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The cloning of the 5.4-KB plasmid of Bacillus megaterium

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THE CLONING OF THE 5.4-KB PLASMID OF *BACILLUS MEGATERIUM*

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ABSTRACT (100-200 WORDS): The construction of a stable cloning vector would be very useful to the study of genetics of Bacillus megaterium. It seems reasonable to assume that a vector made with a natural resident plasmid of this species will will be stable. This is the rationale behind cloning the 5.4-kilobase plasmid of B. megaterium. The 5.4-kb plasmid and the vector were each cut with a restriction endonuclease that produced blunt ends. These two plasmids were ligated using the enzyme T4 DNA ligase. The recombinant plasmid was transformed into E. coli. Transformant DNA was then analyzed using restriction enzymes. Because of errors in the restriction analysis data used, only part of the 5.4-kb plasmid has been cloned. However, this hybrid plasmid may still be useful in constructing cloning vectors for use in B. megaterium. and other bacilli.

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INTRODUCTION

Research in the Vary laboratory is concerned with the genetics and mechanisms of sporulation and germination in the bacterial species *Bacillus megaterium*. *B. megaterium* is a Gram-positive, aerobic, rod-shaped, spore-former. When nutrients are absent or scarce, this bacterium has the ability to differentiate from a vegetatively growing cell to a dormant spore. This process is called sporulation. The spore is resistant to heat, dessication, dyes, and radiation. In this way, the organism can survive until harsh environmental conditions come to an end and essential nutrients become available. Sporulation consists of seven stages. In the second stage, a septum forms dividing the cell into two compartments: the forespore and the mother cell. Signals must cross this septum in order for sporulation to proceed. When nutrients are once again available, the dormant spore grows into a vegetative cell through a process called germination. Signals must pass through the spore coat, cortex, and two membranes in order to trigger germination. This laboratory is interested in cell signaling that occurs during sporulation and germination and the genetics that control these two processes (1).

Various plasmids have been used as vectors in *B. megaterium*, and some have been found to be stable (2). One such plasmid, pEG7, is a shuttle plasmid made up of DNA from *E. coli* and from *B. megaterium* (3). However, some plasmids are not stable (4). The stability problem is probably due to the

fact that most plasmid vectors are from species such as *Escherichia coli*. It seems reasonable that vectors created using plasmids from *B. megaterium* will be stable in this species. *B. megaterium* wild type strain QM B1551 contains seven resident plasmids. These plamids are of molecular weights 3.5, 5.3, 13.0, 32.0, 46.0, 70.0, and 109.0 megadaltons(md). The relative copy number of these plasmids have been found to be 135, 76, 45, 16, 10.5, 3.6, and 1, respectively. The plasmids have been found to be stable. At least 11% of the cellular DNA consists of plasmid DNA. Very little is known about the genes contained on these plasmids. Data has been produced suggesting that a gene for one form of megacin resides on one of the three larger plasmids (2). Recent studies performed in this lab suggest that a germination gene exists on one of the plasmids.

A technology that is very useful to this laboratory as well as many other laboratories is gene cloning. This process is a way of making many copies of a particular fragment of DNA. Recombinant DNA technology can be used to construct various plasmids useful for genetic analysis of bacteria such as *Bacillus megaterium*. The smallest plasmid of *B. megaterium*, the 5.4-kilobase(kb) plasmid (3.5md), has been chosen for the construction of recombinant plasmids that can be of use in the genetic analysis of *B. megaterium*.

The first major step in creating these plasmids is to clone the 5.4-kb plasmid. Various mutant strains have been created that lack certain plasmids. Some of these cured strains were screened to determine if they contained the 5.4-kb plasmid.

Because of problems with the mini-prep DNA extraction protocol, it was not possible to accurately determine which strain contained the smallest plasmid. It was then decided to use wild type strain QM B1551 DNA containing all seven plasmids to obtain the smallest plasmid. DNA was isolated from QM B1551 by cesium chloride preparation, and the plasmids were separated by size using agarose gel elecrophoresis. The band containing the 5.4-kb plasmid was cut from the gel, and the plasmid DNA was removed from the agarose using the GeneClean(TM) procedure. The 5.4-kb plasmid and the *E. coli* plasmid pUC19 were each cut with a different restriction endonuclease leaving blunt ends. The two blunt-ended plasmids were ligated to each other, and the recobinant molecule was transformed into *E. coli* strain JM83 to make multiple copies of the molecule. This new plasmid can be modified to make various stable plasmid vectors for use in studying the genetics of *B. megaterium*.

MATERIALS AND METHODS

Mini-prep DNA extraction

Minipreps of *B. megaterium* were performed using the "Miniscreen for B. subtilis Plasmids" of Rodriguez and Tait (5) & Roy Doi. SNB media was use instead of TBAB. In some preps, cells were taken directly from plates instead of being grown in broth. Also, an extra ethanol wash of the DNA pellet was performed in some preps. Minipreps of *E. coli* were performed

using the Magic Minipreps (TM) DNA Purification System kit from Promega Corporation.

Cesium Chloride Plasmid Preparation

Cesium Chloride preparations were performed using the protocol of Lovett and Keggins (6).

Agarose Gel Electrophoresis

Most electrophoresis was performed using 0.7% agarose in TBE (tris-borate-EDTA) buffer. Preparative gels were performed using 0.7% agarose in TAE (tris-acetate-EDTA) buffer. Gels were run using a power source at various voltages for various times until the loading dye reached the bottom of the gel. DNA in the gels was stained with ethidium bromide and illuminated using a ultra-violet light source. Photographs of the gels were taken using a Polaroid MP4 land camera. For preparative gels, bands were cut from the gel, and the DNA was separated from the agarose using the GeneClean kit from Bio 101 (La Jolla, CA).

Restriction Endonuclease Digestion

DNA was digested using enzymes and buffers from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Reactions were performed in a total volume of 20 microliters (ul) with 2 ul of the appropriate 10X-concentrated buffer. Amounts of enzyme,

DNA, and double-distilled water varied. Reaction mixtures were incubated at 37 degrees C for two hours. Heat-inactivation-resistant enzyme reactions were stopped by adding 2 ul stop load to each reaction. Heat-inactivation-sensitive enzyme reactions were stopped by placing the reaction tubes in a 65 degree C water bath for ten minutes. In-gel restriction endonuclease digestions were performed according to the protocol of the FMC Corporation which was provided with their low melting point agarose.

Ligations and Vectors Used

The vector used was the *E. coli* plasmid pUC19. This plasmid contains an ampicillin resistance gene which means that only cells that were successfully transformed with this plasmid will grow on medium containing ampicillin. The plasmid also contains a multiple cloning site (MCS) in a lacZ gene. Cells that have a piece of DNA cloned into the multiple cloning site will not be able to produce the product of this gene. The product of this gene is beta-galactosidase which cleaves lactose into glucose and galactose and cleaves the synthetic compound X-

gal releasing a blue compound. Therefore, if a cell is transformed with pUC19 and plated on medium containing X-gal, the colonies will be blue. If a cell is transformed with pUC19 with a DNA fragment cloned into its MCS, the colonies will be white. In this way, one can select for cells which contain pUC19 with the cloned fragment (7).

Transformation of E.coli

E. coli strain JM83 was transformed using Protocol II of Maniatis et al (8). LB media was used instead of SOB, and LB with 20 mM glucose was used instead of SOC. Transformed cells and control cells were plated on LB plates with 50 ug/ml ampicillin and 100 ul 1% X-gal per plate, and plates were incubated overnight.

RESULTS

Screening of Mutant Strains

Plasmid DNA from wild type *B. megaterium* strain QM B1551 and from various mutant strains cured of some of the seven plasmids with ethidium bromide were was extracted using the mini-prep protocol. This DNA was electrophoresed to identify a strain which contained the 5.4-kb and few others plasmids. Problems arose with the mini-prep protocol. Little DNA was obtained, and the DNA that was extracted was mostly chromosomal.

Figure 1 shows a photograph of one of the gels from the screening process. Lanes 1 and 2 are *E. coli* strain V517. Lanes 3-8 are various mutant strains, and lanes 9 and 10 are QM B1551. Chromosomal DNA is represented by the thick fuzzy band. Also, the lanes are constricted due to salts present in the DNA solution. The problems with the mini-preps may be due to the fact that the protocol followed uses 1.5M potassium acetate solution while other protocols use 3.0M sodium acetate (5) or KAc that is 3.0M K and 5.0M Ac (9). The protocol will probably have to be changed in order to solve the problems.

DNA Extraction and Restriction Endonuclease Digestion

Because of the problems with the mini-prep procedure, strain QM B1551 was used to obtain the 5.4-kb plasmid. The CsCl plasmid prep was used to extract all seven plasmids and the DNA was electrophoresed on a preparative gel (Figure 2). The band containing the smallest plasmid was cut out of the gel. The DNA was removed from the agarose using the GeneClean procedure. The small plasmid was cut using the enzyme *Hinc*II which cuts the small plasmid at only one site leaving blunt ends (11). The small plasmid cut with *Hinc*II was electrophoresed on an agarose gel and produced only one band, however, this band was very faint and was difficult to see.

pUC19 DNA was also cut with *Hinc*II which cuts this plasmid at only one site in the MCS (7). The cut DNA was electrophoresed on a preparative gel to test the GeneClean kit.

A photograph of the gel can be seen in figure 3. The first lane contains cut pUC19, and the second lane contains uncut pUC19. Uncut pUC19 appears as multiple bands because of multimers. The lowest band represents single closed-circular plasmids. When the plasmid is cut wiht a restriction enzyme, linear fragments of the size of a single plasmid are produced resulting in a single band on the gel. The band for the cut plasmid is higher than the lowest band for the uncut plasmid because linear DNA fragments move slower though a gel than closed-circular plasmids.

Ligation and E. coli Transformation

The vector used for the ligations was pUC19 cut with *Sma*I and phosphatased. This DNA was used instead of the DNA cut with HincII because phosphatasing a linear fragment removes the phosphates from the ends. Without phosphates on the vector, the two ends of the plasmid cannot ligate to each other. This increases the chance that the vector will be ligated to the foreign DNA (the 5.4-kb plasmid) (10). SmaI also cuts pUC19 at only one site in the MCS leaving blunt ends (7).

In the first trial, 77ng of pUC19 DNA and approximately 15.4ng of 5.4-kb plasmid DNA was used for the ligation reaction. The reaction mixture contained a total of 92.4ng of DNA. One transformation reaction tube contained 18.48ng of DNA from the ligation mixture. Another tube contained 40ng of pUC19 DNA alone. A third tube contained no DNA. The last two tubes are

controls. In the second trial, 77ng of pUC19 DNA and about 84ng of the small plasmid was used for the ligation - a total of 161ng of DNA. One transformation tube contained 53.7ng of DNA from the ligation mixture. A second tube contained 154ng of pUC19 DNA alone. The third tube contained no DNA. More pUC19 DNA was used in the second transformation because DNA of a higher concentration was mistakenly used.

The results of the transformations can be seen in Table 1 and Table 2 at the end of the report. Transformation efficiencies are given in number of transformants per ng of DNA. Ligation efficiencies are given in percent white colonies. The first transformation had a higher transformation efficiency than the second. In fact, it had an efficiency approaching that of the pUC19 transformation. This may have been because too much DNA was used in the second transformation. The protocol states that less that 40ng of DNA should be used in transformations. However, in the second transformation, 154ng of pUC19 produced so many transformants that growth was almost confluent (TNTC). There may be some differences in the transformation efficiency of DNA from different species. The second trial produced a higher ligation efficiency. This is most likely due to the higher amount of 5.4-kb plasmid DNA used in the second ligation.

To determine the size of the cloned fragment of DNA, mini-preps of bacteria grown from white colonies were performed. DNA from white colonies from transformation one and pUC19 DNA alone was cut with *Eco*R1 and electrophoresed with lambda cut with *Hind*III. The bands from the transformant DNA were very close to

the bands from pUC19 alone and could not be distinguished. DNA from three colonies that appeared to have slightly larger plasmids was cut with *Eco*R1 and *Bam*H1 to remove the cloned fragment. This DNA (lanes 1-3) was electrophoresed with a lambda standard (lane5) and a 123bp ladder (lane 4), and the results can be seen in Figure 4. Since fragments were at various positions on the gel and the lambda standard did not show the correct pattern, there is reason to believe that the gel did not run correctly. It is possible that the agarose gel contained the wrong concentration of buffer. DNA from trial two white colonies was also digested with *Eco*R1 and *Bam*H1 (lanes 1-10) and run on a gel with a 123bp ladder (see Figure 5). The ladder is in lane 11. Since all colonies produced the same pattern of bands, only one fragment of the small plasmid was cloned. The largest band is pUC19. The two smaller bands are about 1700bp and 500bp. Since these do not add up the the full size of the small plasmid, either only part of the plasmid was cloned and/or the plasmid contains *Eco*R1 or *Bam*H1 sites.

Further Restriction Analysis

In order to determine what happened with the cloning experiment, the small plasmid was digested with *Hinc*II (lane 1) using the in-gel digestion protocol and electrophoresed (Figure 6) with a lambda standard (lane 2). Two bands were produced corresponding to sizes of about 3900bp and 1150bp. To account for the entire plasmid, a 350bp fragment must have run off of the gel. In fact, the 500bp fragment of lambda did run off of the gel.

DISCUSSION

A major problem throughout this project was the low amount of 5.4-kb plasmid DNA available to work with. In order for blunt-end ligation to work well, an insert to vector ratio of 10:1 should be used. The ratios used in these experiments ranged from about 1:5 to 1:1. In order to improve the results, more DNA must be made available. This can be accomplished by using one or two liters of cells in a cesium chloride prep.

Another problem was that the entire cloning strategy was developed using the information from the restriction analysis of the 5.4-kb plasmid (11). The results of this project suggest that this analysis is erroneous. This analysis shows that *Hinc*II cuts the plasmid only once and that *Ecc*R1 and *Bam*H1 do not cut the plasmid at all. The results of cutting the plasmid with *Hinc*II indicate that there are three cut sites for this

enzyme producing three fragments of sizes 3900kb, 1150kb, and 350kb. The results of the digestion of the transformant DNA make sense only if the 3900kb fragment was cloned. There are probably two cut sites within this fragment for either *Eco*R1 or *Bam*H1. This would produce two 1700bp fragments and one 500bp fragment corresponding to the two lower bands in Figure 5. In order to verify these explanations, further restriction analysis must be performed on the 5.4-kb plasmid.

The cloning of part of the 5.4-kb plasmid leads to other possible projects that involve the construction of various plasmids that can be used to study genetics in *B. megaterium*. The first plasmid that can be made is a shuttle plasmid. This plasmid is made up of DNA from two species. This plasmid can be used to compare the expression of a cloned gene in either species (Crabb). It will have to be determined if the cloned fragment of the small plasmid contains the origin of replication of the plasmid. This can be accomplished by transforming *B. megaterium* and seeing if the hybrid plasmid can replicate. There also must be a way of selecting for the plasmid in *B. megaterium*. An antibiotic resistance gene for *B. megaterium* can be inserted into the hybrid plasmid. Because it can replicate in *E. coli* and *B. megaterium*, the shuttle plasmid can be used to make many copies of an inserted gene. The plasmid can then be used to trasform *B. megaterium* in order to express the gene.

Integrative plasmids can also be made. These plasmids are similar to shuttle plasmids in that they contain DNA of two species. The difference is that integrative plasmids must

contain genomic DNA of the species in which the integration will take place do not contain an origin of replication for this species. This will only work with the pUC19/5.4-kb hybrid plasmid if it does not contain an origin of replication for *B. megaterium*. These plasmids integrate at the site of the gene that is homologous or identical to the foreign gene or DNA fragment that they carry. Because they do not contain an origin of replication for *B. megaterium*, the only way they can remain a part of the organism is if they integrate into the chromosome. If a fragment of a gene is used, an integrative plasmid can be used to inactivate that gene. This will give the organism a phenotype similar to that of a mutation of that gene (12). If an entire gene is used and if the plasmid contains an antibiotic resistance gene for the species into which it integrated, the plasmid can be used to map that gene using cotransduction studies.

Another plasmid is called a promoter probe, which can be used to determine if a particular fragment of DNA is a promoter. These plasmids use what are called reporter genes, a gene whose function can be assayed easily. An example of a reporter gene is lacZ, which was explained in "Materials and Methods" above. If the cloned piece of DNA is a promoter, it will express the lacZ gene, and colonies grown on X-gal will be blue (13). Once a promoter is found, these plasmids can be used to determine what factors affect the promoter and, therefore, regulate the gene that corresponds to that promoter

A very useful type of plasmid is an expression vector which is used to make the protein coded for by a particular gene. Expression vectors contain a promoter for a gene from the organism in which expression is to occur. *E. coli* expression vectors usually use the *lac* UV5 promoter or the tryptophan promoter. To make an expression vector for use in *B. megaterium*, a promoter for a gene from this organism can be inserted into the pUC19/5.4-kb hybrid plasmid. The protein that is expressed can then be studied to determine its function. The expression plasmid is also used in industry to make large amounts of a protein for use in research, medicine, and other applications.

TABLE 1

Transformation One

DNA Type	Plate #	Cells plated(ul)	Blue Colonies	White Colonies
no DNA		100	0	0
pUC19	1	100	TNTC	0
pUC19	2	100	TNTC	0
pUC19	3	50	554	0
Transformation Efficiency				13.85
pUC19+p5.4	1	100	6	1
pUC19+p5.4	2	100	7	0
pUC19+p5.4	3	100	6	0
pUC19+p5.4	4	100	12	0
pUC19+p5.4	5	100	5	0
pUC19+p5.4	6	100	3	0
pUC19+p5.4	7	100	7	0
pUC19+p5.4	8	100	5	0
pUC19+p5.4	9	100	14	0
pUC19+p5.4	10	100	126	6
		Total Colonies	191	7
		Transformation Efficiency		10.34
		Ligation Efficiency		3.54%

TABLE 2

Transformation Two

DNA Type	Plate #	Cells plated(ul)	Blue Colonies	White Colonies
no DNA		100	0	0
pUC19	1	100	TNTC	0
pUC19	2	50	TNTC	0
pUC19+p5.4	1	100	13	2
pUC19+p5.4	2	100	11	2
pUC19+p5.4	3	<100	2	0
pUC19+p5.4	4	100	7	1
pUC19+p5.4	5	100	16	1
pUC19+p5.4	6	100	10	0
pUC19+p5.4	7	100	9	1
pUC19+p5.4	8	100	8	1
pUC19+p5.4	9	100	3	1
pUC19+p5.4	10	100	12	1
		Total Colonies	91	10
		Transformation Efficiency		1.88
		Ligation Efficiency		9.90%

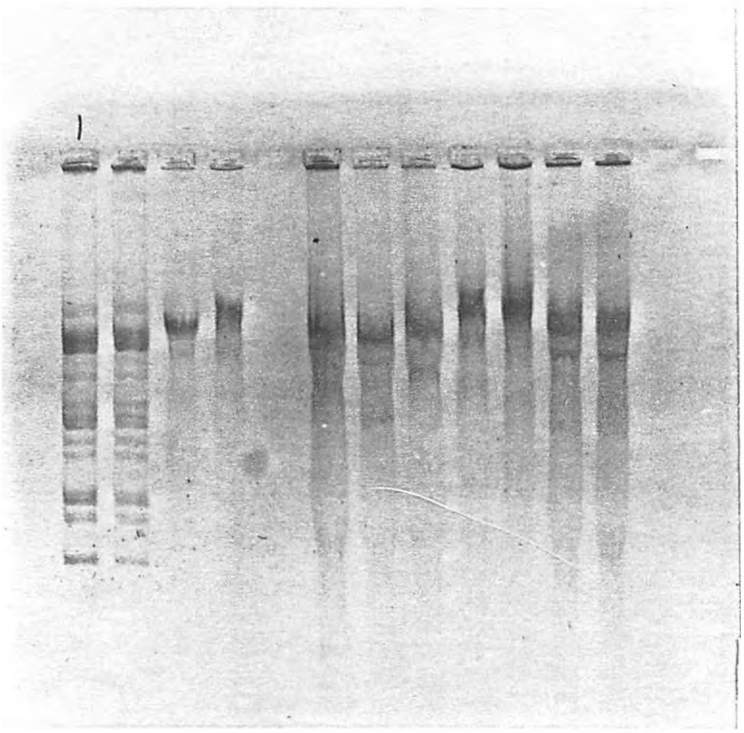

FIGURE 1

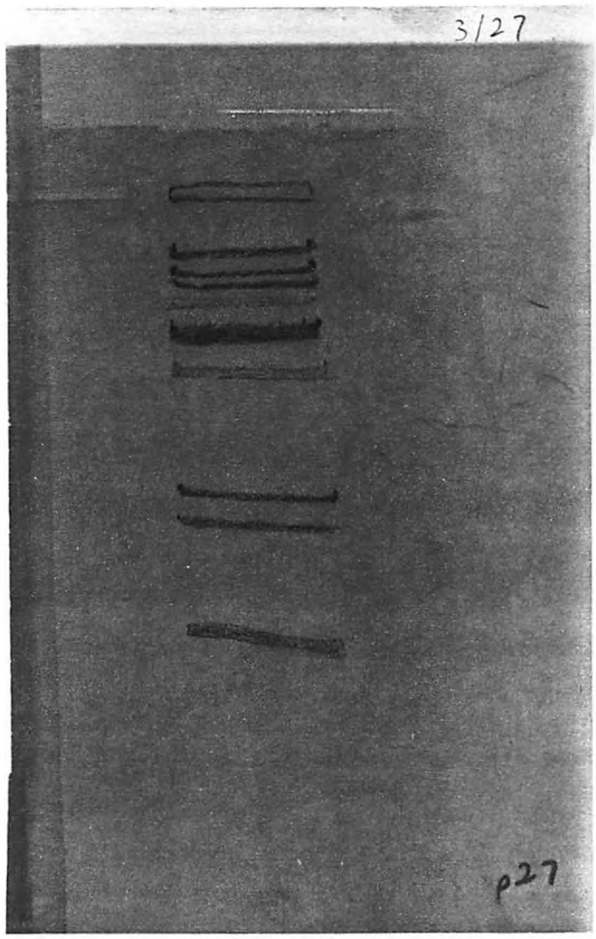

FIGURE 2

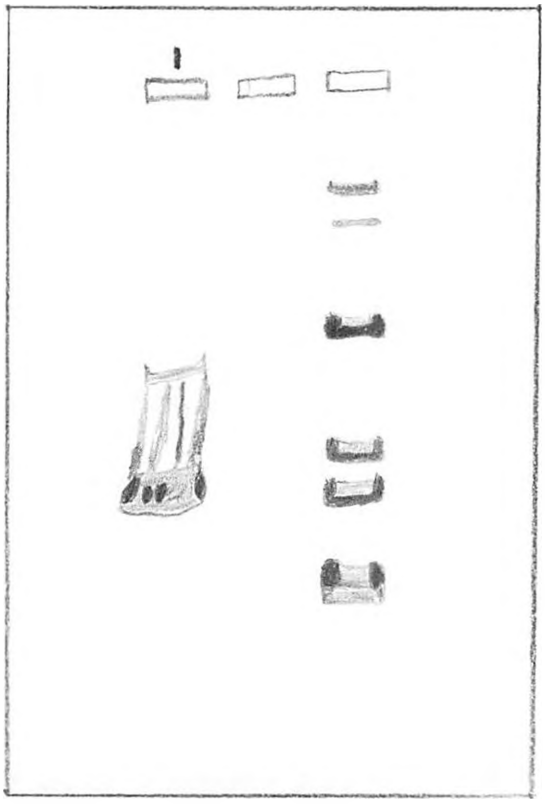

FIGURE 3
(tracing)

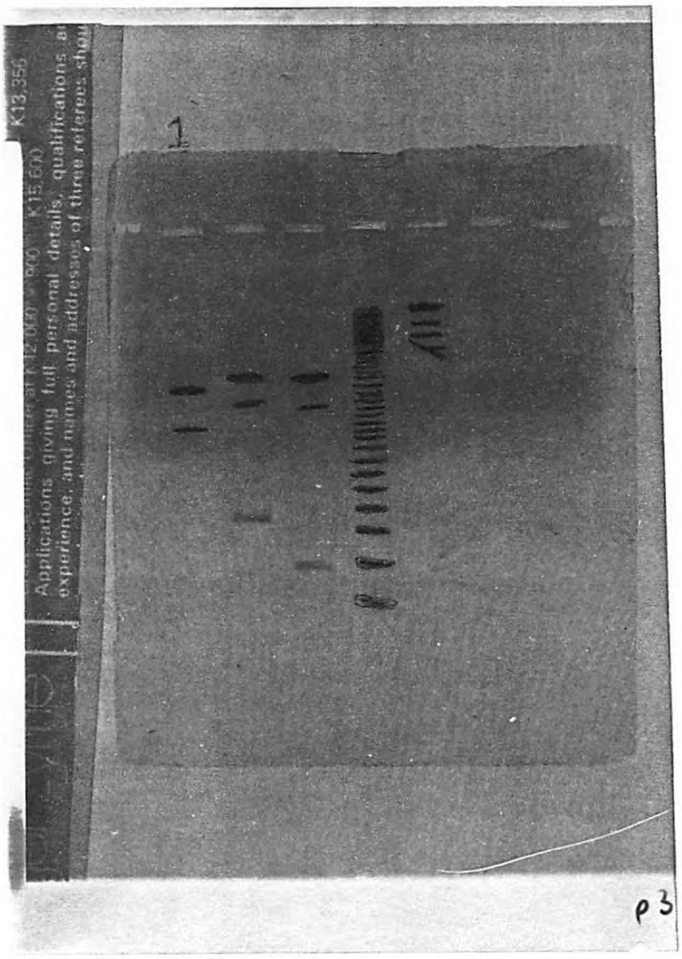

FIGURE 4

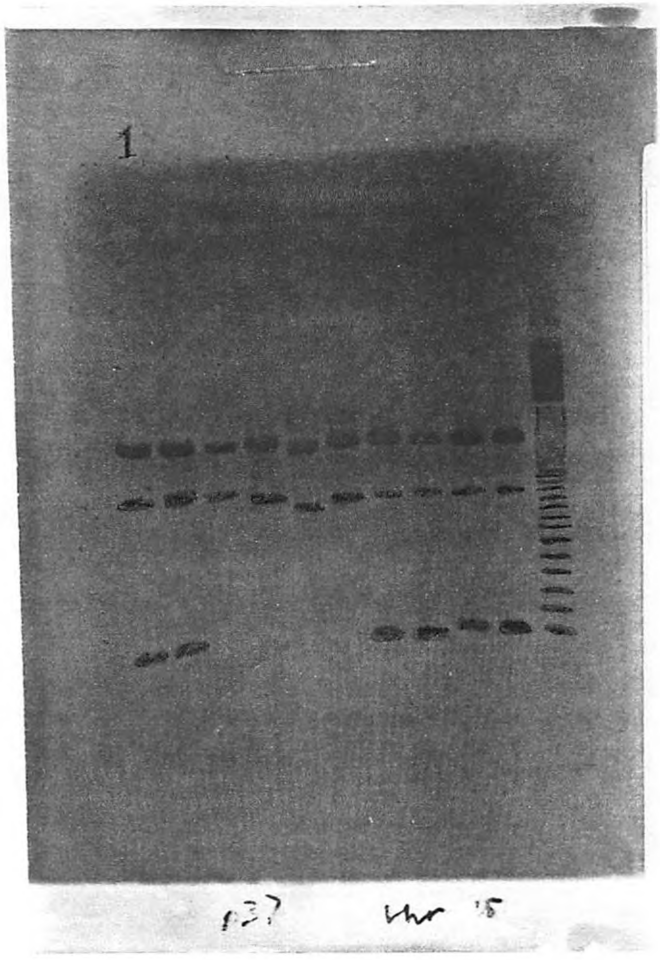

FIGURE 5

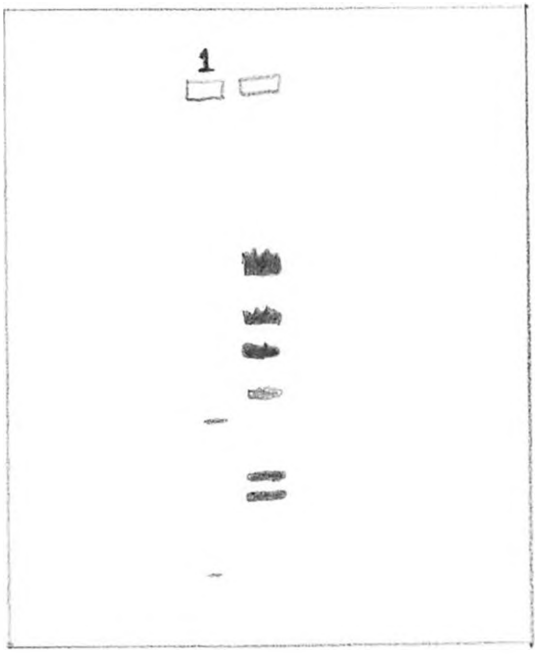

FIGURE 6
(tracing)

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