THE ROLE OF RECOMBINATION IN GONOCOCCAL PILIN GENE VARIATION

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ABSTRACT

Pili show remarkable variation in *Neisserria gonorrhoeae* . This variation is due to variable pilin, the primary protein component of pili. The variation in pilin is in turn due to events at the genetic level. Intragenic recombination (i.e., recombination from a homologous duplex located on the same chromosome) is one means by which variation could be brought about. The presence of the *recBCD* gene wpuld be consistent with such a mechanism since the presence of this gene is involved in recombinational events. This past summer at the Rocky Mountain Labs under the direction of Stuart Hill, I attempted to identify a *recBCD* gene in gonococcus.

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INTRODUCTION

The method by which the gonococcal pilin gene undergoes variation has been a topic of study for sometime. One possible explanation is that intragenic recombination is occurring (Swanson and Koomey, 1989). The precise mechanism for this recombination is not known. One possible recombination mechanism involves the RecBCD protein. The RecBCD protein has important functions in *E.coli* recombination (Watson et al., 1987). If a *recBCD* gene could be found in gonococcus it would provide a possible mechanism for the recombination involved in pilin gene variation.

Gene cloning is one way of determining if a gene is present in the genome of an organism. The procedure is accomplished by breaking the genome up into small fragments. These fragments are ligated into plasmids to create a genomic plasmid library. The plasmid library is then transformed into another organism that is known to lack the gene of interest. If one of the transformants expresses the function of the gene of interest then it can be concluded that the gene is present in the organism from which the genomic fragment was obtained (Fig. 1). This was the procedure that Hill and I used in our attempt to identify the *recBCD* gene in gonococcus.

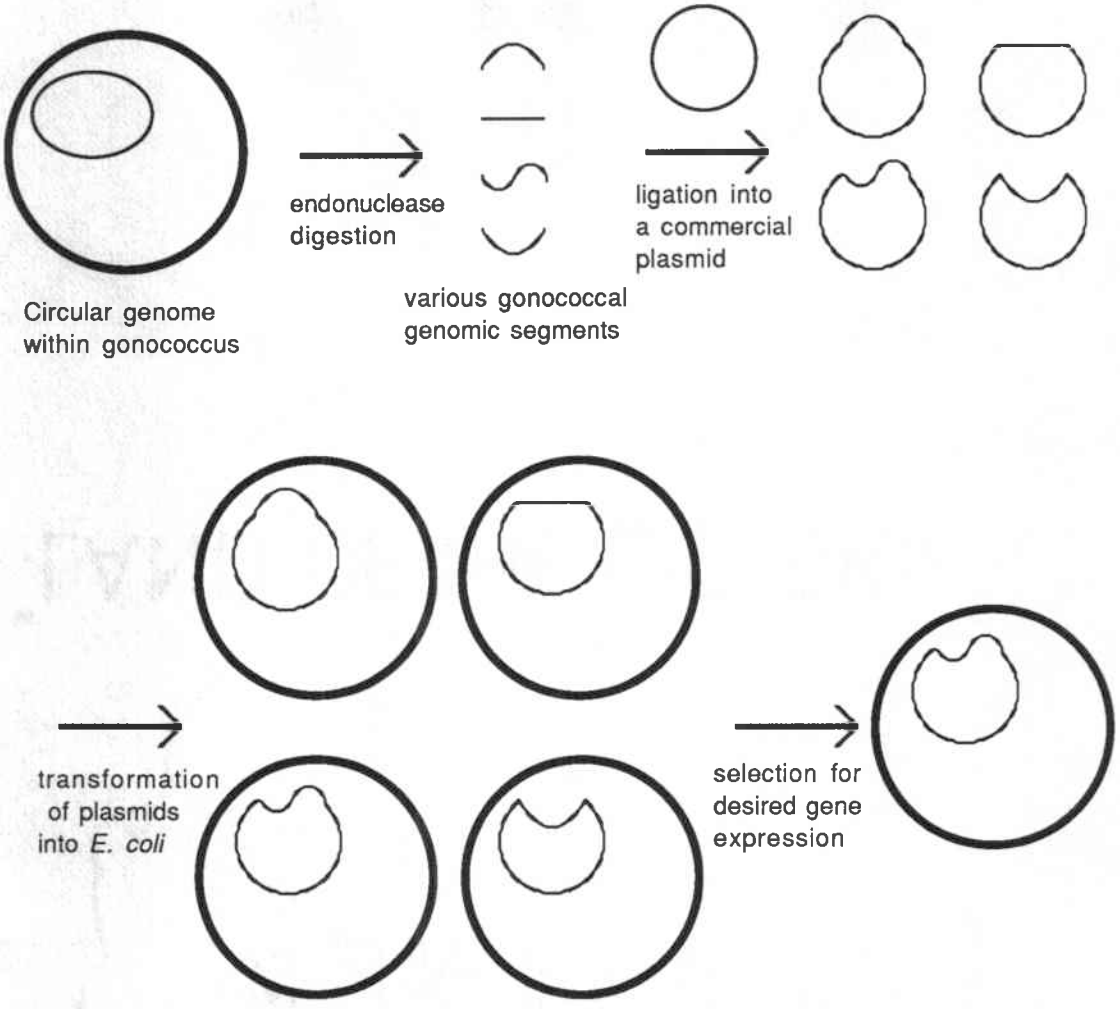


Figure 1. Using the process of gene cloning in *E. coli* to identify a desired gonococcal gene. A plasmid library is first produced using gonococcal genomic segments and a commercially prepared plasmid. The plasmid library is then transformed into *E. coli* . The resulting transformants are subsequently tested for expression of the desired gene.

LITERATURE REVIEW

Gonococcus

According to Pelczar's Microbiology (1986), *Neisseria gonorrhoeae* is the gram negative bacteria responsible for gonorrheal urethritis in humans. This gonococcal bacteria is sexually transmitted. It attaches to the epithelium of the urinary tract via pili. Pili are small hollow, nonhelical, filamentous appendages and are thinner, shorter and more numerous than flagella. Their primary function seems to be adherence. Their attachment prevents pathogenic bacteria from being washed away from the area of infection.

Researchers have shown that gonococcus grown *in vivo* manifest various morphologies (Kellogg et al., 1963; Kellogg et al., 1968). It has been shown that some of these variations in morphology are due to the presence or absence of pili. It was later shown that gonococci that have pili ($pilus^+$) are pathogenic whereas gonococcus without pili ($pilus^-$) are nonpathogenic (Jephcott et al., 1971). This is thought to be due to the inability of the $pilus^-$ to properly adhere to the host tissue. This was proven when male volunteers were challenged with either $pilus^+$ or $pilus^-$ gonococcus. Those inoculated with $pilus^+$ developed gonorrhea 64% of the time whereas not one of the volunteers challenged with the $pilus^-$ bacteria developed the infection (Swanson and Koomey, 1989).

Investigators can determine the piliation of a gonococcal colony

by the colony's size, shape and edge morphology. Pilus$^+$ colonies are small and domed while pilus$^-$ colonies are large and flat. Even small changes in the pilus subunit polypeptide can cause variation in colony morphology (Swanson et al., 1982; Swanson et al., 1983; Swanson et al., 1985).

Transition from pilus$^+$ to pilus$^-$ occurs frequently *in vitro* (Swanson and Koomey, 1989). The rate of transition depends on the media and other environmental factors. The transition between pilus$^+$ and pilus$^-$ will occur in from 0.1 to 1% of the progeny of pilus$^+$ strains. Reversion (pilus$^-$ to pilus$^+$) is equally as common; however, some pilus$^-$ can never revert back to pilus$^+$.

Pilin

Pili are made up of polypeptide subunits known as pilin. Different strains of gonococcus produce different types of pilin, and different types of pilin lead to different types of pili (Schoolnik et al., 1984). However, a cell will typically only produce one type of pilin and therefore only one type of pilus at a time. Different types of pili cause the cells to aggregate in different ways (Swanson and Barrera, 1983; Swanson et al., 1985). This is what accounts for the various colony morphologies and is probably due to variation of the hydrophobic and hydrophilic regions of the pilin molecule (Swanson and Koomey, 1989).

The different types of pili are the result of variations in the amino acid sequence of the polypeptide (Swanson and Koomey, 1989). The pilin molecule is made up of many different regions, some of

which are common to the pilin produced by almost all the strains of gonococcus. These regions are known as common regions. Other unique regions vary greatly from strain to strain. One region that shows great diversity from strain to strain is the "hypervariable" region located near the C terminus of the molecule (Haas and Meyer, 1986). Some strains differ with respect to every amino acid in this "hypervariable" region (Haas and Meyer, 1986; Hagblom et al., 1985).

The Pilin Gene

The gonococcal genome has one expressed, complete pilin gene that codes for the pilin polypeptide. Also located on the genome are several silent, partial pilin genes (Meyer et al., 1984; Meyer et al., 1982; Segal et al., 1986; Swanson et al., 1986). The pilin gene consists of a long open reading frame that codes for the prepilin polypeptide which must be processed to become pilin proper. Seven bases upstream of this open reading frame is a sequence that codes for a strong ribosomal binding site on the primary transcription product (Bergstrom et al., 1986). This allows for the ribosome to attach and consequently for transcription to occur. The transcription initiation site has a proper -10 sequence (TATAAT). This -10 sequence is common to most genes. Another common sequence, the -35 sequence, is not present. The absence of this sequence typically indicates that certain regulatory factors are involved in the initiation of transcription (Raibaud and Schwartz, 1984).

Positioned at several silent loci throughout the genome are 12

to 20 partial pilin genes (Haas and Meyer, 1986; Koomey and Falkow, 1985). Several of these genes have been cloned and sequenced (Haas and Meyer, 1986). They are typically found in clusters on the genome, and they lack the the 5' terminal one-third of a complete pilin gene. Many of the partial genes are homologous to sequences in the complete pilin gene (Haas and Meyer, 1986).

Pilin Gene Variation

Numerous investigations have been undertaken to elucidate the process of pilin variation. One series of investigations involves a strain of gonococcus known as MS11 (Haas and Meyer, 1986; Koomey and Falkow, 1985). This particular strain has a complete pilin gene in its genome, along with a group of 6 partial pilin genes, known collectively as pilS1 copies1-6. When pilus$^+$ MS11 are grown in culture many of the progeny will be pilus$^-$ (Haas and Meyer, 1986). By analysis of mRNA produced during transcription, researchers have determined that in these progeny a segment of the complete pilin gene is replaced with a sequence that resembles pilS1 copy 5 (Swanson, 1988). This new pilus$^-$ strain will produce pilus$^+$ progeny or revertants when grown in culture. Analysis of these revertants shows that all or part of the copy 5 sequence in the complete pilin gene has been replaced by another partial pilin gene (Swanson et al., 1987). If the revertant has only a portion of the copy 5 sequence replaced by a partial pilin gene, then the revertant is actually the product of two serial transitions (Fig. 2). These are: the initial transition, where copy 5 replaced a portion of the

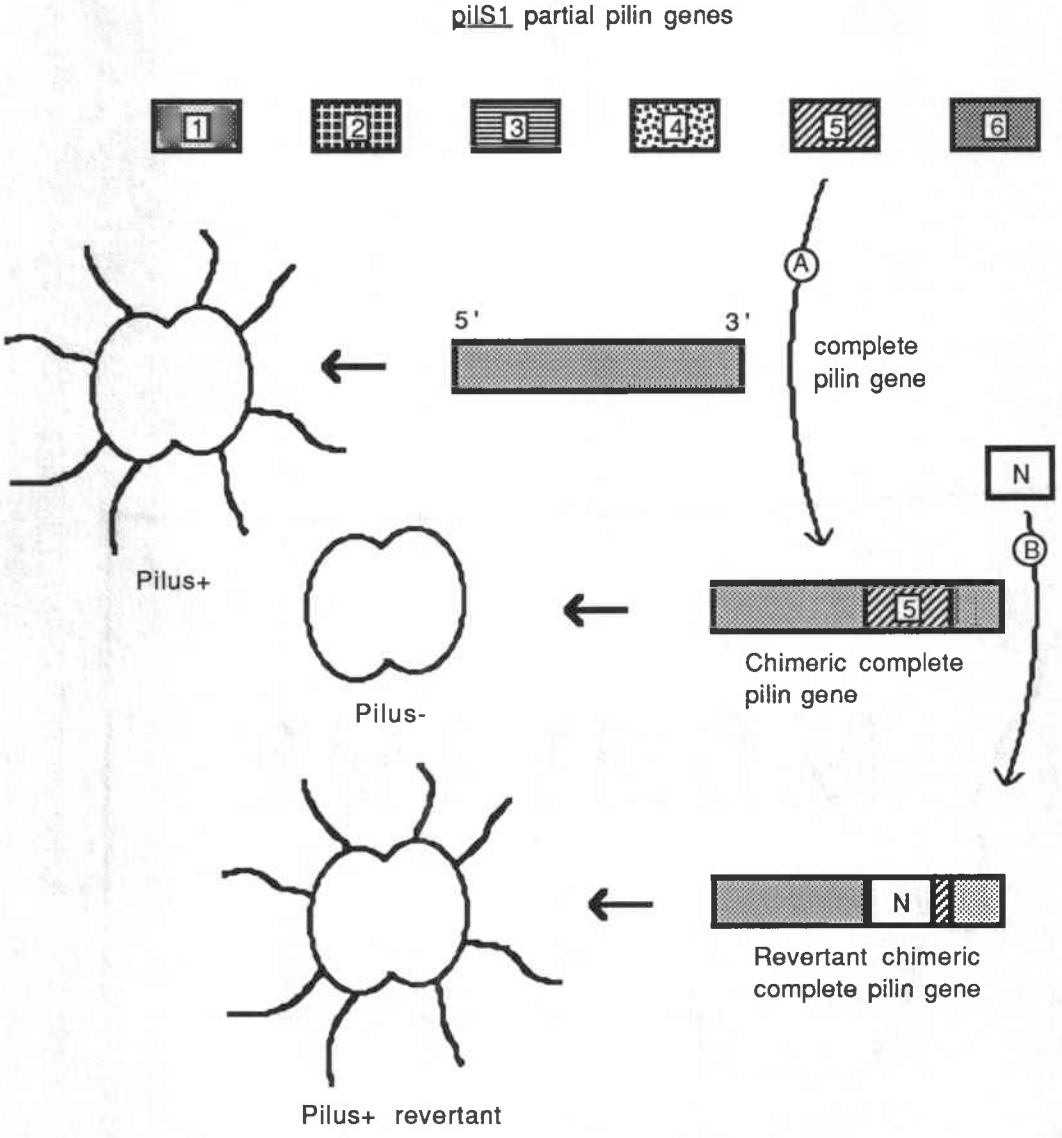


Figure 2. Pilus variation in response to pilin gene variation. Serial transitions in the pilin gene are represented. In event (A) intragenic recombination occurs in which copy 5 is incorporated into the complete pilin gene. The resultant chimeric complete gene looses the pilus+ phenotype due to a phase transition (Meyer et al., 1982). In event (B) the pilus+ phenotype is restored due to the recombinational placement of copy N into the complete pilin gene.

original pilin gene, and the subsequent transition, where a portion of copy 5 was replaced by another partial pilin gene. The large number of partial pilin genes present in the genome along with the possibility of serial transitions allows for a multitude of new pilin gene combinations (Swanson and Koomey, 1989).

Recombination

Recombination is a chemical process by which DNA strands on homologous chromosomes are exchanged. Recombination provides a probable explanation for what is occurring in pilin variation.

Recombination is best explained as it occurs in diploid cells. A diploid cell contains two copies of each chromosome, one from the father and one from the mother. When this cell divides, the maternal and paternal chromosomes exchange genetic information. Each of these chromosomes is made up of two complementary strands of nucleotides. Because the nucleotide sequences on the chromosomes vary only slightly from the maternal chromosome to the paternal chromosome therefore the chromosomes are said to be homologous. This homology is what makes recombination possible. For example, if the paternal chromosome contains genetic information for brown eyes and the maternal chromosome contains the information for blue eyes, the structures of these genes differ only by the minute section which codes for color. In the process of recombination one strand from one DNA molecule will exchange loci with a strand from the homologous DNA molecule. The resultant products are called heteroduplexes, for their complementary strands are not

exact matches. The heteroduplex that was derived from the maternal chromosome is changed only in one small segment of the strand that has been replaced by a portion of the paternal DNA. The same is true of the paternal chromosome; it remains identical except for the small portion that was replaced by the segment from the maternal chromosome. Each heteroduplex now consists of two strands, one strand that is unaltered from the original state, and the "hybrid strand" that contains the segment of DNA from the other parent (Fig. 3).

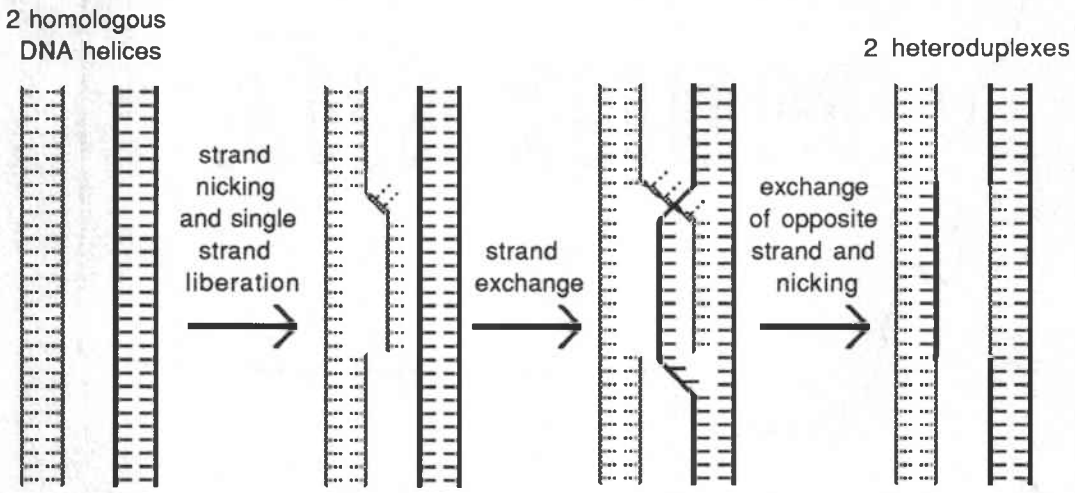


Figure 3. Generalized Recombination. Formation of heteroduplexes from homologous DNA Helices.

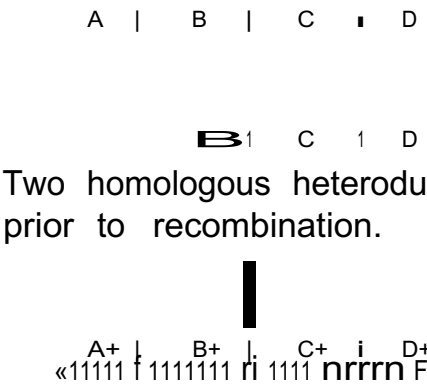
After recombination occurs, the cell cycle will continue, and replication will occur. As replication proceeds, the two strands of the heteroduplex are separated and complementary strands are formed. The progeny are now normal DNA duplexes not heteroduplexes (Fig. 4)(Watson et al., 1987).

Homologous chromosomes do not necessarily come from parents.

heteroduplex. This heteroduplex then undergoes repair via one of the numerous repair mechanisms present in the cell. The repair mechanism utilizes one of the strands of the duplex to correct any inconsistencies in complementation in the opposite strand. If this process occurs using the original strand as the template of repair, then the repaired gene will be exactly like the original gene. However, if repair occurs using the hybrid strand of the heteroduplex as the template, then the repaired gene will be entirely new; it will show no signs of the gene segment that was replaced by the incoming single strand during recombination. In the second case the recombinational process is referred to as gene conversion (Fig. 5). Gene conversion is thought to occur in gonococcal pilin gene variation (Swanson and Koomey, 1989).

Enzymes of recombination

There are many theories about recombination and the enzymes involved in the pathway. These theories have been derived from studies done primarily on *Escherichi coli.* The RecA protein and the RecBCD protein are two enzymes that may play a particularly important roles in gonococcal pilin gene variation. These proteins have been identified and characterized both functionally and structurally in *E. coli* (Amundsen et al., 1986).



♦ Figure 5. Gene conversion. Reciprocal and nonreciprocal recombination.

RecA Protein

There are several different recombination possibilities. These are: (1) two single stranded DNA molecules coming together; (2) a single strand replacing a strand on a double stranded molecule; (3) a double stranded linear molecule coming together with a single stranded circular molecule leaving a double stranded circular molecule and a single stranded linear molecule; and (4) a linear double stranded molecule with a break invading another double stranded molecule to leave two hybrid double stranded molecules (Smith, 1988) (Fig. 6). In each of these cases the strands that recombine are homologous to each other. All of four possibilities have one aspect in common: in one way or another a single strand of DNA is attached to another strand of DNA. In cases 1 and 2 the single strand is unpaired from the onset but in case 4 the strand needs to be broken away from its complementary strand. The RecA protein participates in all of these processes (Smith, 1987).

The function of the RecA protein is to bind to a single strand of DNA. If the strand does not exist singly *in vivo* it it must be nicked by another enzyme and separated from its complementary strand. Once the protein is bound to the strand it then invades a homologous double stranded DNA duplex with the single strand. The protein finds a portion of the DNA duplex that is homologous to the single strand and initiates the process of annealing the single strand to one of the strands in the DNA duplex. This process results in a new DNA duplex (Watson et al., 1987) (Fig. 7).

(a) (b) (c) (d)

Figure 6. Reactions promoted by RecA protein. Single stranded DNA (ssDNA) and double stranded DNA (dsDNA) are represented by single and double lines, (a) Annealing of complimentary ssDNA. (b) ssDNA incorporation into dsDNA. (c) Double stranded linear molecule recombining with single stranded circular molecule, (d) Reciprocal strand-transfer and branched migration (Smith, 1987).

RecBCD protein

The RecBCD protein has been found to have two important functions in recombination in *E.coli.* The enzyme unwinds the DNA duplex forming loops of single stranded DNA. It then nicks these single stranded loops. This allows for the RecA protein to begin pairing the single strand to a double strand. The RecBCD enzyme cuts the single stranded loops at locations known as Chi sites. These Chi sites are segments of DNA with the sequence 5'G-C-T-G-G-T-G-G-3' (McKittrick and Smith, 1989). Although RecBCD activity has been well characterized in *E.coli* and other enteric bacteria, it

has yet to be seen in gonococcus.

Recombination in pilin gene variation

Researchers have suspected that the pilin gene variation occurs in gonococcus involves via RecBCD-mediated recombination. To test this hypothesis an enzyme similar to RecBCD must be found in the gonococcus. Locating the gene that codes for the RecBCD enzyme in gonococcus was the basis of my research at the Rocky Mountain Labs Because *recBCD-Wke* genes have been found in other bacteria, procedures for determining the presence of such a gene have already been established.

Cloning the *recBCD* Gene

N.H. McKittrick and G.H. Smith (1989) conducted extensive studies to determine the presence of *recBCD* genes and Chi sites in enteric bacteria and in pseudomonads.

The work done by McKittrick and Smith Centered around the conservation of Chi sites. Their cloning of the *recBCD* gene from enteric and non-enteric bacteria served as a model in our attempt to clone the *recBCD* gene from gonococcus.

Chi sites, which have been well characterized in *E.coli,* stimulate the RecBCD pathway of homologous recombination in *E.coli.* Recombination occurs at a great frequency in the area of the Chi site. The frequency of recombination decreases as the distance

Figure 7. Activity of RecA protein as demonstrated in vitro in a model reaction using DNA fragments (Watson et al., 1987).

from the Chi site increases.

The mechanism of recombination involves the RecBCD enzyme attaching to the chromosome at an area of a double stranded break. The enzyme then moves along the double stranded DNA. It apparently unwinds the double stranded DNA, separating the strands and cutting the strand containing the Chi sequence. This cutting is the most essential portion of the stimulation, for without this nick, no recombination will occur. The nick liberates a single strand of DNA. The Rec A protein can then insert the single strand into another double stranded DNA, and recombination proceeds.

McKittrick and Smith suspected that enzymes similar to the RecBCD independent nuclease were present in other bacteria. If these RecBCD-like enzymes could be found in other bacteria it would suggest that Chi sites have been conserved as a recombination stimulating site in the enteric bacteria.

In an attempt to gain experimental evidence that Chi sites have been conserved in other bacteria, McKittrick and Smith cloned genes coding for many of the ATP-dependent nucleases with activities similar to RecBCD from enteric bacteria into *E.coli.* They then tested the clones to determine if they had activation of Chi hotspots.

McKittrick and Smith hoped to remove the genes that encoded for the RecBCD-like enzymes from the enteric and non-enteric bacteria being studied and clone them into *E.coli.* This particular species was selected because of the ease with which it transforms and conjugates.

One problem that arises is that most strains of *E.coli* already have a functional RecBCD enzyme. This makes it difficult to test for

the function of a RecBCD-like enzyme encoded by the gene present in a foreign plasmid. In order to make the experiment work the genomic *recBCD* gene must be deleted from the *E.coli* strain. These deletion mutants differ phenotypically from wild-type *E.coli.* The mutants have a decreased cell viability and an increased sensitivity to ultraviolet light and mitomycin C. They also support altered plaque formation by several phages. If when injected with a foreign plasmid via transformation the function of a RecBCD enzyme is observed in this strain the only possible explanation would be that the *recBCD* gene is present on the foreign plasmid.

The *recBCD* gene in *E.coli* is located between the *thyA* gene and the *argA* gene. These genes, *thyA-recBCD-argA,* are all located on a strand of DNA that can be extracted with BamHI endonuclease. For this reason, McKittrick and Smith used BamHI to cleave the genomes of six enteric bacteria and three pseudomonads. They then ligated the acquired fragments into the plasmid pBR322 and transformed the plasmid into *E.coli* strains. This process worked well for organisms closely related to *E.coli.* Slightly more involved procedures were used for the more distantly related organisms.

The next step was to test the clones for RecBCD-like characteristics. This was done by growing up the *E.coli recBCD* deletion mutants that contained the plasmid with the candidate fragment on plates with low levels of mitomycin C or limited exposure to ultraviolet light. The clones were also exposed to certain bacteriophages. The clones that contained *recBCD-Wke* genes showed resistance to mitomycin C and ultraviolet light. They showed no plaques due to phages.

Recombination proficiency and Chi activity were tested next. Recombination proficiency was measured in lambda *Red' Gam'* vegetative crosses and in Hfr conjugations. McKittrick and Smith could determine through these tests, the amount of recombination that occurred in these systems and thus determine the level of Chi activity. Results showed all but one of the strains of enteric bacteria had markedly increased recombination frequencies over the control *recBCD* deletion mutants. The non-enteric bacteria exhibited increased recombination proficiency yet did not show activation at Chi sites. This indicates that some other sequence must signal for nicking in these type of bacteria.

Next enzyme assays were run on RecBCD-like enzyme extracts from the various clones to determine whether these enzymes' activities matched up to the RecBCD enzymes activity. With the exception of one minute variation in all cases of the enteric bacteria, the activities of the RecBCD-like enzymes matched activity of the RecBCD enzyme's.

Having performed these various tests, McKittrick and Smith were reasonably confident that Chi sites have been conserved as a recombination stimulation site in enteric bacteria but are not necessarily conserved for this purpose in non-enteric bacteria.

The Chi sites of the enteric bacterial genome are essential components in stimulating homologous recombination via the RecBCD pathway. These sites have not, however, been found in gonococcus (Hill, personal communication). It is possible that another sequence could activate the pathway, as is thought to happen in the pseudomonads that McKittrick and Smith tested. If a *recBCD* gene is

present in gonococcus, it would help to understand the mechanism involved in recombination.

MATERIALS AND METHODS

The method used for the gene cloning was similar to that used by McKittrick and Smith (1989) to clone *recBCD* in enteric bacteria and pseudomonads.

(a) Growth media

LB broth and agar plates, tryptone broth (TSB) and agar plates have been described (Smith et al., 1986). Drug-resistance transformants were selected and grown in media containing ampicillin (100pg/ml). Mitomycin C resistance was measured on LB agar plates containing mitomycin C (1pg/ml).

(b) Cloning of the DNA fragments

Bacterial DNA was prepared by the method of Saito and Miura (1963). Plasmid DNA was prepared using the protocol given in Appendix A. Plasmid and chromosomal DNA was thoroughly digested using the appropriate restriction enzyme, mixed and ligated. The ligated DNA was used to transform *E.coli* strain V277 *(recC22- hsdR, SupE, Sup F, YacY1, trp R, met, arg A21)* using the procedures of Hanahan (1983). Transformation frequency was tested on plates containing ampicillin. Five separate cloning attempts were made.

The first cloning attempt was made with a cosmid library that wes acquired from Robert Belland of Rocky Mountain Labs. A cosmid is a virally derived particle similar to a plasmid but considerably larger. The genomic segment within the cosmid was approximately

4.0kb in length. The transformation was performed and the transformants were plated onto mitomycin C plates.

The second cloning attempt also used the cosmid library. The transformants were then plated onto ampicillin plates.

The third cloning attempt was done using a plasmid library acquired from Kolari S. Bhat of Rocky Mountain Labs. The genomic segments in this library had been cut from the gonococcal genome using Sau 3A restriction endonuclease and ligated into the Bam site of ptz18 commercially prepared plasmid to arrive at segments approximately 5.0kb in length. The transformants were then plated onto mitomycin C plates.

In the fourth cloning the Bhat plasmid library was again used. The transformants were then incubated in an an ampicillin solution (100jig/ml LB broth) for 3 hours. After the incubation they were grown up on mitomycin C plates. The survivors were then grown on ampicillin plates. The ampicillin resistant colonies were then analyzed to determine the size of the segment present in the plasmid. To analyze the transformed plasmid it was first necessary to purify the the plasmid from a large number of clones (Appendix A). The plasmids were then cleaved with an endonuclease to linearize them and then run across an electrophoretic gel.

The fifth cloning used a plasmid library that we constructed. This library was constructed from gonococcus segments cut by Sau 3A for three minutes. This produced segments that were approximately 10.0 kb in length. These fragments were then ligated (Appendix C) into the Bam Hl site of pBR322. The transformants were incubated for 3 hours in ampicillin solution and then plated on

mitomycin C plates

RESULTS AND CONCLUSION

The results of the first cloning attempt were negative. No mitomycin C resistant colonies were found. This result indicated that either the transformation was unsuccessful or the *recBCD* segment (which should encode mitomycin resistance) was not present on a transformed plasmid. To determine the transformation frequency we performed the second transformation. The second group of transformants were tested for ampicillin resistance. The commercially prepared plasmid ptz18 confers ampicillin resistance to the recipient organism. Since no ampicillin resistant colonies were observed it was construed that no transformation occurred. There are many possible explanations for this. We suspected that the reason no transformation occurred was because the cosmid is too large. The cosmid was thus abandoned as a vector for transformation.

The next two cloning attempts involved the Bhat plasmid library. The first transformant showed what appeared to be mitomycin C resistance. These colonies however did not show substantial ampicillin resistance. This lack of ampicillin resistance indicates that little or no transformation was actually occurring. The mitomycin C resistance could have been due to dead cells separating the "resistant" colonies from the toxic media. Another possible explanation is that the resistance to mitomycin C was due to reversion of the *recC*- mutation in the *E.coli* genome.

In the next cloning attempt we decided to screen the transformants for transformation frequency prior to testing them

for mitomycin C resistance. We did this by incubating the transformants in an ampicillin solution prior to growing them up on mitomycin C media. Ideally only cells that had undergone transformation would survive through the incubation. The incubation solution was then spread across mitomycin C plates. Nineteen colonies showed resistance to mitomycin C. These colonies also showed resistance to ampicillin when spread on ampicillin plates. The plasmids from these colonies were cleaved with endonucleases and run through electrophoresis gels. Analysis of these gels showed plasmids of sizes 7.0 and 6.0 kb. Another clone showed two fragments of sizes 4.0 and 3.5 kb for a total of 7.5 kb. Subtracting the length of the ptz18 fragment, 2.7 kb, from 7.0, 6.0, and 7.5 kb fragments leaves gonococcal genomic segments of 4.3, 3.3, and 4.8 kb respectively. A typical *recBCD* gene fragment from *E.coli* is approximately 10.0 kb. Therefore the fragments obtained from this transformation were too small to be complete *recBCD* genes. They could possibly contain partial or complete *recC* genes. (Fig. 8)

Our last cloning was an attempt to clone the entire *recBCD* gene from gonococcus. We used a plasmid vector that we constructed from 10 kb gonococcal genome segments cleaved by Sau 3A and ligated into the Bam H1 site of pBR322. The transformants were incubated in an ampicillin solution and then grown up on mitomycin C plates. No resistant colonies were observed probably due to the large size of the fragments. Fragments of this size would have difficulty transforming.

Future investigations may use an *E. coli* strain with a *recBCD* deletion mutation in order to test for the function of the entire

recBCD gene.

Figure 8. Results of fifth transformation. Compared to l ladder (cleaved with Hind III). Clones 1 - 3 were identified with plasmid fragments of 4.0 kb and 3.5 kb for a total of 7.5 kb. Clones 4 - 6 contained plasmid fragments of 6.0 kb. Clone 7 contained a 7.0 kb plasmid fragment. The plasmids were cleaved with Eco Rl, Pst, Hind III, and Bam H1 endonucleases to linearize. The circular ptz 18R plasmid has a length of 2.7 kb. Subtracting this length from the measured fragments gives gonococcal genomic segment sizes of 4.8 kb for clones 1 - 3, 3.3 kb for clones 4 - 6, and 4.3 kb for clone 7.

APPENDIX A

Protocol for plasmid preparation

1) Resuspend culture in 800pl 10% STE.

* STE: O.IMNaCI
10mM Tris pH 8.0
1mm ethylenediaminetetraacetic acid (EDTA)

2) Centrifuge in a tabletop Beckman centrifuge for 30 sec at max speed at 2°C.

3) Deposit supernatant in Rocal.

4) Resuspend in 10Opl Solution I.
Solution I: 50mM glucose
25mM Tris pH 8.0
10mM EDTA

5) Vortex.

6) Add 200JJ.I Solution II, shake, ice for 5 min.
Solution II: 0.2N NaOH
1% lauryl sulfate, sodium salt (SDS)

7) 150pl 3M potassium acetate, pH 4.8, ice for 5 min.

8) Centrifuge for 10 min 2°C; save supernatant.

9) Phenol extract:
Add an equal volume of phenohchloroform.
Vortex several times.
Centrifuge for three minutes.

10) Keep top fraction.

11) Add 600pl 99% ethanol (let sit for 15 min).

12) Centrifuge 10 min. 4°C (take off supernatant).

13) Wash with 500JLI 70% ethanol; centrifuge 5 min.

14) Remove supernatant.

15) Centrifuge open in a vacuum.

16) Resuspend in 20JJI TE (Tris, EDTA) for storage.

Enzyme digest (Endonuclease or restriction enzymes)

1) 10jil TE DNA solution.

2) Add 2jil MediumlOX buffer.

MediumlOX buffer: 50mM NaCl
10mM Tris Cl (pH7.5)
10mM MgCl2
1.0mM dithiothreitol

3) Incubate 37°C for at least one hour.

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APPENDIX B

Transformation Protocol

1) Inoculate 20ml of LB broth with 75jil of overnight bacterial culture.

2) Grow approx. 2 hours 37°C with shaking until OD=0.15.

3) Ice for 1/2 hour.

4) Centrifuge for 10 min at 4°C.

5) Pour off supernatant.

6) Add 10ml TSB, ice for 10 min.

7) Centrifuge for 10 min at 4°C.

8) Resuspend in 1 ml TSB.

9) Add 35pl of dimetylsulfoxide (DMSO); ice 5 min.

10) Add 2pl B-mercaptoethanol; ice 10 min.

11) Add 35pl DMSO, ice 5 min.

12) Add 2pl of plasmid solution (in TE) to 200(il competent cell solution.

13) Ice 30 min.

14) Heat Shock 11/2 min. at 42°C; ice 2 min.

15) Add 0.8ml LB.

20) Incubate 40 min 37°C with slight shaking.

APPENDIX C

Ligation Protocol

1) Add the following:

< 3pJ Ligase buffer(McKittrick and Smith, 1989)
1.2jil digested gonococcal DNA
12.0p.l commercial plasmid
1.0pl ligase enzyme

2) Incubate overnight at 14°C.

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