathways and genes on Tn5 insertion. The data seen on Tables 3 and 4 show the insertion means of the two sets of strains tested. In order to determine whether there were any statistically significant differences among these means (within sets only), an Analysis of Variance (ANOVA) (29) was employed followed by a Student-Newman- Keuls Analysis (29) to locate where the differences resided (Appendix I). For the first set, consisting of JC5176, SDB1311, JC4845, and HF4733, the ANOVA indicated that a significant difference exists among the means of insertion frequencies of these strains, ($.0001 < p \leq .005$, $F=7.53$). The Student-Newman-Keuls test was employed utilizing Least Significant Ranges and the critical value of Q. By analysing comparisons between every possible pair, differences can be located. This test revealed that the almost 8 fold difference in insertion frequency between SDB1311 and JC4584 is the only significant difference seen among these strains. All other comparisons are 5 fold lower or less, and are not significantly different. These data, when translated into pathway and enzyme effects indicate that the RecBC/E (SDB1311) is different from

the Rec- pathway (JC4584); and that is the only statistically significant difference, among these strains. RecBC/E strains produce both ExoV (the RecBC enzyme) and ExoVIII. JC4584, the Rec- strain has neither. These two strains are therefore the least similar in this set. The other two strains in this set produce either ExoV or ExoVIII.

Analysis of the means derived from the second set of strains indicate that the strain JC7623 utilizing RecF showed 10 times higher insertion frequency than the wild type strain (AB1157) and 18 times higher insertion than the Rec- strain (JC5519). However, it was important to determine whether this enhancement would be eliminated if the pathway were rendered inoperative. Since the RecF pathway of recombination is dependent on the recF gene product, recF mutants derived from the above strains were examined, (Table 4).

Analysis of Variance performed on the data from strain Set II (see Appendix 2), revealed that there were differences among the means, ($F=14.595$; $.0001<p\leq.005$). In order to locate these differences, the Student-Newman-Keuls test was applied using the Critical value of Q to determine Least Significant Ranges (Appendix 2). As a result of this analysis, it was determined that the strains could be grouped into three separate blocks or cells, according to their means (Figure 1). One block consisted of the RecF strain, JC7623, which was different from and significantly higher than any of the other strains (mean= 2.7). The second block consisted of three strains with equivalent means. These were: the RecBC strains JC8814, and AB1157, and the Rec- strain, JC5519 (means= 0.24, 0.26, and 0.15, respectively). The third group consisted of the two Rec- and RecF strains that were also recF, JC8824 and JC8111 (means= .07,.08, respectively). The insertion frequency of the RecF strain JC7623

was 12 times higher than the average mean of the RecBC and Rec- strains from block 2 (JC8814, AB1157 and JC5519), and 36 times higher than the average mean from block 3 (JC8824 and JC8111).

Discussion

We have analysed the influence of general recombination pathways in E. coli on transposition of Tn5. Transposition, an illegitimate recombination event, was thought to be independent of these pathways and the gene products associated with them, because studies indicated that transposition was recA independent (18). The RecA protein is required for the alignment of homologous segments of DNA during general recombination (25). Although the E. coli synaptase may not be required during transposition, it may be involved in this process, because recently it has been found that transposition may be reduced in recA mutants (1). In addition to recA, E. coli possesses a battery of recombination enzymes including both ATP-dependent and ATP-independent DNases that process donor and recipient DNA during recombination (11) and define the recombination pathway the strain will utilize. It is clear that there are host functions which influence transposition of several elements.

One element, Tn903, is entirely dependent on host recombination for transposition (33). Other host factors which influence transposition are polA (10,26); top (31); gyr (21) and tnm (31,20). Therefore, although the element carries the essential machinery required for transposition, i.e. the transposase, the physical state of the DNA influences transposition efficiency. For this reason the effects of ExoV, ExoI and ExoVIII on Tn5 transposition were studied.

The insertion frequency data from this study show that HF4733, (utilizing the RecBC or wild type pathway of recombination), is as efficient in its ability to support transposition as the Rec- strain JC4584. HF4733 has ExoV activity. The Rec- strain lacks ExoV activity . The loss of

ExoV activity does not seem to impair this strain's ability to support transposition. Strain JC5176 utilizes the RecE pathway of recombination. This strain lacks ExoV, but has ExoVIII. It is also not significantly different in its transposition activity from the wild type or the Rec- strain. Since these three strains are not significantly different from each other in their ability to support transposition (Table 3, Appendix I) one can conclude that the impact that either ExoV and ExoVIII has on transposition are equivalent, and that the presence of neither of these enzymes is required for transposition.

The genotype of strain SDB1311 provides for the expression of both ExoV and ExoVIII, designating the RecBC/E pathway. The phenotype of this strain is recombination proficient and the presence of both these exonucleases provides no advantage to the element when compared with the RecBC, and the RecE pathways, which each contain either ExoV or ExoVIII. The presence of both ExoV and ExoVIII in the same cell is not advantageous to the insertion of the element over the presence of either ExoV or Exo VIII alone. The only statistically significant difference in insertion frequencies is observed when the insertion frequency of SDB1311 is compared to that of the Rec- strain, JC4584. These two strains are the least similar to each other in this set since SDB1311 has both ExoV and ExoVIII, and JC4845 has neither. Comparisons of these two strains indicate significantly different transposition efficiencies. The RecBC/E pathway is able to support transposition insertion of Tn5 almost eight fold higher than the Rec- strain, (4.59 vs. 0.59×10^{-4} respectively). It is only the combination of ExoV and ExoVIII which provides a more efficient environment than when both are absent.

There are differences among the insertion means of strain set II;

AB1157, JC7623, JC5519, JC8814, JC8824, and JC8111.The Student-Newman Keuls Analysis applied to these strains (Appendix 2), revealed that they can be grouped into three different blocks (Figure 1). Within blocks strains are equivalent in their capacity to support insertion. The first block contains JC7623. It has a significantly higher insertion frequency than any of the other strains. In the second block are JC8814, AB1157 and JC5519. The third block consists of JC8111 and JC8824.

These data show that the RecF pathway is the preferred pathway for insertion. The wild type RecBC pathway is lower than RecF but higher than Rec- and RecF strains which harbor a recF mutant allele. It appears that transposition efficiency in a Rec- strain is dependent on the presence of the recF gene product. This implies that under circumstances where recF is not functional, ExoV may be important in the process of transposition.

The enhancement of transposition seen in strains that are recB/recC-sbcB- is lost when the recF gene is mutated. This indicates that this enhancement is truely a RecF pathway effect since this pathway is dependent upon a functional recF gene product. The RecF pathway is primarily considered a repair pathway (23). It does not have a functional sbcB gene and therefore lacks ExoI. Since ExoI mutants have more stable single stranded DNA, any mechanism, including transposition, requiring regions of single stranded DNA as a possible intermediate in a recombination event may proceed better in RecF strains. RecF strains do indeed predominantly catalyse the integration of single stranded DNA during recombination (22).

Recently it was shown that Tn5 may transpose via a replicative pathway (1). In replicative models of transposition, a staggered nick is

introduced in the target molecule, presumably by the transposase. Nicks are also required at specific sites at the ends of the element. The polarity of these nicks is not known nor is the order in which they are made. It is believed that the target strands, assuming they have opposite polarity are then ligated forming a χ shaped intermediate. Semiconservative replication from the 3' hydroxly ends may result in long single stranded regions that are displaced during DNA synthesis (27). This intermediate may be protected from degradation in a RecF strain, although it could not simply be due to the fact that ExoI is not present, because in two strains in which ExoI is present, AB1157, and JC5519, there are higher insertion frequencies than in one where ExoI is absent (JC8111).

Table 1. STRAINS USED IN THESE EXPERIMENTS

Strain	Recombination Phenotype	Relevant [a] Genotype	Relevant Enzymes	Source or Reference
AB1157*	Wild type	recB+/recC+ sbcB+	ExoV, ExoI	(3)
JC7623	RecF	recB21/recC22 sbcB15	—	A.J. Clark
JC5519	Rec-	recB21/recC22 sbcB+	ExoI	"
JC8824	Rec-	recB21/recC22 sbcB+ recF143	ExoI	"
JC8814	RecBC	recB+/recC+ sbcB15 recF143	ExoV	"
JC8111	Rec-	recB21/recC22 sbcB15 recF143	—	"

* Denotes parent of set.

a Additional Characteristics:

F- thr leu B1 lacY galK ara xyl mtl proA his argE str tsx

Table 1a

STRAINS USED IN THESE EXPERIMENTS

Strain	Recombination Phenotype	Relevant Genotype [a]	Relevant Enzymes	Source or Reference
HF4733*	Wild type	recB+/recC+ sbcA+	ExoV	(4)
SDB1311	RecBC/E	recB+/recC+ sbcA6	ExoV, ExoVIII	S.D. Barbour
JC4584	Rec-	recB21/recC22 sbcA+	___	A.J. Clark
JC5176	RecE	recB21/recC22 sbcA6	Exo VIII	A.J. Clark

* Denotes parent of set.

(a) Additional characteristics:
These strains are males.

JC5176 & SDB1311 are thi gal JC4584 is thi gal his

HF4733 is thi gal thy

Table 2

Average Plating Efficiencies of Lambda::Tn5 on Host Strains

Host	pfu/ml
JC5176	2.0×10^8
SDB1311	2.4×10^8
JC5485	0.8×10^8
HF4733	1.1×10^8
AB1157	2.9×10^8
JC7623	4.7×10^8
JC5519	2.9×10^8

Averages were derived from 2-3 phage titers, by mixing host and phage in soft agar overlays. Plaques were counted after 24 hours incubation at 37^0.

Table 3

Tn5 Insertion Frequencies in Strains Utilizing Different Recombination Pathways

Insertion/Viable Cells at Plating Time

Strain	Pathway	Insertion Frequency[a]	Total Kan^r Colonies[b]
JC5176	RecE	3.09×10^{-4}	2423
SDB1311	RecBC/E	4.59×10^{-4}	3140
JC4845	Rec-	0.59×10^{-4}	559
HF4733	RecBC	2.00×10^{-4}	1034

a) For procedure and description of frequency calculation see Materials and Methods

b) Data derived from 12 individual experiments.

Table 4

Insertion Frequencies in Strains Utilizing Different Recombination Pathways
Insertions/Viable Cells at Plating Time

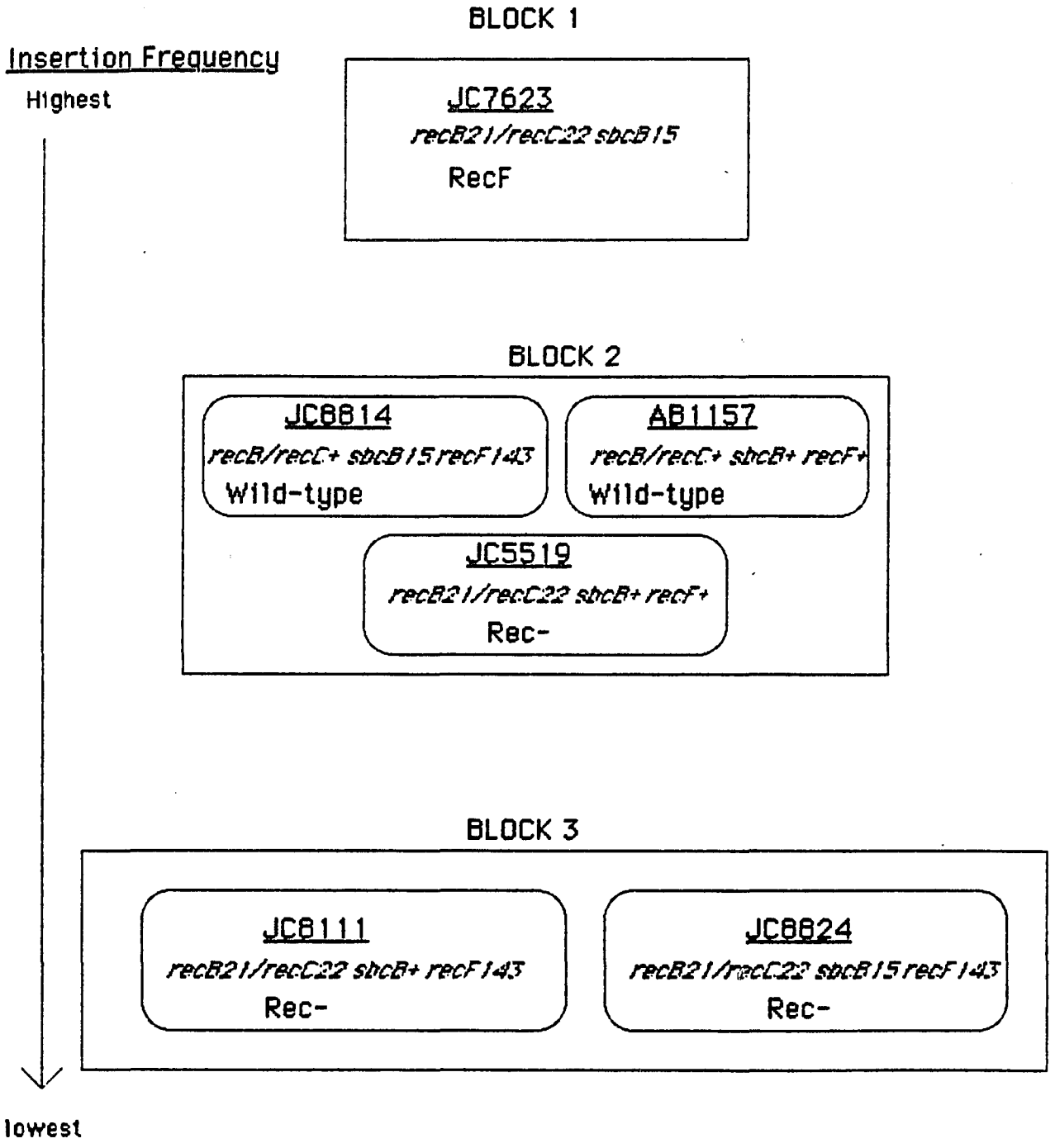
Strain	Pathway	recF143	Insertion Frequency [a]	Total Kan^r Colonies [b]
AB1157	RecBC	+	0.26×10^{-4}	475
JC8814	RecBC	-	0.24×10^{-4}	1380
JC7623	RecF	+	2.70×10^{-4}	514
JC8824	RecF	-	0.07×10^{-4}	66
JC5519	Rec-	+	0.15×10^{-4}	625
JC8111	Rec-	-	0.08×10^{-4}	60

a) For procedure and description of frequency calculation see Materials and Methods

b) Data derived from 2-6 individual experiments.

Figure 1.

BLOCKS OF EQUIVALENT STRAINS WITH RESPECT TO TN5 INSERTION



Appendix 1.

Student-Newman-Keuls Analysis ofJC5176, SDB1311, JC4584, and HF4733

SDB1311
One Way Analysis of Variance
F=7.539
$.0001 < p \leq .005$
$MS_{within}=.284$
df=38
$\alpha=.05$

SDB1311	JC5176	HF4733	JC4584
$\bar{Y}_1$	$\bar{Y}_2$	$\bar{Y}_3$	$\bar{Y}_4$
2.462	2.294	1.787	1.499

k=	2	3	4
Q=	2.864	3.451	3.802
LSR=	.8134	.9800	1.0800

k=4

2.462-1.499=.963 significant

k=3

2.462-1.787=.675 not significant

2.294-1.499=.795 not significant

Appendix 2

Student-Newman-Keuls Analysis of Strains AB1157, JC7623
JC5519, JC8824, JC8814, and JC8111

ANOVA

F= 12.561
$p \leq .0001$
$MS_{within} = .079$
df=16
$\alpha = .05$

Means:

JC7623	JC8814	AB1157	JC5519	JC8111	JC8824
2.311	1.355	1.302	1.166	0.908	0.841

k=	2	3	4	5	6
Q=	2.998	3.649	4.046	4.333	4.557
LSR=	0.237	0.288	0.319	0.342	0.410

k=6
2.311-0.841=1.47 sig.

k=5
2.311-0.908=1.403 sig.
1.355-0.841=0.514 sig.

k=4
2.311-1.166=1.145 sig.
1.355-0.908=0.447 sig.
1.302-0.841=0.461 sig.
1.302-0.908=0.394 sig

k=3
2.311-1.302=1.009 sig.
1.355-1.166=0.189 not sig.
1.302-0.908=0.394 sig.
1.166-0.841=0.325 sig.

k=2
2.311-1.355=0.965 sig.
1.166-0.908=0.258 sig
0.908-0.8411=0.067 not sig.

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Chapter 2.

Title:
Precise Excision of Tn5 is Inhibited in Strains
That Utilize The RecF Pathway of Recombination in Escherichia coli.

Running Title:
RecF Inhibits Tn5 Excision

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Abstract

The effects of the general recombination pathways of Escherichia coli on precise excision of Tn5 were investigated. A set of four strains were constructed that differed from each other only with respect to the recombination pathway utilized: RecBC, RecF, RecBC/F and Rec-. These strains have chromosomal deletions of their lactose operons and harbor the F'lacZ202::Tn5 plasmid. A precise excision event in a cell of a colony cultured on MacConkey agar resulted in the formation of a Lac+ papilla. Several different types of papillae were observed. These were purified and the activity of the lacZ gene was analysed to determine which were the result of a precise excision. A RecF strain exhibited decreased excision compared to RecBC, RecBC/F and Rec- strains. Excision of Tn5 is not dependent on the activities of ExoV nor ExoI, but it is influenced when both these activities are lacking as found in the RecF strain.

Introduction

Transposable elements can excise from the site into which they have inserted. Tn5 excision frequency in Escherichia coli, from the lacZ gene varies from 10^{-6} to 10^{-5} depending upon whether the excision is precise or imprecise (2,7). Precise excision leads to the restoration of a functional gene because the entire element is deleted along with one set of the nine base pair duplicated target sequences (9). Nearly precise or imprecise excision events occur ten times more frequently. These result in part of the element remaining at the target site and thus function of the affected gene is not restored. Precise excision of an imprecise excision remnant has been observed (9).

Excision of transposable elements appears to be independent of insertion because transposase which is required for insertion, is not required for excision (7), and excisions are more rare than transpositions. Excision is not necessarily correlated with movement to a new site (3), although this study demonstrates that in cells that undergo precise excision, the kanamycin resistance determinant is present, and in most cases is found on the chromsome.

Excision is recA independent (7). However, other E. coli recombination functions have been found to affect excision including the mutant tex alleles of recB/recC (11,15). Efficient excision requires the presence of inverted repeats which are present at the ends of most transposable elements. When the inverted repeats of Tn5 are converted to direct repeats, excision is drastically reduced (7). Excision frequency is site dependent (6,7), as well as dependent on the length of the palindromes within the segment to be deleted (6).

Current models of excision are adapted from models proposed to explain spontaneous deletion formation in DNA similar to those observed in the lacI gene (8). Spontaneous deletions can occur during DNA synthesis, due to copy errors in segments with inverted repeats or palindromes (4, 5, 7), as well as with direct repeats (1,10, 21). It has been found that palindromic DNA sequences are not stable when cloned in E. coli (4).

Currently, there are two molecular models that describe transposon excision (6,7). Both models involve DNA synthesis and repair. In one model, "slipped mispairing" (21) occurs during synthesis of the direct repeats which have been brought into close proximity in a single stranded template, presumably because a stem and loop structure has formed due to the palindromic symmetry created by the inverted IS50 ends. In the other, hairpin loops containing the element in double stranded cruciforms are cleaved. Subsequently, resectioning and reannealing of the segment containing the direct repeats occurs, followed by synthesis and repair of the resulting gap.

We have calculated precise excision frequencies in a set of isogenic strains that differed with respect to the pathway of recombination they use, and found that excision is decreased in a recB21/recC22 sbcB15 mutant (RecF), relative to strains which are Rec-, or utilize the RecBC or combination of RecBC and RecF pathways.

Materials and Methods

Strains. Table 1 lists the strains constructed and used in this study. Parental strain E42-202 was mutagenized with N-methyl-N'-nitro-N- nitrosoguanidine (19) and a His- mutant was selected after a double ampicillin enrichment (19). The resulting strain, SC709, was treated with trimethoprim to select spontaneous Thy- mutants (19). These mutant alleles were used as handles for positive selection after P_1 transduction to introduce recBC and sbcB alleles into the strains (19) and create a set of isogenic strains which differ only with respect to their Rec pathways.

Precise excision assay. Precise excisions were determined by a modification of the procedure of Egner and Berg (7). Stationary phase cells stored on slants in the cold, were eluted into 1.0ml of 1x minimal salts (17) and diluted and plated out on MacConkey agar (Difco) and were incubated at 37^0 for 43-48 hours. Precise excisions were identified as red papillae on the surfaces of Lac- colonies, because a precise excision of Tn5 from the lacZ202 allele on the F'lac plasmid harbored by these strains results in the restoration of the gene (3). The Lac+ phenotype generated, is due to an excision event since the chromosomal lactose genes have been deleted, and cannot revert.

Papillae were counted at the surfaces of the colonies with the aid of a dissecting microscope and hand held counter. Each colony possessed from 0 to confluent numbers of papillae. The precise excision frequency was calculated by dividing the number of papillae by the total number of cells. In cases where the surfaces of the colonies were confluent with papillae the precise excision frequency was estimated as 10^{-6}. This

frequency was determined by plating strain SC720, a Trp+ derivative of SC709, on minimal medium (17) with lactose as a sole carbon source which selected cells that had undergone precise excision.

Determination of the number of cells/colony. The number of cells per colony were determined in the following way. The strains were eluted from the slants, serially diluted and plated out on MacConkey agar at the dilution necessary to achieve approximately 10 colonies per plate. After 48 hours of incubation at 37^0, 10ml of minimal salts (17) were used to wash the colonies off the plate. The surface of the plate was scraped to insure thorough washing of the cells. This 10ml aliquot of cells was plated out on penassay medium for viable counts.

Papillae characterization. After 43-48 hours of growth on MacConkey agar (Difco), colonies exhibited different types of papillae morphology; lightly pigmented (pink), dark pigmented (red), and non-pigmented papillae. Samples of each type of papillae were purified and analysed for genetic characteristics. These included kan^r, the presence of the F', the synthesis of β-galactosidase, the ability to transfer lacZ and/or kan^r via mating. Cells from totally smooth colonies or non-papillated colonies were also analysed. Since papillae are very small in comparison to the rest of the colony, an inoculating needle with a very fine wire and a dissecting microscope was used during purification procedures.

β-galactosidase assays were performed according to Miller (19). A precise excision was determined to have occurred in those cases where the isolates displayed wild type levels of enzyme activity.

Results

Characterization of papillae. Excision frequencies were calculated in four strains that were isogenic except with respect to the recombination pathway utilized. The strains, SC709, SC711, SC703 and SC704 utilzed the RecBC, RecBC/F, RecF and Rec- pathways respectively. Red, light pink and non-pigmented papillae as well as entirely smooth or non-papillated colonies were observed to occur after cells were plated on MacConkey agar and colonies reached maturity. Light pink and non-pigmented papillae, remained so even after one week. It was necessary to determine which kind of papillae arose as the result of a precise excision and to determine whether smooth colonies had lost the plasmid that harbored Tn5. Inocula from 21 red pigmented papillae, 9 lightly or pink pigmented papillae, 22 non-pigmented papillae and 12 smooth colonies (non-papillated) were analysed to determine the condition of the lacZ allele on the plasmid and the location of the element in the cell since all isolates purified on MacConkey agar were always kanamycin resistant.

To determine whether the plasmid was present in these isolates they were cultured on minimal medium that lacked proline. All of the pigmented (both light and dark) and non-pigmented papillae were Pro+ and therefore harbored the plasmid. Smooth colonies for the most part were due to plasmid segregation since of 12 smooth colony isolates tested, 7 were proline auxotrophs and 2 isolates were Pro+. Three did not grow on either unsupplemented minimal medium or minimal medium supplemented with proline. Since all isolates tested were kanamycin resistant, it is

possible that Tn5 transposed to a gene resulting in another auxotrophic requirement.

To determine the location of Tn5 in cells from papillae (red, pink, and non-pigmented) as well as from smooth colonies, conjugations were carried out using these strains as donors and a kanamycin sensitive female as host. Two levels of kanamycin transfer were observed and scored based on the numbers of transconjugants. These were: 1) weak levels of transfer which had from 0-30 transconjugants on a plate, and 2) high levels of transfer which had thousands to near confluent numbers of transconjugants on a plate. Low level transfer of kan^r (or none at all) was mediated by cells from all but one of the pigmented papillae and the lightly pigmented papillae. In comparison, high levels of Kan^r transfer were exhibited by cells from the non-pigmented papillae and the smooth colonies that were Pro+. Cells from one of the pigmented papillae also promoted high levels of Kan^r transfer. In this strain, Tn5 may have precisely excised from the lacZ gene and hopped to another site on the plasmid. Alternatively, the strain may carry two plasmids one which harbors the transposon and one which had undergone an excision.

To determine the conditon of the lacZ gene on the plasmid, β-galactosidase assays were conducted. The results of these biochemical assays indicate that cells of non-pigmented papillae produce no β-galactosidase, cells from pigmented papillae produce β-galactosidase, and cells from lightly pigmented papillae produce β-galactosidase at slightly elevated levels with respect to normally pigmented papillae and wild-type controls (Table 2). Perhaps lightly pigmented papillae contain cells that had undergone duplications of all or portions of lacZ enabling the cell to produce the enzyme in excess. Alternatively, the regulatory

region of this operon may have undergone rearrangement creating an over-active promoter. In both the case of the non-pigmented (where the assay did not detect any enzyme) and lightly pigmented papillae, DNA rearrangements cannot be ruled out to explain these results since rearrangements are associated with the presence of transposable elements. When Tn5 excises, imprecise excisions occur, and it is possible that cells in the non-pigmented papillae had undergone imprecise excision, leaving behind enough active protein so that papillae could form, but not enough to be able to cause a color shift in the MacConkey agar, nor to detect in the assay as performed here.

Isolates from the papillae were also analysed for their ability to transfer Lac+ to Lac- hosts. Pro+ isolates from purified papillae were mated along with the original parental strains SC709, SC703, SC711 and SC704. The results of the matings were scored in the same way as the kanamycin matings. In the parental strains as expected, there was no lactose utilization capabilty transferred. This was true for cells grown from smooth colonies as well as non-pigmented papillae. Pigmented papillae and lightly pigmented papillae on the other hand, transferred lactose with great efficiency.

Thus, cells of pigmented papillae both dark and light, produce β-galactosidase, transfer lac genes, have lost the kanamycin resistance determinant in most cases from the plasmid but in all cases from the lacZ gene and arise as the result of precise excision. Lightly pigmented papillae were rare. Cells of non-pigmented papillae, on the other hand, are usually Lac-, can transfer kanamycin resistance on mating but cannot transfer Lac+. They remain non-pigmented even after a week of incubation at 37^{0}. Non-pigmented papillae, although abundant in some strains were not

precise excisions, and were not characterized further.

Comparison of precise excision in strains that utilize different recombination pathways. SC709, which utilizes the wild type RecBC recombination pathway, and SC711 that utilizes both RecBC and RecF pathways (RecBC/F), both exhibited colonies confluent with papillae and thus wild type excision frequencies; $\geq 10^{-6}$ (Table 3). This value was derived under conditions where a Trp+ derivative of SC709 was grown in liquid minimal medium and then plated on minimal lactose medium, where Lac+ colonies represent excision events. This is in agreement with the frequency of excision from this allele, that has been previously reported (7).

It appears that the RecBC and the RecBC/F pathways are equivalent in the ability to support precise excision. However, in the strain that utilized RecF alone (SC703), precise excision is reduced by approximately 2 orders of magnitude. The Rec- strain (SC704) showed a decreased precise excision frequency after 48h of colony growth. It is not clear however whether this is truely a pathway effect or whether it is due to smaller colony size, since after 48 hours of incubation at 37^0 SC704 colonies contained at least 10 fold less cells than other strains. When allowed to incubate an additional 24 hours at 37^0, SC704 colony size increased and the excision frequency increased to 4.7×10^{-7}. After 96 hours the papillae were confluent.

Discussion

The host environment plays a major role in determining the frequency with which a particular element will excise. Genes directing the conformation and condition of the bacterial chromosome such as uvrD (16), texA (15), mutD5 (16) affect precise excision. There are many E. coli mutations that affect precise excision of various transposons (Appendix 2). It was therefore our intention to determine whether precise excision of Tn5 was influenced by the particular pathway of genetic recombination that a strain utilized. Although pathway effects were observed for the insertion of Tn5, (Fuchs, PhD. thesis), no predictions could be made about precise excision based on this information, since these two are separate events. We have found that Tn5 insertions are elevated in a RecF strain, (Fuchs, PhD. thesis), and in this study that precise excisions are significantly reduced in a RecF strain.

Cloned palindromic sequences are 5-10 fold more stable in a recB21sbcB15 host (5). This is consistent with results presented here that precise excisions are about 100 fold lower in SC703. Excision may be reduced in a RecF strain because single stranded regions may be stabilized by increased binding of RecA (14), and therefore may be unable to form the secondary structure purportedly required for an excision event to occur (6,7). This hypothesis is consistent with the finding that ssb mutants show elevated levels of excision (16). This stability is not due however to the loss of ExoI in these strains as hypothesized by Collins (5), since strains SC709 and SC711 which are isogenic except with respect to ExoI

production display equivalent precise excision frequencies.

We have found that the RecBC enzyme is not required for precise excision since in a Rec- strain (recB21/recC22) wild type excision is seen, when colonies are allowed to mature an additional 48 hours. It appears from this and other findings (5,15) that the recombination functions of the RecBC enzyme are not essential for precise excision. Recombination proficient mutant alleles of RecBC designated as texA and texB stimulate excision presumably by altering the interaction between the direct repeats by the helicase activity of the enzyme (11,15). Current models of recombination propose that the RecBC enzyme may be responsible for providing single strand substrate at the initiation of excision (6,7). The data presented here do not conflict with this proposal since it may be possible that excision occurs in this way in the wild type (SC709) and RecBC/F (SC711) strains. In the Rec- strain (SC704), precise excision may be carried out from double stranded template as proposed in an alternative model (6).

In summary strains that utilize the RecF pathway of recombination are decreased in their ability to support precise excision of Tn5. This may be due to the stabilization of single stranded regions of DNA in these strains.

Table 1.

STRAINS USED IN EXCISION STUDIES

Strain	Relevant genotype/phenotype	Recombination Pathway	Source/Ref
E42-202[a]	Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	RecBC	D. Berg
SC703	*recB21/recC22 sbcB15* Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	RecF	This study
SC704	*recBC21/recC22* Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	Rec-	"
SC709	Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	RecBC	"
SC711	*sbcB15* Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	RecBC/F	"
SC720	Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	RecBC	"
ZIP514	Δ *lac3*	—	B Bachmann
W3110	Lac+	—	(2)

[a] E42-202 is the parent of the SC strains listed in this table. In addition to the relevant markers it also harbors ΔtrpE5. SC709 and SC704 are His- because they were transduced to auxotrophy to manipulate the sbcB allele (See Materials and Methods). SC720 has been transduced to Trp+.

Additional markers harbored by Zip514 are trp49 relA1 rpsL150 tsx-93 spoT1 λ-

See Appendix 1 for the strategy employed in the construction of these strains

Table 2. Beta-Galactosidase Assays of Purified Isolates From Various Sources

Source of sample cells [b]	Average Units of Activity [a]	Range of Activity	[c]
Red Pigmented Papillae	6586	4845-9894	(12)
Pink Pigmented Papillae	10,420	8625-11,923	(4)
Non-pigmented Papillae	<50	0-53	(11)
Smooth Colonies	<10	0-23	(4)
ZIP514	<10	0-9	(2)
W3110	5205	4066-6250	(3)

(a) Specific activity was calculated according to Miller (19) in the following way:

$$\text{Units} = 1000 \times \frac{OD_{420}}{\text{time} \times \text{volume} \times OD_{600}}$$

OD_{420} read from reaction mixture.

OD_{600} read from cell density before assay.

Uninduced levels of activity have been subtracted from the average units of activity.

(b) Enzyme assays performed on purified clones of cells from above sources.

(c) The numbers in parentheses are the number of individual clones tested.

ZIP514 carries a lactose operon deletion. W3110 is wild type.

Table 3.

Precise Excision Frequencies of Tn5 From Hosts Utilizing Different Pathways of Recombination

Strain	Pathway	Precise Excision[a] Frequency (48h)			Total Colonies
SC709	RecBC	$\geq 10^{-6}$ [b]			1592
SC711	RecBC/F	$\geq 10^{-6}$			340
SC703	RecF	3.0×10^{-8}			401
		48h	72h	96h	
SC704	Rec-	1.9×10^{-9}	4.7×10^{-7}	$\geq 10^{-6}$	357/178/308

(a) The excision frequency was calculated in the following way:

$$P.E. = \frac{\text{\# papillae}}{\text{\# cells/col} \times \text{\# colonies}}$$

The number of cells/colony are as follows: SC709: 12.1×10^{8} SC703: 3.0×10^{8} SC711: 2.2×10^{8} SC704: 0.2×10^{8}

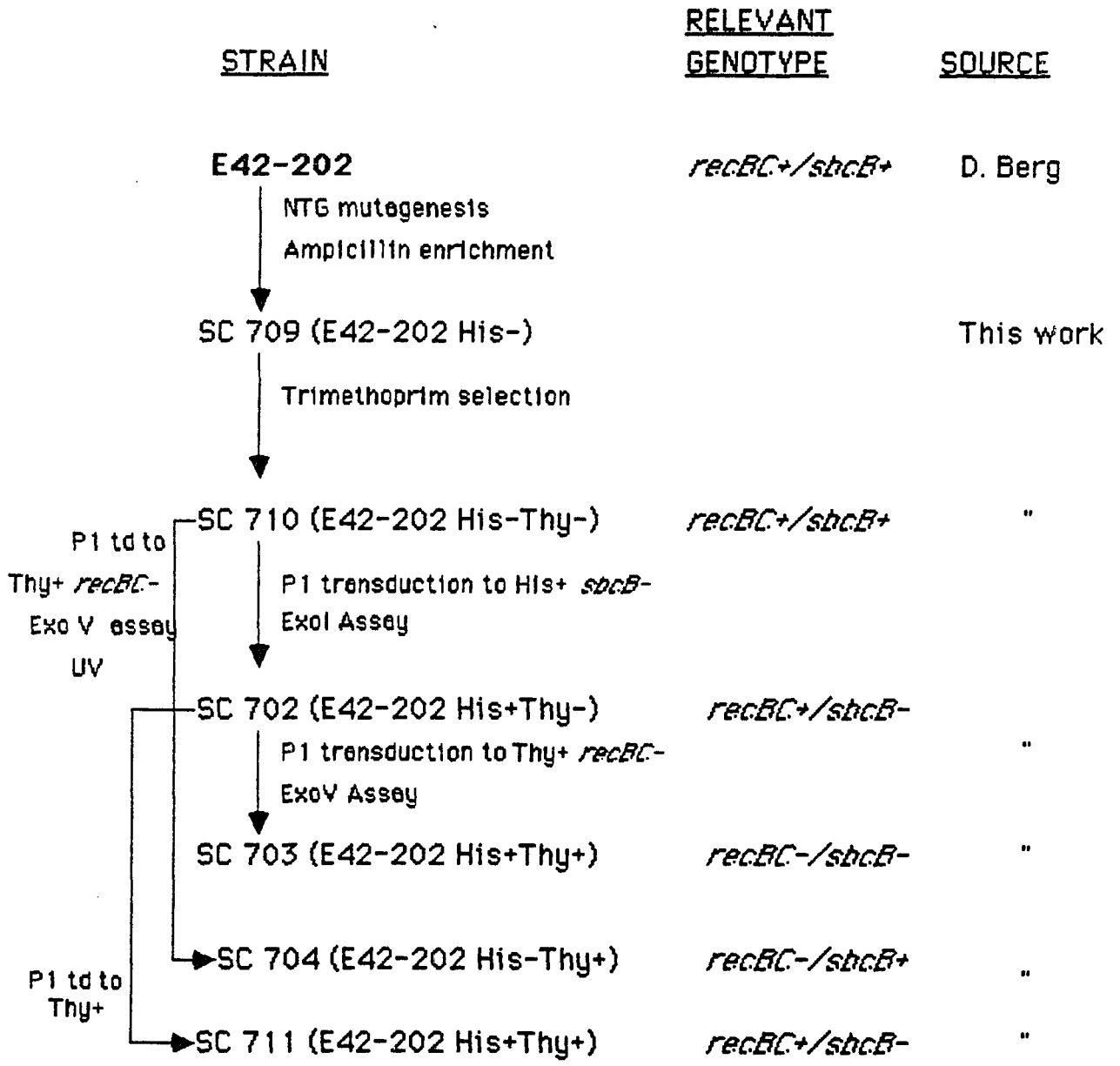
After 48 hours SC704 colonies were immature and grew larger upon further incubation. The other three strains were mature after 48 hours.

(b) When a Trp+ derivative of SC709 was grown in minimal medium and plated on minimal lactose plates the excision frequency was calculated as 10^{-6} which was defined as the lower limit of precise excision when papillae were confluent.

Papillae were counted after 43-48 hours of growth except for SC704 which was also analysed after 72 and 96 hours.

Appendix 1.

STRAIN CONSTRUCTION STRATEGY



All strains have their chromosomal lac operon deleted and contain *F'proIacZ202::Tn5*.

Appendix 2

E. COLI. MUTATIONS AFFECTING PRECISE EXCISION OF TRANSPOSONS

Mutation	Gene/Prod.	Effect on Excision	Reference
uup	unknown	stimulation Tn5,Tn10	12
him	host integration prod. for λ	reduction Mu	18
recBCsbcB	ExoV,ExoI (RecF)	reduction Tn5,Tn10	5, This study
thyA	thymid. synth.	reduction Tn5	T. Fuchs (PhD. thesis)
polA	Polymerasel	reduction Tn5	"
texA,texB	RecBC	stimulation Tn10,Tn5	15
del	unknown	reduction IS1	20
ferA	sex factor gene	elimination of stimulation by ferB	13, 22
ferB	sex factor gene	stimulation Tn5,Tn10	as above

Appendix 2 continued.

Mutation	Gene/Prod.	Effect on Excision	Reference
ssb-113	single strand binding protein	stimulation Tn10	16
dam	DNA adenine methylation	stimulation Tn10	"
mutD5	PolymeraseIII	stmulation Tn10 helicase	"
uvrD	DNA helicase II	stimulation Tn9, Tn10, Tn5	"
mutH	mutator gene mismatch repair	stimulation Tn9,Tn10	"
mutS	"	stimulation Tn9,Tn10	
mutL	"	stimulation Tn10	"

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Chapter 3.

Title:
Precise Excision of Tn5 is Inhibited in thyA and polA Mutants of Escherichia coli.

Running Title:
Excision is inhibited in thyA and polA mutants.

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Abstract

Precise excision of Tn5 from the lacZ202::Tn5 allele harbored by an F'lac plasmid was studied in thyA and polA mutants. Strains were plated on MacConkey agar where precise excisions give rise to red papillae in Lac- colonies. The number of papillae were scored for each strain and excision frequencies were calculated. Thy- strains were reduced in precise excision by five orders of magnitude. In addition papillae were observed only after the initial 24 hours of incubation when colonies had already reached maturity. Excision frequencies were lower at 30^0 than at 37^0 or 42^0 even though colonies grown at 30^0 were mature by 48 hours of incubation. The polA mutants were reduced in excision but the extent of inhibition was allele dependent. The thyA effect was reversible with the addition of thymine to the medium of Thy- strains. Excision inhibition was also seen in wild type strains when thymidylate synthase, the thyA gene product, was inhibited by 5-fluorouracil and trimethoprim. These observations suggest that excision occurs specifically during repair synthesis mediated by DNA Polymerase I in mature colonies. Elements of current models of transposon excision have been strengthened by these findings.

Introduction

Transposable elements can excise from the site into which they have inserted. Tn5 excision frequency, from lacZ varies from 10^{-6} to 10^{-5} depending upon whether the excision is precise or imprecise (12). Precise excision leads to the restoration of a functional gene because the entire element is deleted along with one set of the nine base pair duplicated target sequences (14). Efficient excision requires the presence of inverted repeats which are present at the ends of most transposable elements. When the inverted repeats of Tn5 are converted to direct repeats, excision is drastically reduced (12). Excision frequency is site dependent (10,12), as well as dependent on the length of the palindromes within the segment to be deleted (10).

Current models of excision are adapted from models proposed to explain spontaneous deletion formation in DNA similar to those observed in the lacI gene (13). Spontaneous deletions can occur during DNA synthesis, due to copy errors in segments with inverted repeats or palindromes (7, 8,12), as well as with direct repeats (1,15, 24). It has been found that palindromic DNA sequences are not stable when cloned in E. coli (7).

Currently, there are two molecular models that describe transposon excision (10,12). Both models involve DNA synthesis and repair. In one model, "slipped mispairing" (24) occurs during synthesis of the direct repeats which have been brought into close proximity in a single stranded template, presumably because a stem and loop structure has formed due to the palindromic symmetry created by the inverted IS50 ends. In the other, hairpin loops containing the element in double stranded

cruciforms are cleaved. 3'-5' exonucleolytic degradation then occurs resulting in resectioning and reannealing of the segment containing the direct repeats. This is followed by synthesis and repair of the resulting gap. It is not known which polymerase may be responsible for the events described and until now DNA polymerase I has not been investigated in this capacity. The activity of DNA Polymerase I shown here to be involved in excision strenghtens current models of excision implicating 3'-5' exonucleolytic activity and DNA synthesis in the formation and repair of gaps, respectively.

In addition the observation that Tn5 is more stable in Thy- hosts has practical applications. Since transposons are often used as tools for mutagenesis in the construction of insertion mutants, instability of these mutations can be significantly reduced if the strain also harbors a thyA mutant allele.

Materials and Methods

Strains. Table 1 lists the strains constructed and used in this study. For the construction of these strains (Appendices1-3) nitrosoguanidine mutagenesis, ampicillin enrichment, generalised transduction, trimethoprim selection of spontaneous Thy- mutants, and matings have been described in Miller (20).

Precise excision assay. In order to quantify precise excisions, an assay was modified which was originally reported by Egner and Berg (12). To determine precise excision frequency, stationary phase cells stored on slants in the cold, were eluted into 1.0ml of 1x minimal salts (19) and then diluted and plated out on MacConkey agar (Difco). Different concentrations of thymine, fluorouracil or trimethoprim were added to the MacConkey agar prior to pouring. Plates were incubated at 30^0, 37^0 and 42^0 for 43-48 hours. Different papillae types including red pimented, lightly pigmented and non-pigmented were characterized (Fuchs PhD. thesis) and it was determined only red pigmented papillae were the result of a precise excision event. The Lac+ phenotype generated, is due to an excision event from the lacZ202 allele on the F'lac plasmid harbored by these strains (3). The chromosomal lactose genes have been deleted, and cannot revert.

Papillae were counted at the surfaces of the colonies with the aid of a dissecting microscope and hand held counter. Colonies possessed 0 papillae to confluent numbers. In most cases each strain had either colonies that were confluent or colonies that had countable numbers of papillae with only rare confluent colonies present. For strains where the number of papillae were countable the precise excision frequency was

calculated by dividing the number of papillae by the factor of the total number of colonies and the number of cells per colony, (i.e. the total number of cells, see below). In strains with confluent papillae the precise excision frequency was estimated as 10^{-6}. This frequency was determined by plating strain SC720, a Trp+ derivative of SC709, on minimal medium (19) with lactose as a sole carbon source which selected cells that had undergone precise excision.

In experiments using polA strains (Table 1) plates contained a mixture of colonies with confluent and countable papillae. In this case, excision was reported on the basis of % confluent colonies. Colonies that were not confluent usually had countable numbers of papillae. In some experiments using these mutants, the cells were spread as confluent lawns on MacConkey agar and paper disks were applied to the center of the lawn containing either methyl methane sulfonate (MMS) or minimal salts, (SSA). In these cases zones of cell and papillae inhibition or growth were measured to determine the effects of MMS on excision.

Determination of the number of cells/colony. The number of cells per colony were determined in the following way. The strains were eluted from the slants, serially diluted and plated out on MacConkey agar at the dilution necessary to achieve approximately 10 colonies per plate. After 48 hours of incubation at 37^0, 10ml of minimal salts were used to wash the colonies off the plate. The surface of the plate was scraped to insure thorough washing of the cells. This 10ml aliquot of cells washed from the MacConkey agar was plated on penassay medium, and the viable count determined.

Growth curve. The growth curve of SC709 was measured. SC709 was grown overnight in LB medium (10g tryptone, 5g yeast extract, 5g

NaCl per liter of water) at 30^0, 37^0 and 42^0 and diluted to 1/100 in fresh medium. The cells were incubated in a rotary waterbath at the appropriate temperature, and growth was monitored by measuring OD in a Klett Summerson colorimeter every 20-40 minutes for 5-7 hours.

The generation time was calculated from the growth curve. The minimum doubling time (MDT) defined as the fastest interval required for a population doubling was recorded for each temperature (Figure 1).

Results

Calculation of precise excision. Excision frequencies were determined at three different temperatures and were found to be between 100-1000 times lower at 30^0 than at 37^0 or 42^0 (Table 2). The minimal doubling times (MDT) were calculated to determine whether or not reduced excision frequencies observed at 30^0 were due to slower growth rates. SC709, in fact, showed a longer MDT at 30^0 (47') than at 37^0 (27') and 42^0 (27') (Figure 1). However, at 48 hours, the time the colonies were observed for papillae, all colonies were mature since there was little variation in the number of cells/colony for SC709 and SC711 at the three temperatures (SC709, $6\text{-}12 \times 10^8$; 711, $2\text{-}3 \times 10^8$). However due to the slower growth rate at 30^0 they reached maturity later at this temperature.

Effect of <u>thyA</u> mutation on excision. Strains that were Thy- (SC710 and SC702), rarely produced pigmented papillae, and colonies were generally smooth. When these strains were incubated for a week, there was no significant change in their appearance. The excision frequencies were thus determined to be less than 10^{-11}. In two isogenic sets of Thy+ and <u>thyA</u> strains the precise excision frequencies of the <u>thyA</u> strains were

approximately five orders of magnitude lower than the Thy+ strains. When the thyA mutant allele harbored by SC702 was transduced back to wild type in strain SC711, precise excision was restored. We conclude that the thyA effect was not an artifact of trimethoprim selection for the thyA mutant, but truely a Thy- effect (Table 3).

To determine whether these cells retained the plasmid, inocula were cultured on minimal medium that lacked proline. Smooth colonies from all the strains were tested for proline auxotrophy. In strains SC702 and SC710, all of the smooth isolates tested were Pro+. Therefore the smooth Thy- colonies are not the result of plasmid segregation, but in fact, an excision inhibition phenomenon. In their Thy+ counterparts, (SC709 and SC711), the rare smooth colonies seen on MacConkey agar are due to plasmid segregation since the smooth colonies tested were proline auxotrophs.

It was observed that both Thy- and Thy+ colonies were mature after 48 hours and had equivalent numbers of cells/colony (SC702, 0.9-1.0x10^8; SC711, 2-3x10^8; SC709, 6.2-12x10^8; SC710, 1.5-3.0x10^8) at three temperatures. Thus it can be concluded that there is enough thymine available to support the growth of Thy- mutants. It was necessary to test whether the addition of excess thymine to the medium of Thy- strains would enable them to overcome precise excision deficiency. Media were supplemented with thymine with concentrations from 10-80µg/ml. When thyA mutants were plated on these media they were able to overcome their inability to support precise excision in a dose dependent manner. The addition of uracil to the medium did not allow thyA strains to restore excision. This was expected since uracil cannot be utilized by thymine requiring strains in the synthesis of deoxythymidylate,

the thyA gene product (Table 4).

Two methods were used to determine whether the effect of a thyA mutation on precise excision was reversible in mature colonies. It is possible that mutations accumulate as thymine is depleted from the medium thus causing irreversible changes in the cell which prevent excision. Thymine mutants in a thymine depleted environment can undergo thymineless death and thymineless mutagenesis (for a review see 18).

MacConkey agar was seeded with strains SC710 (thyA) and its wild type parent SC709. After 24 hours of incubation at 37^0 both strains produced mature smooth colonies on MacConkey agar. No papillae were present in either strain. After 48 hours strain SC709 produced confluent colonies and 100% of SC710 colonies were smooth. To these plates a total of 2mg of thymine from a stock solution was delivered to the agar by injecting thymine into three different spots on the bottom of the perti plate. In a second method, holes were bored into the top of the agar with a sterile cork borer and 1.2-1.8 mg (total) of thymine in solution was added to the wells created by the borer. The plates were placed back at 37^0 for an additional 24 hours. The next day they were observed for papillae. The results were as follows. Strain SC709 retained colonies with confluent papillae. As expected, this occurred on both supplemented and unsupplemented plates. When thymine was injected, or added to holes bored into the agar containing the SC710 colonies, a gradient of papillae was observed; colonies closest to the source of thymime, produced more papillae than those further away. The control plate of strain SC710 which was not injected, produced only smooth colonies. It can be concluded that precise excision inhibition in Thy- strains is not irreversible.

Effects of thymidylate synthase inhibitors on excision.

The chemical inhibitors 5-fluoruracil (FU) and trimethoprim (TMP) of the thyA gene product were used to determine whether wild-type strains with blocked thymidylate synthase activity would inhibit precise excision. Trimethoprim (TMP) indirectly inhibits thymidylate synthase by inhibiting dihydrofolate reductase (9). 5- fluorouracil, (FU), is metabolized to 5-fluorouridylate, an inhibitor of thymidylate synthase (6).

When colonies of Thy+ strains were grown on medium supplemented with 0.2µm/ml fluorouracil, pigmented papillae were not seen (Table 5). To reduce the possibility that FU was inhibiting the induction of β-galactosidase and not excision, W3110 (Lac+ Thy+) was grown on MacConkey + FU medium. Incorporation of high levels of FU into E. coli B can block the induction of this enzyme as well as some constitutive enzymes (17). When W3110 was grown in the presence of FU, the cells grew into Lac+ red colonies, indicating that FU did not interfere with the induction of the enzyme.

FU may have inhibited papillae formation because it selectively inhibits rapidly growing cells. FU inhibits transformed eucaryotic tumor cells (16,6). Two strains MA214 (Ara-) and CSH34 (Lac-), which readily form papillae on MacConkey medium produced abundant papillae on medium containing FU. Thus, fluorouracil did not inhibit papillae formation that occurred as the result of a point mutation but did inhibit papillae formation when the papillae formed as the result of an excision event.

To determine whether the effects of fluorouracil could be counteracted, two sets of Thy+ and thyA strains were grown in the presence of thymine, uracil, FU, or FU+thymine. Strains were plated as single colonies and plates were observed after 48 and 72 hours of incubation at 37^0. As seen on Table 6, the wild-type strain SC709, was

able to overcome the effects of FU after 72 hours of incubation. However, the wild-type strain from the other isogenic pair, (SC716), was unable to do so. This difference observed may be due to unknown strain effects. When thymine was added (60µg/ml), in the presence of FU, once again strain effects were observed. Both wild-type strains were able to recover excision under these circumstances however only one of the Thy- strains (SC710), was able to do so. It does not appear that thymine was the reason for excision recovery in SC710 in the presence of FU, since it was the isogenic Thy+ partner to this strain (SC709) that was able to recover excision in the presence of FU alone. Uracil was unable to restore excision capacity to a Thy- strain, (SC710) either in the presence or absence of FU. This was to be expected since dUMP cannot be utilized by a thyA mutant for the production of dTMP (Table 4).

TMP was added to MacConkey agar to asses it effects on precise excision at doses from 0-5µg/ml, and was found to show a dose dependent inhibition of precise excision (Table 7). Although TMP is routinely used to select thyA mutants, the concentrations of TMP used in this experiment were well below those used in mutant selection (5µg/ml). In addition, Thy- colonies on plates containing TMP were normal sized, but the Thy+ strains were very slightly reduced. When 1.0µg/ml of TMP are added together with 60µg/ml of thymine, both the wild-type and the Thy- strains formed confluent papillae (Table 7).

Effect of polA mutations on excision. In order to study the effects of DNA polymerase I on precise excision, two DNA polymerase I mutants were used. One SC728, harbored the polA12 temperature sensitive allele, and the other SC727, the polA1 ochre suppressible allele. These strains were tested for their sensitivity to the alkylating agent methyl

methane sulfonate (MMS). Both strains were more sensitive to the mutagen (as measured in a disk assay) than a wild type strain, and the sensitivity of the polA12 allele was more sensitive at the non permissive temperature (42^0), than at the permissive temperature (30^0) (Table 10). When these strains were tested on MacConkey agar, inhibiton of precise excision was observed but was stronger in the polA1, mutant because at no time were confluent papillae observed in this strain (Table 9). Individual fresh isolates of the temperature sensitive mutant showed different temperature effects from complete inhibition to slightly elevated excision at the non-permissive temperature. Overall, a slight temperature dependent inhibition of precise excision occurred 75% of the time with these isolates (Table 8). In wild type strains excision is not optimum at 30^0 (Table 2). Since at 30^0, 75% of the polA isolates show higher excision than at 45^0, the decreased excision frequencies observed in these mutants is a true polA effect. The constraint of having to grow the temperature sensitive mutant at 30^0 for a permissive phenotype, masks the potential to form confluent papillae on colonies within 48 hours. This may explain the less obvious excision reduction observed in the polA12 mutant compared to the polA1 allele. In addition, the strain with the polA12 allele retains a residual mutant phenotype at the permissive temperature. Thus the Polymerase I effect may not be as pronounced in this strain.

Effect of methyl methane sulfonate on excision. Since polA mutants are sensitive to methyl methane sulfonate, we asked how the presence of MMS would affect precise excision. Cells were spread to confluency on MaConkey agar and the mutagen was introduced on sterile paper disks.

SC728 and SC709 were tested. Although these two strains are

not isogenic; the location of Tn5 in these strains is identical since both strains harbor the same F'lacZ202::Tn5 plasmid. Therefore, excisions are occuring from the same locus, eliminating location as a factor which can influence excision frequency.

After 48 hours of incubation at 30^0 and 42^0, the plates were observed and several zones were measured and recorded. These were: the diameter of the zone of killing as measured from the center of the disk in the presence of 10-15µl MMS, the zone of papillae (excision) inhibition as measured from the end of the zone of killing and the relative density of pigmented papillae on the rest of the plate. The relative papillae density was scored from 1-3, with 1 being the least dense. Results can be seen in Table10 and Figure 2.

As expected there was more killing in the polA12 mutant at the non-permissive temperature. However the zone of papillae or excision inhibition in the presence of MMS was significantly larger in the wild type strain. The wild type strain showed a higher density of papillae outside the zone of inhibition (relative denisity of 3) than the mutant (relative density 2 at 30^0 and 1 at 42^0).

It appears that MMS inhibits precise excision, in both polA+ and polA strains but the wild-type strain is more sensitive to the effects of the mutagen with respect to precise excision.

Discussion

We have found that precise excision is reduced by five orders of magnitude in thyA mutants (Table 3) and is significantly reduced in polA mutants (Table 9) although the extent of polA inhibition is allele-dependent. This together with other results presented here has led us to conclude that precise excision occurs in mature colonies during DNA repair synthesis.

The 100,000 fold reduction in thyA strains was evident even after prolonged incubation periods. Thy- colonies were the same size as Thy+ colonies on MacConkey agar and were as numerous. MacConkey agar can adequately support the growth of Thy- strains. The Thy- effect was reversed in a dose dependent manner by addition of exogenous thymine to the medium of actively growing cells (Table 4) as well as in mature colonies. Therefore as thymine was depleted there was no irreversible. damage accumulating and preventing excision. Thy- mutants when supplied with thymine, utilize a salvage pathway for the production of deoxythymidylate (dTMP) using thymidine kinase. When uracil was added to the medium, excision was not restored (Table 4).

Experiments which confirmed the requirement for the thyA gene product, thymidylate synthase in precise excision included the inhibition of excision by two inhibitors of thymidylate synthase, 5-fluorouracil (FU), and trimethoprim (TMP) (Tables 5 and 7). In the case of TMP, thymine was able to reverse the inhibition, but it is not clear whether it was able to do so in the presence of FU (Tables 6 and7) Therefore it has been shown that dTMP is necessary for excision whether it is synthesized from dUMP by thymidylate synthase or from thymidine in a salvage pathway. This

suggests that DNA synthesis is required for precise excision and strenghtens current models of excision (Appendices 4 and 5) proposed by Egner and Berg (12) and DasGupta, et. al. (10).

Three observations led us to conclude that repair synthesis is the mode of synthesis involved in excision. First, papillae were only observed only after the first 24 hours of incubation, never within this time period, even though colony sizes were mature by this time. Nor was this delay due to the need for a longer time period for papillae to become visible. When thymine was injected into mature colonies on MacConkey agar, pigmented papillae were produced overnight. It appears that excision occurs in mature colonies. When Thy- colonies reached maturity they had presumably depleted the available thymine and therefore were unable to carry out DNA repair synthesis. The addition of thymine to mature colonies was able to restore excision up to wild type levels indicating that excisions were not predominantly occuring during DNA replication involved in colony formation.

The second observation was that at 30^0, precise excision frequencies were significantly reduced (Table 2). Although the generation time of a wild type strain was slower at 30^0 (Figure 1), by 48 hours the 30^0 colonies were mature since they had roughly the same numbers of cells at all temperatures. We feel, the reason precise excision was reduced at this temperature was because colonies were mature for a shorter period of time at 30^0 than at 37^0 or 42^0 and thus had less of an opportunity to undergo repair synthesis and excision.

Our third observation strongly suggests that excision occurs during the repair of DNA. Isogenic sets of strains bearing two different mutant <u>polA</u> alleles were significantly reduced in precise excision. <u>polA</u>

is the structural gene for DNA Polymerase I which repairs short single strand gaps (22). In both of these backgrounds excision was reduced with respect to a wild type strain although the extent of the reduction was allele dependent (Table 9). In SC727, the polA1 mutant which harbors a point mutation, there were no confluent colonies. Most had a few countable papillae. In SC728, harboring a temperature sensitive polA12 allele, the reduction was temperature dependent, with inhibition occurring usually at the non-permissive temperature (Table 8). The reason for the allelic effects can be accounted for by the different levels of activity of the enzyme in these two strains (11,21).

In an attempt to inhibit the use of DNA Polymerase I during excision we treated wild type and polA12 strains with methyl methane sulfonate (MMS) in MacConkey medium, and observed the effects on papillae formation. The plates were incubated at 30^0 and 42^0 for 48 hours. A zone of inhibition with respect to papillae formation and therefore excision, was 30 times larger for the wild type strain than the mutant (Table 10). This is to be expected if DNA Polmerase I is involved in excision, because in a polA+ strain, a mutagen would inhibit precise excision to a greater extent than in a polA mutant. In the wild type strain, Pol I may be unavailable for excision as it is involved in the repair of MMS induced lesions. In addition, any excision occuring in the polA strain may be occurring by a polA- independent, mutagen-independent process. Under these circumstances, the addition of a mutagen would not affect the excision frequency. If the polA12 mutant strain showed a reduced excision phenotype at the permissive temperature compared to the non-permissive temperature, this argument would have been strengthened. As seen on Table 10 this was not the case. A possible explanation for equivalent

excision capacity at both temperatures in the presence or absence of MMS is that the difference in wild type and mutant activity may be masked, because the enzyme does not have a completely wild type phenotype at the permissive temperature (21) and 30^0 incubation temperatures do not permit wild type levels of excision within 48 hours (Table 1). Alternatively, strain effects cannot be ruled out to explain the difference in size of the zone of inhibition, observed in SC709, and SC728, because these strains are not isogenic. However, excision in these strains was studied from the same allele (lacZ202), ruling out possible site dependent effects.

The finding that DNA Polymerase I is involved in precise excision of Tn5 is consistant with current models proposed to explain excision. In both, repair synthesis is implicated in the model. This study confirms the role of polA in precise excision of Tn5 perhaps in the capacity illustrated (Appendices 4 and 5).

DNA rearrangements such as transposition, excision, inversion, deletion and duplications are mediated by palindromic or repeated sequences, and transposable elements present in procaryotic and eucaryotic genomes. Rearrangements of segments of DNA are significant in the regulation of gene expression (for a review see 4), and are believed to act as switches such as in phase variation in Salmonella, controlling elements in maize, mating type switches in yeast, rearrangement of mammalian immunoglobulin genes, as well as in the activation of a murine cellular oncogene (23). It appears that DNA Polymerase I is involved in both transposition (5), and excision, (this study). The availablity of deoxythymidylate may modulate the ability of DNA Polymerase I to repair gaps created during DNA rearrangements and thus may be involved in the

regulation of gene expression.

From a practical point of view, it is important to note the increased stability of Tn5 in strains harboring thyA mutations. Transposable elements are used as tools for insertion mutagenesis. In constructs where excision is causing instability, the use of hosts with a thyA mutation or the addition of 5-fluoruracil to the growth medium could be useful in maintaining the insertion mutation.

Table 1

Strains Used For Excision Studies

Strain	Parent	Relevant genotype/phenotype[a]	Source / Ref.
E42-202	___	Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	D. Berg
SC702	E42-202	Thy- *recBC+/sbcB15* Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	This study
SC709	E42-202	His- Δ *(lac/proB)* F' *lacZ202::Tn5/pro*	"
SC710	SC709	Thy-	"
SC711	SC702	Thy+	"
Zip514	___	Δ *lac3 trp49*	B. Bachmann
SC716	Zip514	Thy^+Kan^r F' *lacZ202::Tn5*	This study
SC717	Zip514	Thy^-Kan^r F' *lacZ202::Tn5*	"
SC720	E42-202	Trp+	"
JW164	___	*lacZ53 thyA36 polA1*	B. Bachmann/(2,11)
MM383	___	*lacZ53 thyA36 polA12*	B. Bachmann/(2,21)
SC727	JW164	Thy^+Kan^r F *'lacZ202::Tn5*	This study
SC728	MM383	Thy^+Kan^r F *'lacZ202::Tn5*	"
MA214	___	Ara- *mutT+*	W. Maas
CSH34	E7089	F' $lacZ_{U118}$ *proA+B+* Δ*(lac pro)*	(20)

(a) Additional markers or phenotypes are as follows: E42-202; ΔtrpE5.

Zip514; trp49 relA1 rpsL 150 tsx-93 spoT1 λ-

MM383; rha- and Str^r

MA214; arg met his thi

CSH34; supE thi

Table 2.

Excision Frequencies at Three Temperatures

Strain	Temperature	Excision Frequency[a]	Total Colonies
SC709	30^0	5.5×10^{-9}	1399
	37^0	$\geq 10^{-6}$	1592
	42^0	$\geq 10^{-6}$	1409
SC711	30^0	1.7×10^{-9}	379
	37^0	$\geq 10^{-6}$	340
	42^0	$\geq 10^{-6}$	515

[a] Strains were plated on MacConkey Agar as described in Materials and Methods. Excision frequencies were calculated by dividing the number of papillae by the number of colonies times the number of cells/colony. The value of 10^{-6} was derived by plating SC720 on minimal lactose medium and comparing the number of colonies to minimal glucose medium.

Table 3.

Excision Frequencies In Thy+ and thyA Strains

Strain	Phenotype (Thymidylate Synthase)	Precise Excision Frequency[a]	Total Colonies
SC709	+	$\geq 10^{-6}$	1592
SC710	-	3.9×10^{-11}	1409
SC711	+	$\geq 10^{-6}$	340
SC702	-	$<10^{-11}$ [b]	536

(a) Excision frequencies calculated as described previously.
All colonies were grown on MacConkey agar, incubated at 37^{0} and observed after 43-48 hours.

(b) When no papillae were seen, the frequency of excision was reported as $<10^{-11}$ since this was the lowest frequency observed for the appearance of papillae.

SC709 and SC710 and SC702 and SC711 are isogenic pairs respectively, except with respect to thyA.

Table 4.

Excision Recovery in Strain SC710
Dose Effect of Thymine[a]

Supplement µg/ml		Excision Events[b]	Colonies Observed	Excision Frequency[c]
Thymine	0	0	390	$<10^{-11}$
"	10	36 in total	414	8.8×10^{-8}
"	20	0-confluent/colony	370	——
"	30	Confluent	330	$\geq 10^{-6}$
"	40	"	473	"
"	60	"	344	"
Uracil	40-80	0	1338	$<10^{-11}$

(a) Thymine and uracil were added to MacConkey agar at different concentrations prior to pouring. Strain SC710 was eluted from a slant, diluted and plated. Plates were incubated at 37° for 43-48 hours.

(b) Excision events were the number of papillae counted.

(c) Excision frequencies were calculated as previously described.

Table 5.

Precise Excision of Tn5 in Thy+ and Thy- Strains in the Presence and Absence of Fluorouracil

Strain	Phenotype (thymidylate synthase)	Excision Frequency [a] -FU	Excision Frequency [a] +FU	FU Colonies Screened
SC709	+	$\geq 10^{-6}$	$< 10^{-11}$	1421
SC710	-	$< 10^{-11}$	$< 10^{-11}$	1193
SC711	+	$\geq 10^{-6}$	$< 10^{-11}$	331
SC702	-	$< 10^{-11}$	$< 10^{-11}$	200
SC716	+	$\geq 10^{-6}$	$< 10^{-11}$	280
SC717	-	$< 10^{-11}$	$< 10^{-11}$	130

(a) Excision frequencies calculated as described previously.

Strains were diluted and plated on MacConkey Agar as described in Materials and Methods.

Fluorouracil added to MacConkey agar at a concentration of 0.20µM/ml.

Paired strains are isogenic sets except one is Thy+ and one is Thy-.

Table 6

Precise Excision in Thy+ and Thy- Strains in the Presence of Fluorouracil and Thymine

Medium[a]	Precise Excision Frequencies			
	SC716 Thy+	SC717 Thy-	SC709 Thy+	SC710 Thy-
No Supplement				
48h	$\geq 10^{-6}$	$<10^{-11}$	$\geq 10^{-6}$	$<10^{-11}$
72h	n.c.[b]	n.c.	n.c.	n.c.
Thymine				
48h	$\geq 10^{-6}$	$<10^{-11}$	$\geq 10^{-6}$	$\geq 10^{-6}$
72h	n.c.	$\geq 10^{-6}$	n.c.	n.c.
Fluorouracil				
48h	$<10^{-11}$	$<10^{-11}$	4.0×10^{-11}	$<10^{-11}$
72h	n.c.	n.c.	$\geq 10^{-6}$	n.c.
Thymine + Fluorouracil				
48h	2.0×10^{-10}	$<10^{-11}$	4.7×10^{-11}	6.0×10^{-11}
72h	$\geq 10^{-6}$	n.c.	$\geq 10^{-6}$	$\geq 10^{-6}$
Total Colonies	1200	580	2322	2343

(a) Colonies were grown on MacConkey agar at 37° and the number of papillae recorded after 48 and 72 hours. Thymine added at 60ug/ml, FU added at 0.2uM/ml

(b) Denotes no change in appearance of papillae.

Table 7

The Effects of Trimethoprim on Precise Excision of Tn5

	SC709 (Thy+)		SC710 (Thy-)	
Media[a]	Papillae/Colony	Excision Frequency	Papillae/Colony	Excision Frequency
Unsupplemented	confluent	$\geq 10^{-6}$	0	$<10^{-11}$
+TMP 0.01ug/ml	confluent	$\geq 10^{-6}$	0	$<10^{-11}$
+TMP 0.1ug/ml	0-confl.	——	0	$<10^{-11}$
+TMP 0.5-5.0ug/ml	0	$<10^{-11}$	0	$<10^{-11}$
+Thymine	confluent	$\geq 10^{-6}$	confluent	$\geq 10^{-6}$
+TMP, Thymine[b]	confluent	$\geq 10^{-6}$	confluent	$\geq 10^{-6}$

a Cells were grown on MacConkey Medium and incubated for 48 hours at 37°
Thymine was added at a concentration of 60ug/ml.

b TMP was added at a concentration of 1.0ug/ml.

For SC709 approximately 800 colonies were screened on each type of medium.
For SC710 approximately 600 colonies were screened on each type of medium.

Table 8.

Comparison of Precise Excision in a Temperature Sensitive polA12 Mutant (SC728) at its Permissive and Non-Permissive Temperatures

Isolate Number	% Colonies Confluent with Papillae		Total Colonies
	30 ^{0}	45 ^{0}	
1	60	89	126
2*	80	49	127
3*	100	72	63
4*	73	50	115
5	67	61	232
6*	89	0	70
7*	71	57	146
8*	66	59	108

Colonies that were not confluent had less than 25 papillae.

* Denotes isolates which showed decreased excision at the non-permissive temperature.

Colonies were grown on MacConkey agar at the indicated temperatures for 66 hours.

Table 9.

The Effect of Two Different *polA* Mutations on Precise Excision

Strain	Genotype	% Colonies Confluent with Papillae [a]	Colonies Observed
SC709	*polA+*	96	>1000
SC727	*polA1*	0	>500
SC728	*polA12*	39	482

(a) Numbers represent the % of colonies that displayed confluent papillae. Colonies that were not confluent were either non-papillated or had under 25 papillae.

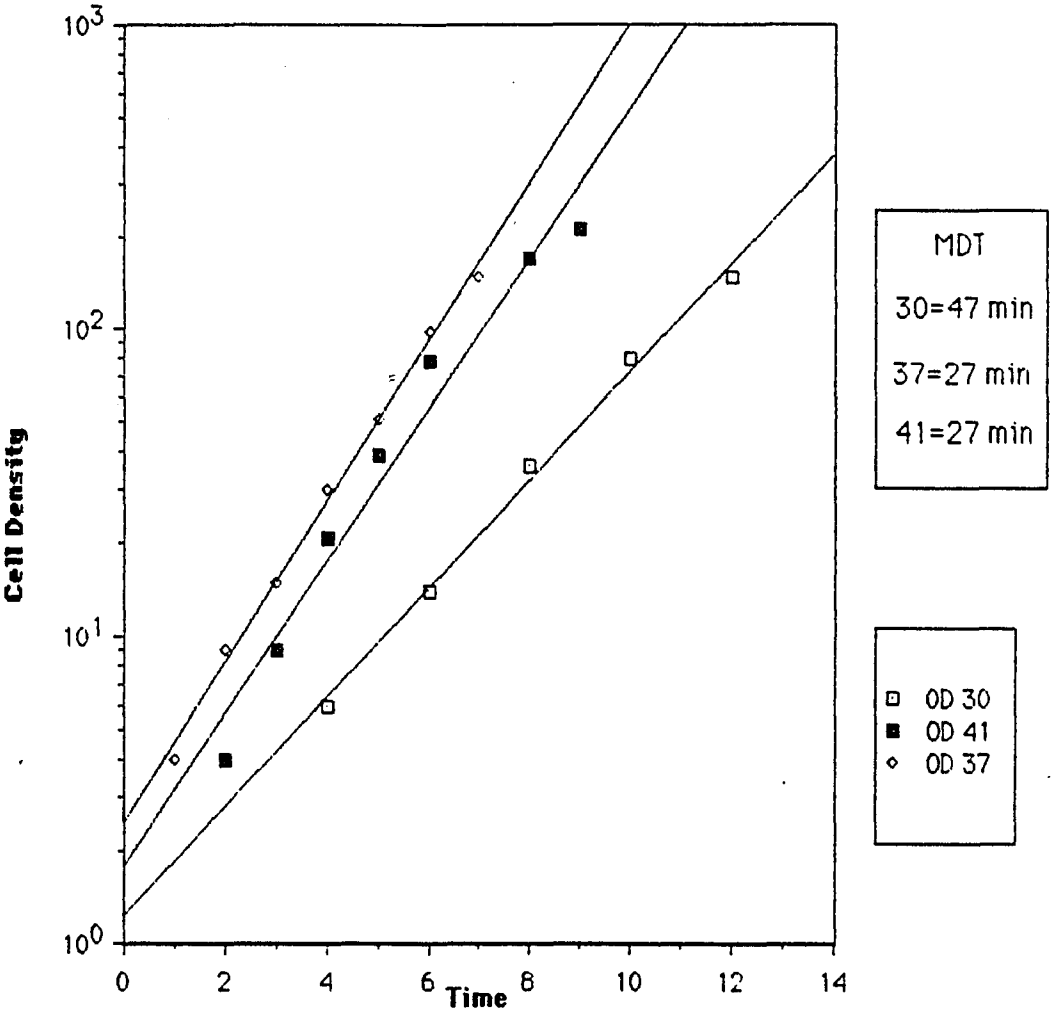
Colonies were grown on MacConkey agar and were incubated at 42° for 48 hours.

Table 10. Excision Inhibition in the Presence of Methyl Methane Sulfonate

Appearance of Growth (Zone)	SC728 (polA12) SSA 30°	SC728 (polA12) SSA 42°	SC728 (polA12) MMS 30°	SC728 (polA12) MMS 42°	SC709 (polA+.) SSA 30°	SC709 (polA+.) SSA 42°	SC709 (polA+.) MMS 30°	SC709 (polA+.) MMS 42°
Zone of Killing (a)	0	0	300mm	500mm	no date	0	no date	250mm
Zone of Papillae Inhibition (b)	0	0	10mm	10mm	no date	0	no date	300mm
Relative Pigmented Papillae (c)	2	1	2	1	no date	3	no date	3

Strains were spread as confluent lawns on replicate MacConkey plates, with disks introduced to the to the center of the lawn after inoculation. (See Materials and Methods) 10ul of MMS used.
All plates were observed after 48 hours of incubation on MacConkey agar.
(a) ZK diameter of zone of killing measured from center of the disk.
(b) ZPI diameter of zone of papillae or excision inhibition measured from the end of ZK
(c) RPD Relative pigmented papillae density scored on a scale of 1-3; 1 is the least dense

Figure 1 Growth Curve of SC709



Legend for Figure 1

SC709 was grown in LB at 37^0 in a rotary waterbath. Optical density was measured in a Klett Summerson Colorimeter with a red filter at 20-40 minute intervals. See Materials and Methods.

Figure 2

Papillae Distribution on Confluent Lawns of a polA Mutant and Wild-type Strain Exposed to Methyl Methane Sulfonate

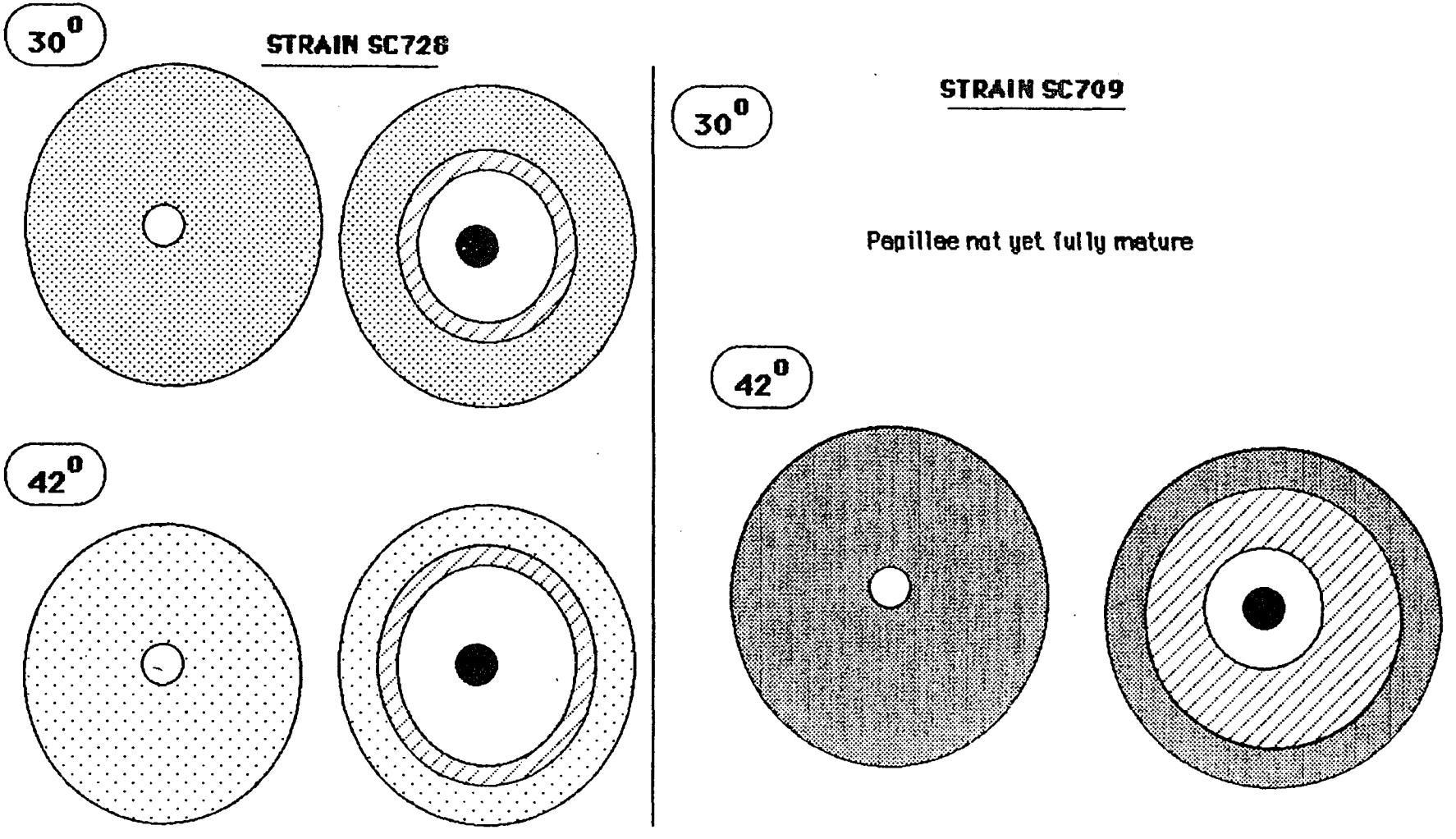
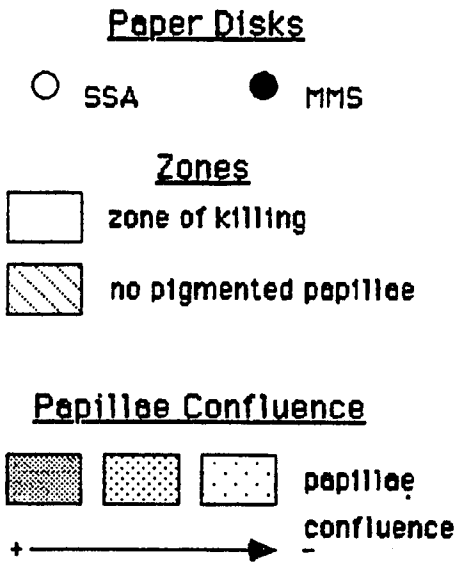


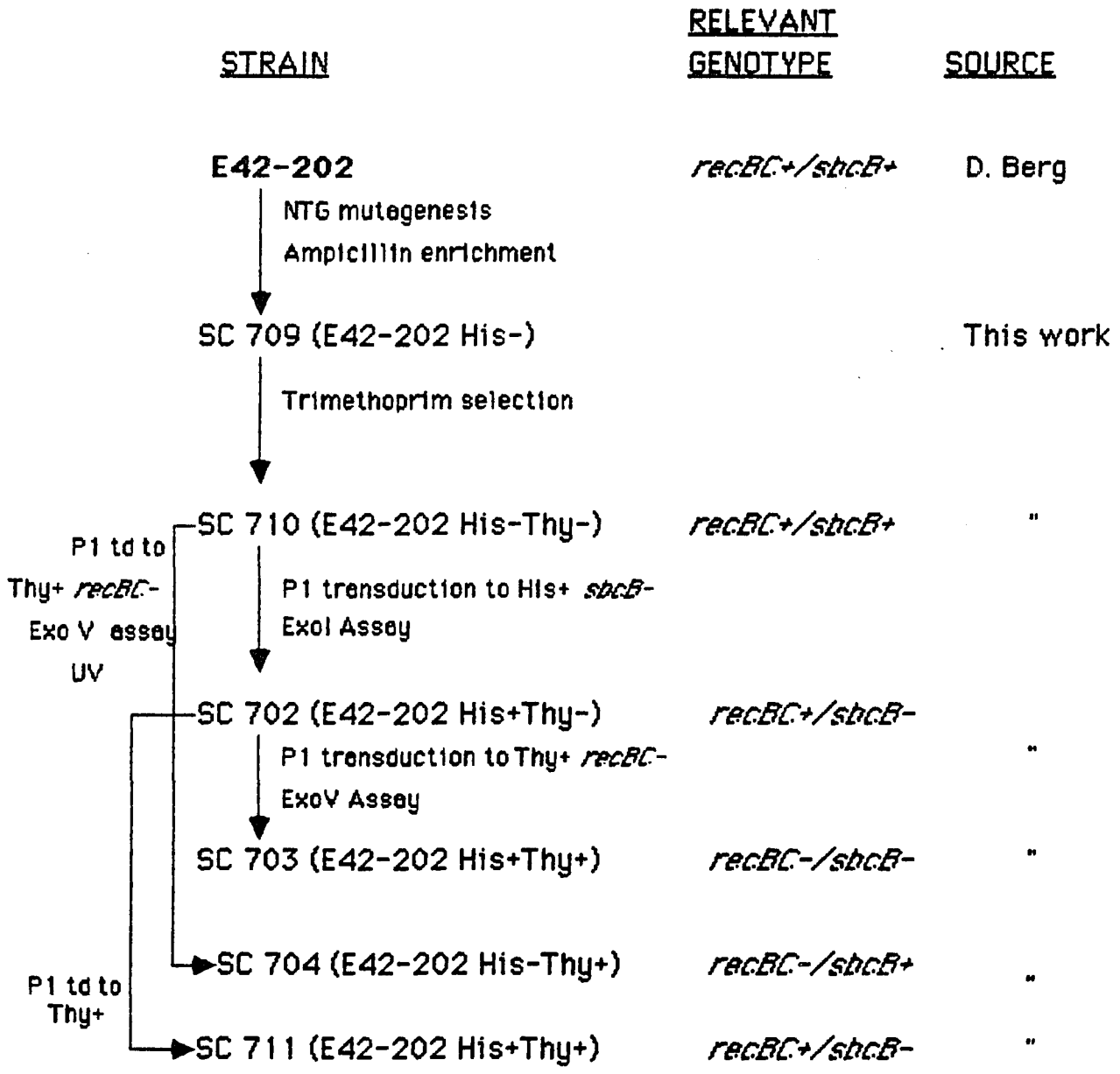
Figure Legend

Figure 2



Appendix 1.

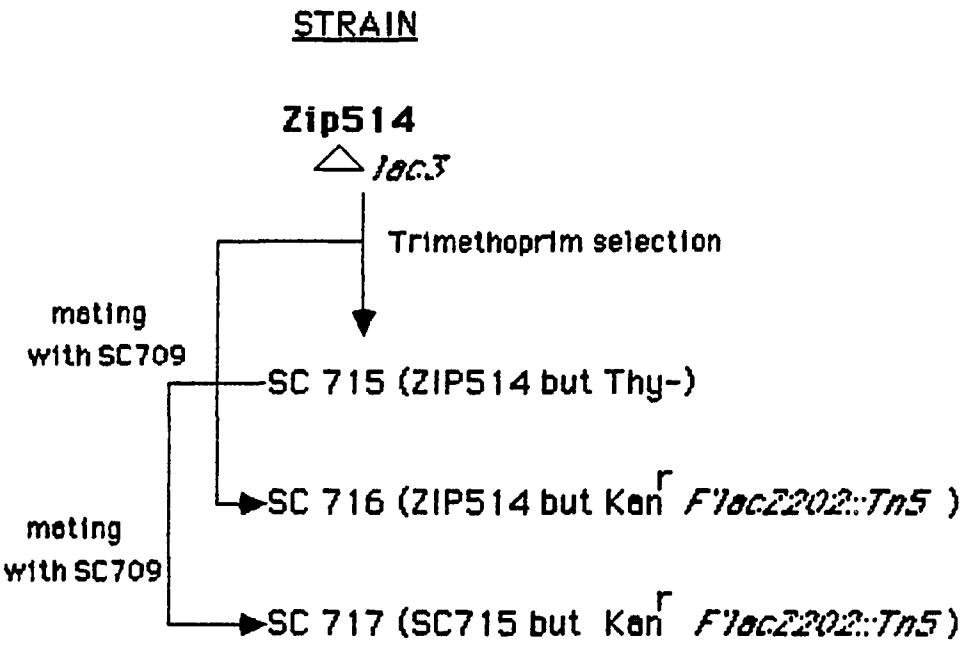
STRAIN CONSTRUCTION STRATEGY



All strains have their chromosomal lac operon deleted and contain *F'proIacI^{q}202::Tn5*.

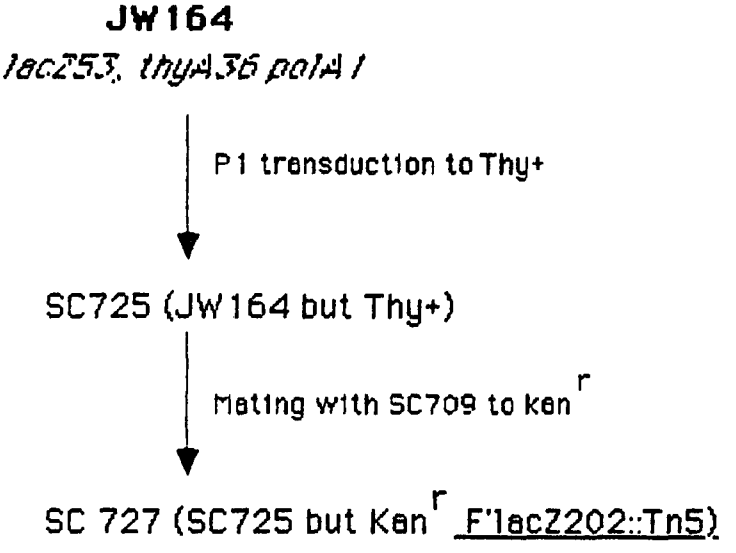
Appendix 2

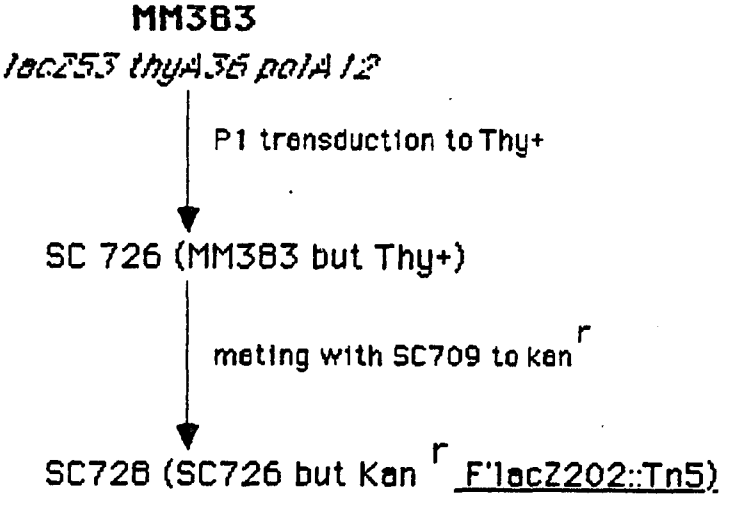
STRAIN CONSTRUCTION II



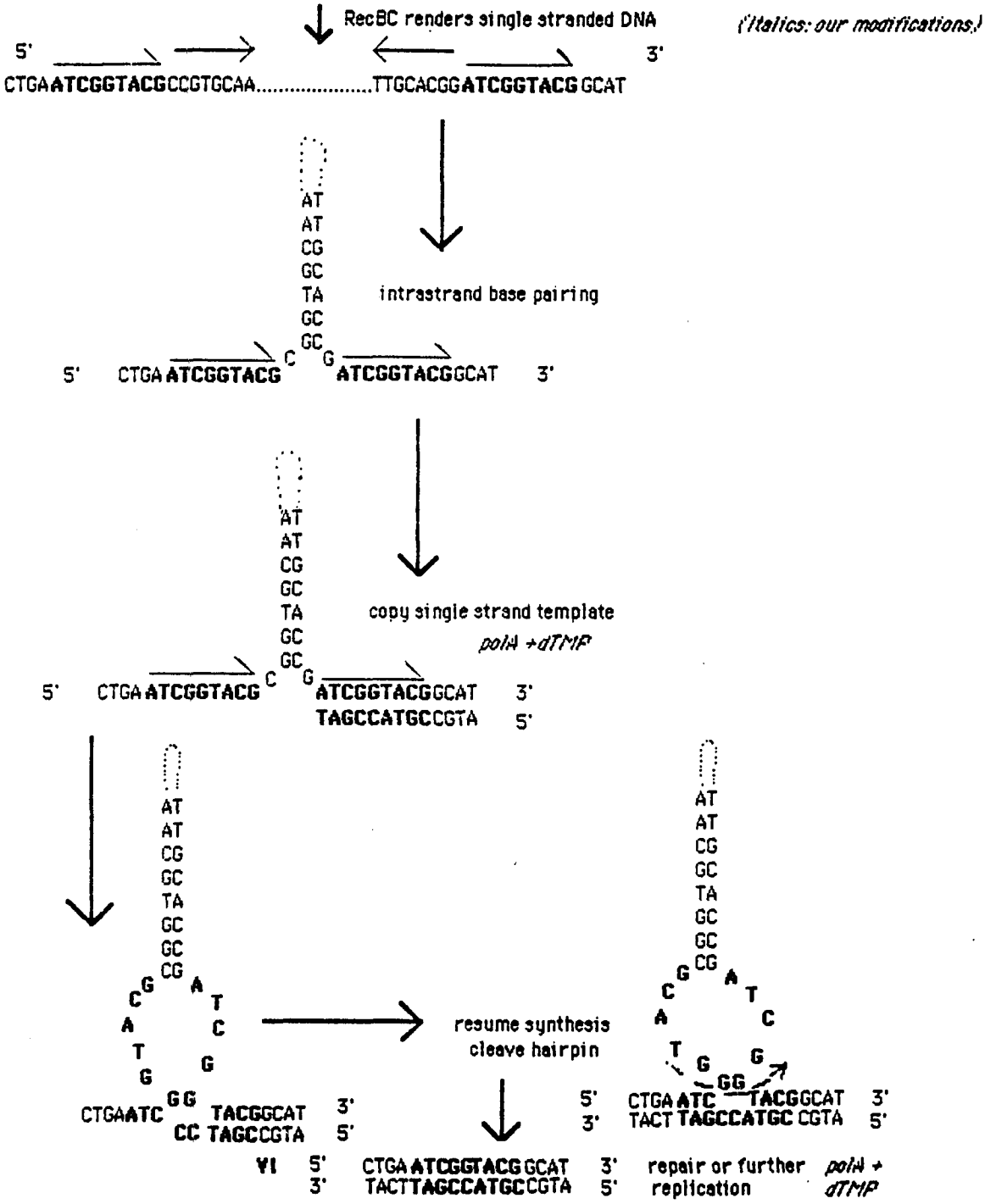
Appendix 3

STRAIN CONSTRUCTION III



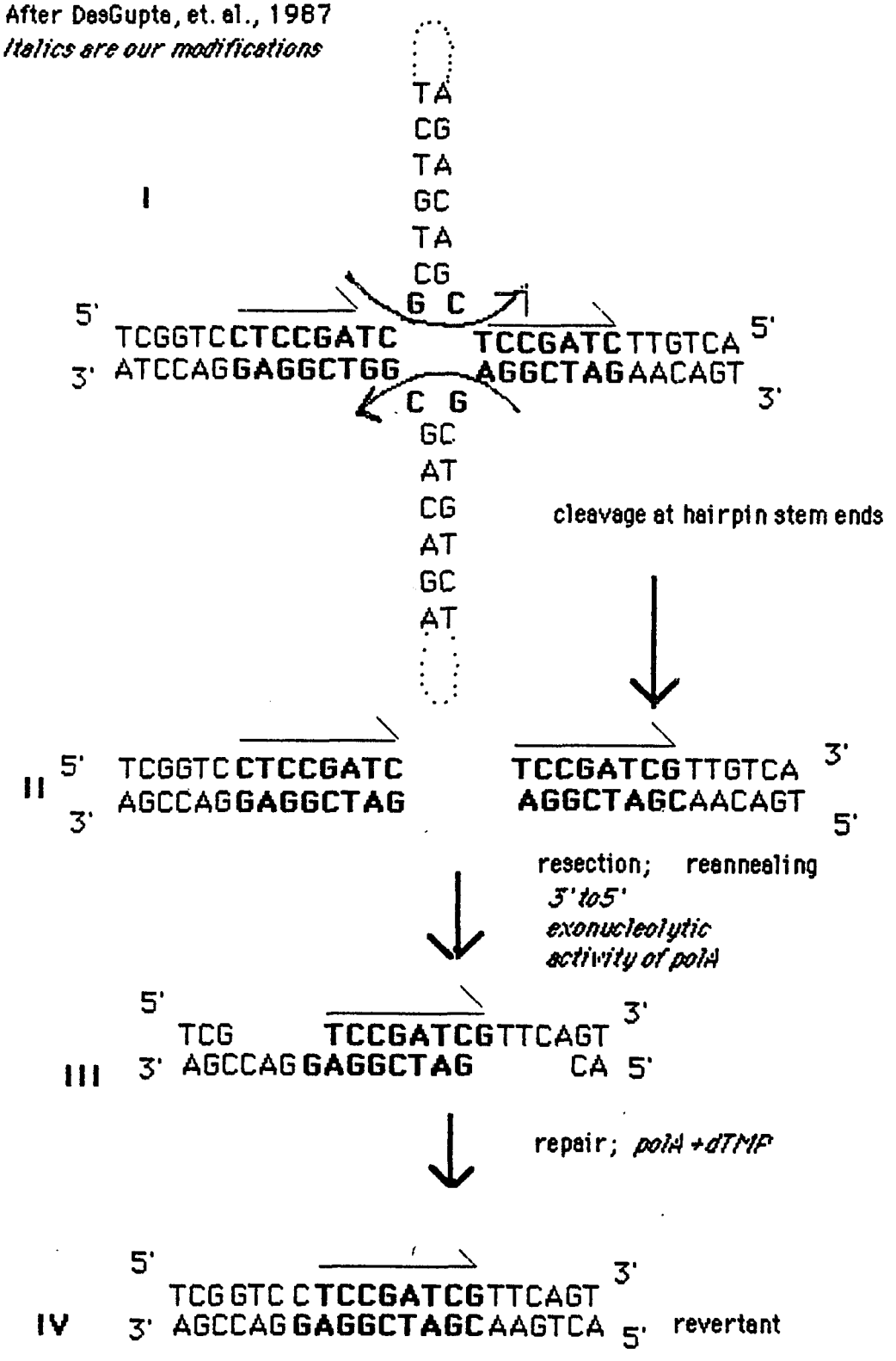


Appendix 4 Model 1 Precise Excision After DasGupta, et. al. 1987



Appendix 5 Model 2 Precise Excision

After DesGupte, et. al., 1987
Italics are our modifications



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