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RESTRICTION DIGESTION OF pUC18 AND pBR322 BY BAMH I AND ECOR I ENZYMES

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ABSTRACT: Restriction digestion of pUC18 and pBR322 will be done using restriction enzymes such as BamH I and EcoR I respectively. The Hybrid plasmid (pUC18 + pBR322) will be constructed by adding T4 DNA ligase to the digested vectors and transformed to the competent cells of DH5 α E.coli strain with the help of Calcium chloride. Blue- white Screening would be done for the transformed cells where the white colonies would be selected and the possible results might be of transformed cells containing either pBR322 alone or the hybrid plasmid. The selected white colonies will be subjected to antibiotic screening by adding ampicillin. The colonies that will be formed after the addition of ampicillin are supposed to be considered as the cells having hybrid plasmids. Further, the transformed DH5 α cells containing the hybrid plasmid would be subjected to stability studies such as determination of growth rate by comparing the growth rates of control DH5 α cells, transformed DH5 α cells containing pUC18 and pBR322 individually with the transformed DH5 α cells containing the hybrid plasmid. Other stability studies such as thermal resistance of transformed DH5 α cells containing the hybrid plasmid would be determined. From the above studies, it would be possible to create a novel DH5 α cell strain with added advantages of increased growth rate and thermal resistance.

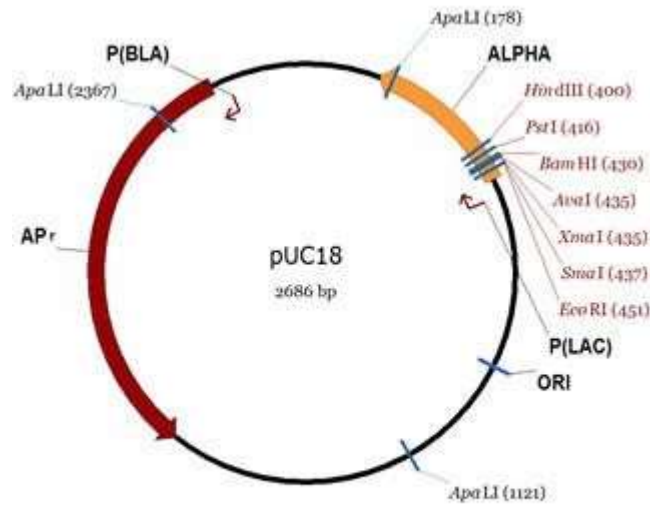
INDEX TERMS - PUC18 AND PBR322, HYBRID PLASMIDS, DH5A E.COLI STRAIN

I. INTRODUCTION

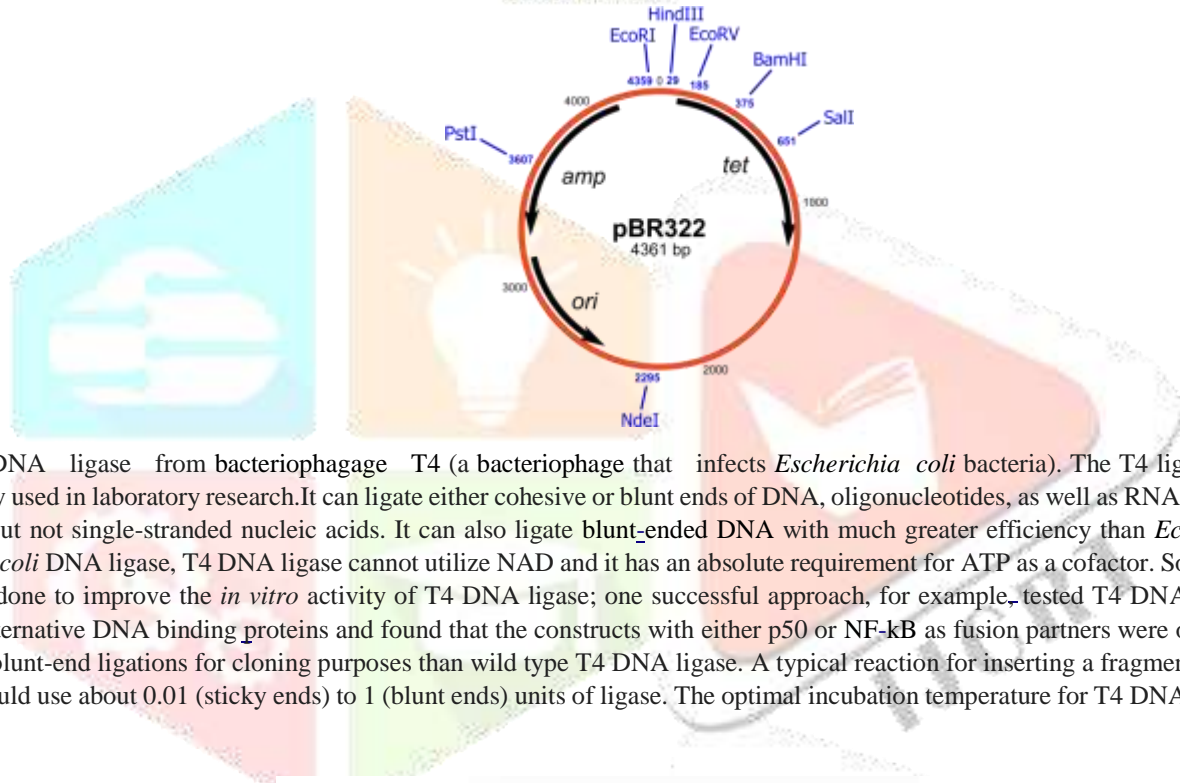
A plasmid is a circular dsDNA molecule a few hundred or thousand base pairs in circumference. The artificial plasmid pUC18 has been genetically engineered to include a gene for antibiotic resistance to Ampicillin (*amp^R*), and a gene (and its promoter) for the enzyme beta-galactosidase (*lacZ*). The *lacZ* gene contains a polylinker region, with a series of unique restriction sites found nowhere else in the plasmid. Digestion with any one of these endonucleases will make a single cut that linearizes the circular plasmid DNA, and allow it to recombine with foreign DNA that has been cut with the same endonuclease.

pBR322 is 4361 base pairs in length¹ and has two antibiotic resistance genes the gene *bla* encoding the ampicillin resistance (Amp^R) protein, and the gene *tetA* encoding the tetracycline resistance (Tet^R) protein. It contains the origin of replication of pMB1, and the *rop* gene, which encodes a restrictor of plasmid copy number. The plasmid has unique restriction sites for more than forty restriction enzymes. Eleven of these forty sites lie within the Tet^R gene. There are two sites for restriction enzymes HindIII and ClaI within the promoter of the Tet^R gene.

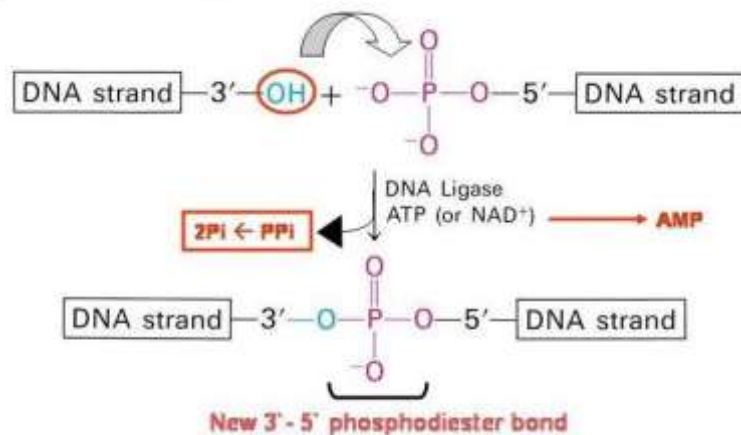
DNA ligase is a specific type of enzyme, a ligase, that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. It plays a role in repairing single-strand breaks in duplex DNA in living organisms, but some forms (such as DNA ligase IV) may specifically repair double-strand breaks (i.e. a break in both complementary strands of DNA). Single-strand breaks are repaired by DNA ligase using the complementary strand of the double helix as a template, with DNA ligase creating the final phosphodiester bond to fully repair the DNA.



pUC18 map



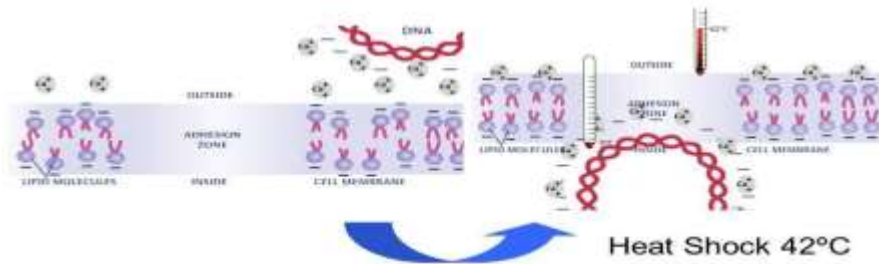
The DNA ligase from bacteriophage T4 (a bacteriophage that infects *Escherichia coli* bacteria). The T4 ligase is the most commonly used in laboratory research. It can ligate either cohesive or blunt ends of DNA, oligonucleotides, as well as RNA and RNA-DNA hybrids, but not single-stranded nucleic acids. It can also ligate blunt-ended DNA with much greater efficiency than *E. coli* DNA ligase. Unlike *E. coli* DNA ligase, T4 DNA ligase cannot utilize NAD and it has an absolute requirement for ATP as a cofactor. Some engineering has been done to improve the *in vitro* activity of T4 DNA ligase; one successful approach, for example, tested T4 DNA ligase fused to several alternative DNA binding proteins and found that the constructs with either p50 or NF-κB as fusion partners were over 160% more active in blunt-end ligations for cloning purposes than wild type T4 DNA ligase. A typical reaction for inserting a fragment into a plasmid vector would use about 0.01 (sticky ends) to 1 (blunt ends) units of ligase. The optimal incubation temperature for T4 DNA ligase is 16 °C.



The process of calcium chloride heat-shock transformation encourages bacterial cells to uptake DNA from the surrounding environment. The exact mechanism of how this process works is still largely unknown, but there are hypotheses on the different aspects of the procedure. The role of calcium ions in the cell suspension is hypothesized to be a cation bridge between the negative charges on phosphorylated lipid A in lipopolysaccharide (LPS), and the phosphate backbone of DNA (1, 2). The ice-cold CaCl₂ solution facilitates binding of DNA to the surface of the cell, which then enters the cell after a short period of heatshock (3). Cells that are successfully transformed are usually identified by selection or screening markers such as drug resistance or fluorescence (4). This technique is commonly used to transform cells with plasmids for various purposes like recombinant protein expression, cloning, and long term storage of the plasmids.

Calcium Chloride Solution

- The addition of calcium ions in CaCl_2 solution shields the negatively charged phosphate groups so that the DNA can pass through the membrane when heat shocked.



DH5-Alpha Cells are *E. coli* cells engineered to maximize transformation efficiency. They are defined by three mutations *recA1*, *endA1* which help plasmid insertion and *lacZM15* which enable blue white screening. The cells are competent and often used with calcium chloride transformation to insert the desired plasmid.

II.METHODOLOGY

A. Restriction Digestion

Restriction enzymes are powerful tools of molecular genetics which recognise specific DNA sequences and cleave DNA at specific location of recognition site. Restriction digestion is commonly carried out at 37c along with the buffering agents and cofactors that help in high fidelity.

Prepare two different reaction mixtures using the following ingredients a)REACTION 1(EcoR I DIGESTION)

INGREDIENT	VOLUME(IN μl)
λ DNA	5
10X cohesive buffer	5
Restriction Enzyme	2
Nuclease Free Water	13

b)REACTION 2(BamH I Digestion)

INGREDIENT	VOLUME(IN μl)
λ DNA	5
10X cohesive Buffer	5
Restriction Enzyme	2
Nuclease Free Water	13

Now incubate the tube at 37c for one hour.

B. Ligation

The following reactions were set up in a microfuge tube. Total reaction volume was made to 20ml

T4 DNA Ligase buffer (10x)	2µl
Vector DNA (4Kb)	50ng
Insert DNA(1Kb)	37.5ng
Nuclease Free Water	6µl
T4 DNA Ligase	2µl

- It was gently mixed by pipetting up and down in the centrifuge
- For cohesive ends , incubated at 16c overnight

NOTE: high concentration of T4 DNA Ligase can be used in 10minutes Ligation

- Heat Inactivated at 65c for about 10minutes
- Chilled on ice and transformed 1-5µl of the reaction into the 50µl of competent cells

C. Transformation

The blue–white screen is a **screening technique** that allows for the rapid and convenient detection of recombinant bacteria in **vector-based molecular cloning** experiments. **DNA** of interest is ligated into a **vector**. The vector is then **inserted** into a competent host cell viable for transformation, which are then grown in the presence of X-gal. Cells transformed with vectors containing recombinant **DNA** will produce white colonies; cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies. This method of screening is usually performed using a suitable **bacterial strain**, but other organisms such as yeast may also be used.

The blue/white screening method works by disrupting this α -complementation process. The plasmid carries within the *lacZ α* sequence an MCS. This MCS within the *lacZ α* sequence can be cut by restriction enzymes so that the foreign DNA may be inserted within the *lacZ α* gene, thereby disrupting the gene that produces α -peptide. Consequently, in cells containing the plasmid with an insert, no functional β -galactosidase may be formed.

The presence of an active β -galactosidase can be detected by X-gal, a colourless analog of lactose that may be cleaved by β -galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. This results in a characteristic blue colour in cells containing a functional β -galactosidase. Blue colonies therefore show that they may contain a vector with an uninterrupted *lacZ α* (therefore no insert), while white colonies, where X-gal is not hydrolyzed, indicate the presence of an insert in *lacZ α* which disrupts the formation of an active β -galactosidase.

C. Process

Selection of cells containing our hybrid DNA is done based on the selectable marker Identification of the recombinants among all the transformed cells is generally done by the insertional inactivation. Insertion of the insert plasmid into the other destroys the integrity of one of the genes present on the molecule. The presence of lacZ gene that codes for the enzyme gets disturbed due to the insertion of insertion plasmid and results in producing white colonies as recombinants and blue as non recombinants. Aseptically aliquot 100 µl competent cells into vials.100ng of the hybrid plasmid was added to the competent cells. Heat shock the cells by placing them at 40c water bath for 2 minutes. Then the vials were chilled.

1ml of LB Broth was added to the vials and incubated for one hour so that the bacteria expresses its antibiotic resistance .Label three LB plates and add 40µl of X-gal and IPTG are added .Spread well using the glass rod. An LB plate was also labelled as control to check for the contamination. The cells that are not transformed were added to it.Incubate the plates over night at room temperature. Check for blue white colonies.

E. Cell extracts

A 40 nd volume of cells at a Klett reading of 100 +- 5 units was chilled on ice, the cells pelleted by centrifugation at 12100 Xg, and washed two times in 0.85% sodium chloride. The cells were stored as frozen pellets for no longer than 48 h. The thawed cells were resuspended in 2.0 nd of 1.0 M phosphate buffer (pH 7.4) and extracts prepared by sonifying 1 time for 5 s with the ES tip (No. 9118) on a Lab.Line Ultratip Labsonic System at a power setting of 80 W. Centrifugation in an eppendoff model 5412 centrifuge (1.5 ml polypropylene tubes) for 5 min in a 4°C cold room removed debris. Extracts were used within 1 h for enzyme assays.

F. Enzyme

STHM activity was determined by the method of Taylor and Weissbach (1965) which monitors the conversion of L-[3-t⁴C]serine to glycine. The results are averages of two or more assays in which the reactions were determined in triplicate for each assay, (f) Protein determinations Protein determinations were made by the method of Lowry et al. (1951).

G. Plasmid isolation

Plasmid DNA was prepared from chloramphenicol amplified cells (Clewell 1972) by an SDS lysis procedure followed by ethidium bromide-caesium chloride equilibrium density gradient centrifugation (Guerry et al., 1973; Selker et al., 1977). To analyze transformants for plasmid DNA, small quantities of DNA were isolated using the procedure described by Cameron et al. (1977) as modified by Williams et al. (1979).

III. CHARACTERIZATION OF PLASMID DNA

Hybrid Plasmids Extracted by the Screening Method. Twenty-eight individual clones of hybrid plasmids containing fragments of coliphage T4 DNA inserted into pBR322 were prepared by alkaline extraction as an illustration of the method (Fig. 1). The results are typical of those that may be expected with the screening procedure. Bands of plasmid DNA are detectable in nearly every slot, although the intensity of the individual band varies. Thirty samples can be handled readily on a 20 cm-wide gel. Overall, the mobility of the CCC form of the plasmid should provide a good indication of the size of the inserted fragment.

IV. MOLECULAR AND GENOMIC CHARACTERISATION OF STRAINS

The multilocus sequence typing (MLST) method based on seven housekeeping genes (<http://bigsdw.web.pasteur.fr/klebsiella/klebsiella.html>) was used to identify the STs of the two *K. pneumoniae* strains. The genomes were extracted with the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the recommended protocol. Whole-genome sequences (WGS) of the two strains were obtained using the paired-end sequencing strategy (2 × 150 bp) in the Illumina HiSeq 2500 and Nanopore MinION (long-read) sequencing with Rapid Barcoding Kit (RBK004) in the R9.4.1 flow cell. Then, the hybrid de novo assembly strategy with the Unicycler tool was used to assemble the genomes, as previously reported [18], and followed by manual confirmation in terms of recombination sites with original long-read by BLASTn for hybrid plasmids. Different databases, including ResFinder and ISfinder, were used to investigate the distribution of plasmid replicons, resistance genes and insertion sequences among the genomes, and the putative virulence genes of KP17-15 and KP17-16 were predicted using the Virulence Factor Database (VFDB; <http://www.mgc.ac.cn/VFs/main.htm>). Together with the genomes of KP17-15 and KP17-16, six genomes, including another four *K. pneumoniae* strains with different STs, were annotated by Prokka (<https://github.com/tseemann/prokka>) and phylogenetic tree was constructed (Figure S1). The default settings in terms of sequence identity (95%) and paralog splitting (turn on) were used during Roary analysis. A total of 4226 core genes (4 134 945 bp in total) were aligned with Roary. SNPs of the genomes were analysed by SNP sites based on the core_gene_alignment file generated by Roary [19].

V. CONJUGATION AND PLASMID ANALYSIS

Conjugation assay was conducted with modifications from a previously described method to evaluate the transferability of virulence plasmids [20]. In brief, the KP17-15/KP17-16 isolates served as the donor, while *E. coli* strain EC600 (rifampin resistant) and ST11 *K. pneumoniae* HS11286YZ6 (hygromycin resistant but susceptible to doxycycline) were used as the recipient strains [21]. Both donor isolates and recipient strains were cultured in LuriaBertani (LB) broth at 37°C for 6–8 h. The cultures of the donor and recipient were diluted 1:25 in fresh LB broth respectively, mixed at a 1:1 ratio and subsequently incubated at 30°C for 24 h. Then, the mixture was inoculated on LB agar plates supplemented with different combinations of antibiotics at 37°C for 24 h. Transconjugants that grew on the selecting medium were picked up and identified by PCR and sequencing. Conjugation frequencies of plasmids that transferred to different recipient strains were compared by using the χ^2 test at a significance level of $P < 0.05$. S1-PFGE and Southern hybridization were used to obtain plasmid profiles and locate the resistance and virulence genes in KP17-15/KP17-16 and its transconjugants [22]. Plasmid comparisons were performed by using BRIG (<http://brig.sourceforge.net/>) and Easyfig (<http://mjsull.github.io/Easyfig/>) tools.

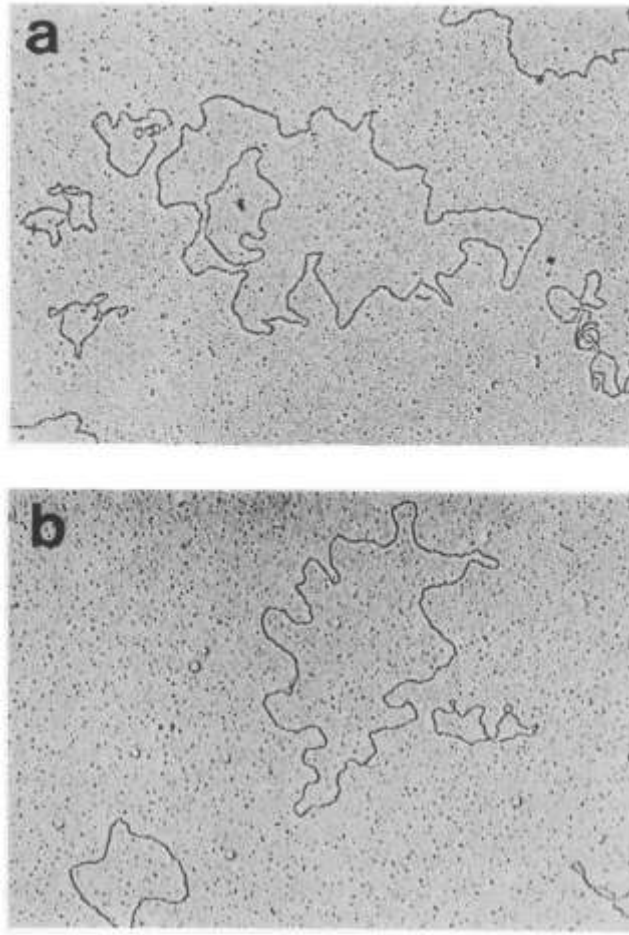
VI. RESULTS

A. Transformation efficiency of annealed hybrid DNA

DNA was trpA58, transformants were Trp⁺ since the MV10 Annealed hybrid DNA was used in several transformations recipient has a wild-type trpA gene.) Forty-five isolates of *E. coli* auxotrophs (Table 2). Col E1 circular plasmid were obtained (4 transformants/ μ g of DNA), twelve of DNA alone, when used to transform strain SB2(C600) to col⁻ which were resistant to colicin E1 and sensitive to colicin E2. Colicin E1 immunity, routinely yielded approximately 6 × 10⁴ The remaining 33 colonies were Trp⁺ but were sensitive to transformants per μ g of DNA on L-broth-colicin E1 plates. both colicins, and presumably were recombinants between The transformants were resistant to colicin E1 but sensitive trp⁺ DNA and the chromosome. They were either lacking to colicin E2 (see Methods and Materials). Annealed hybrid plasmid or the ability to express colicin E1 resistance. We DNA, when used in similar transformations, gave approxi- have not seen this type of recombinant transformant if the mately 103 transformants per μ g of DNA. Again, these trans- *E. coli* recipient is recA, however. formants were resistant to colicin E1, but sensitive to colicin The preparation of annealed hybrid DNA was also used to E2. The Col E1 (LRI exo)-(dT)150 DNA gave less than one transform strain N

It was observed that more transformation was observed in all the petriplates (pUC18, pBR322 and hybrid plasmid). Transformation efficiency is the efficiency by which cells can take up extracellular DNA and express genes encoded by it. This is based on the competence of the cells. It can be calculated by dividing the number of successful transformants by the amount of DNA used during a transformation procedure. The blue coloured colonies are observed more in number than the white colonies (non recombinants) this may be because of higher ligation efficiency. The preparation of annealed hybrid DNA was also used to E2. The Col E1 (LRI exo)-(dT)150 DNA gave less than one transform strain NL20-127 (AaraC recA) and colonies were selected on minimal arabinose plates. Two candidates which could

utilize L-arabinose were obtained at an efficiency of 0.2 transformants/ μ g of DNA, and both were colicin E1 resistant and colicin E2 sensitive. Attempts to obtain a hybrid plasmid carrying the lactose operon were unsuccessful.



There is some evidence, however, for an EcoRI restriction endonuclease site in the lacZ gene (Clarke and Carbon, unpublished work; see Note Added in Proof). Thus, annealed hybrid DNA composed of plasmid and sheared *E. coli* DNA would need to be used to select for hybrid plasmids of this type. Such plasmids, which carry an EcoRI restriction endonuclease site within a well-characterized *E. coli* operon, may be useful for cloning other types of DNA. Characterization of hybrid plasmids

Covalently closed circular plasmid DNA was isolated from both of the Ara⁺ isolates and two of the Trp⁺ isolates by preparing cleared lysates of cells carrying the hybrid plasmids and banding plasmid DNA in CsCl-ethidium bromide gradients (8, 9). The Col E1-ara plasmids were designated pL-1 and pLC3, and the Col E1-trp plasmids, pLC5 and pLC19. Relaxed circular plasmid DNA from each of the four isolates was spread for electron microscopy using relaxed circular Col E1 DNA as a standard (Fig. 2). Projections of the molecules were traced on paper and measured with a map measurer. Both the pLC1 and pLC3 DNAs were approximately 4.3 times the length of Col E1 DNA or about 18×10^6 daltons, while pLC5 and pLC19 DNAs were 3.6 times the length of Col E1 DNA or 15×10^6 daltons. Transformations were carried out using pLC1, pLC3, and pLC19 DNAs and various auxotrophic recipients (Table 3). As anticipated, pLC1 (Col E1-ara) DNA and pLC3 (Col E1-ara) DNA transformed strains NL20-0127 (AraC recA) and NL20-028 (araC rec⁺) to Ara⁺ with high efficiency. Likewise pLC19 (Col E1-trp) DNA transformed strain MV12 (AtrpE5 recA) to Trp⁺ with high efficiency. Further transformations were carried out with pLC1 and pLC3 DNAs, selecting for Ara⁺, using two strains carrying longer deletions, NL20-008 (AraCOIBA) and NL20-047 (AraCOIBAD). As shown in Table 3, pLC1 DNA transformed both strains to Ara⁺ with high efficiency, whereas pLC3 DNA transformed neither. Apparently pLC1 carries the entire arabinose operon, but pLC3 does not. Since the hybrid plasmids were made from EcoRI endonuclease products of *E. coli* DNA, pLC1 and pLC3 should be identical. Indeed, no difference in the length of their DNAs was detected in electron microscopic measurements. Both plasmid DNAs are resistant to digestion with EcoRI endonuclease, so pLC1 was not originally constructed from a partial enzyme digestion product. Several reasons may be offered to explain the differences between the two plasmids.

The *E. coli* DNA fragment from which pLC3 was constructed may have been sheared at a region close to the araC gene prior to EcoRI digestion. On the other hand, the pLC3 plasmid DNA could have undergone a small deletion, excising a portion of the arabinose operon. Finally, the *E. coli* DNA fragment from which pLC3 was originally constructed may have been excessively digested with X-exonuclease, resulting in the loss of several genes. We surmise that the ara region must be close to that portion of the DNA that was treated with X-exonuclease and joined to poly(dA). Both pLC1 and pLC3 DNAs transform strain NL20-000 (leu) to Leu⁺ with high efficiency (Table 3). Since the two plasmids carry pieces of *E. coli* DNA of approximately 14×10^6 daltons and since ara and leu are 50% cotransducible with phage P1 (16) and map very close together on the chromosome (17), the portions of the arabinose and leucine operons carried by the plasmids probably define the extreme ends of the *E. coli* DNA contained in pLC1 and pLC3. Hershfield et al. (2) have shown previously that there are no EcoRI endonuclease sites within the tryptophan operon. The hybrid plasmid pLC19 (Col E1-trp) carries trpE, trpD, trpC, trpB, and presumably trpA58 (Table 3). It was necessary to select for indole utilization when strain DM8 (Atrp EDCBA-tonB) was transformed with pLC19 DNA, because the DNA from which the hybrid plasmid was constructed was isolated from strain CS520 (trpA58), which grows in the absence of tryptophan only when supplied with indole. We have not yet established the presence of the trpA region on pLC19, however. The usefulness of the plasmid Col E1 as a vehicle for amplification of *E. coli* DNA, mRNA, and proteins has been previously demonstrated by Hershfield et al., in that strains carrying the Col E1-trp plasmid pVH15 show greatly elevated trp operon message and enzyme levels (2). We have found similarly elevated levels of trp mRNA and ara mRNA and proteins in strains carrying pLC5, pLC19,

and pLC3. For example, after treatment of the cells with chloramphenicol (8), up to 38% of pulse-labeled messenger RNA in cells harboring pLC3 is specific for arabinose operon genes (Nancy Lee, personal communication).



B. Generation of hybrid plasmid

After comparing the plasmids between KP17-15 and KP17-16, it was found that p17-15-vir was a hybrid plasmid containing both p17-16-vir and p17-16-CTX, a conjugative MDR plasmid in KP17-16 (Tables 1 and 2). Linear alignment of the three plasmids with Easyfig indicated that p17-15-vir was likely derived from homologous recombination of plasmids p17-16-vir and p17-16-CTX by common sequences containing group II intron reverse transcriptase gene and ISShes11. Whether proteins encoded by the two elements take roles in plasmid rearrangements warrants further research. It may have involved three recombination events and resulted in inversions of the R2, R3 regions and the MDR region of R1, along with target gene duplications inversely (Figure 1c). This kind of plasmid recombination is different from the traditional generation of hybrid plasmid mediated by IS26 and ISKpn19 [26,27], which typically involves one element and is mediated by one recombination event. The generation of the hybrid plasmid demonstrates that recombination mediated by insertion sequences and introns plays a pivotal role in the generation of hybrid supersized plasmids carrying MDR and virulence factors. Although MDR hybrid plasmids are common, super hybrid plasmids with multiple replicons and carrying MDR, virulence and conjugational systems have rarely been reported.

VII. CONCLUSION

In this Experiment, for Hybrid plasmid white colour colonies are recombinants, Blue colored colonies (non recombinants) are found less in number. White coloured colonies are observed because the integrity of the lacZ gene in the plasmid is lost. The presence of lacZ gene that codes for the enzyme gets disturbed due to the insertion of plasmid and results in producing white colonies as recombinants and blue as non recombinants. The Hybrid plasmid (pUC18 + pBR322) was constructed by adding T4 DNA ligase to the digested vectors and transformed to the competent cells of DH5α E. coli strain with the help of Calcium chloride.

Thus an artificially created hybrid plasmid (pUC18 + pBR322) has antibiotic resistance genes and also increase in the insert capacity for gene of interest. It was observed that hybrid plasmid was able to sustain the bacterial strain.

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