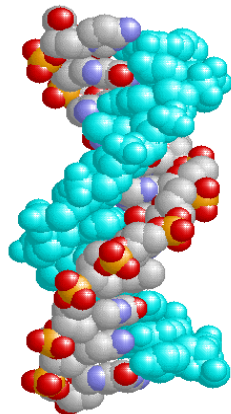


2000/01

**CARDIFF**  
UNIVERSITY

**Cardiff School of Biosciences**

# **Biochemistry**



**Level II:  
Practical  
Manual**

**Molecular Biology I**

**Dr Peter Kille**

## GENE CLONING PRACTICAL

The purpose of this practical is to introduce you to the basic techniques of gene cloning. The techniques that you will use include:

- The preparation of plasmid DNA
- Digestion of DNA with restriction endonucleases
- Analysis of DNA fragments by electrophoresis
- Ligation of DNA fragments to produce recombinant molecules
- Transformation of bacterial cells
- Selection of clones by insertional inactivation of a gene
- Selection of clones by expressed phenotypes
- Amplification of specific DNA fragments by the polymerase chain reaction (PCR)
- Induction of gene expression
- Enzyme activity measurement

Particular attention should be paid to the following points:

- [1] The manual should be used as a guide to assist you with the experiments rather than as a complete recipe book.
- [2] There are certain safety requirements (in addition to those appropriate to normal laboratory practice) that must be adhered to (see next page). **It is a requirement that you read the section on safety before commencing any work.**
- [3] These experiments will work well but only if the advice on page 4 is followed.

## **TIMETABLE**

Practicals will be held in lab. 101W every Tuesday. Sessions will start promptly at 10.00 am and will continue through until 18.00. If you cannot attend any part of the practical please inform a demonstrator or the lecturer in charge.

### **Week 1**

[1] Preparation of plasmid DNA

[2] Digestion of DNA with restriction endonucleases

[3] Analysis of DNA fragments by electrophoresis

[4] Ligation of DNA fragments to produce recombinant molecules

### **Week 2**

[5] Transformation of bacterial cells

[6] Plating out of bacteria on selective media

### **Week 3**

\* Monday before practical (approx. 1 hour)

[7] Plating out of bacteria for insertional and phenotypic screening

\* Tuesday

[8] Selection of clones by insertional inactivation of a gene

[9] Selection of clones by expressed phenotype

[11] Preparation of plasmid DNA from recombinant plasmids

[10] Identification of specific gene by polymerase chain reaction (PCR) and restriction mapping.

**Week 4**

\* Monday before practical (approx. 1 hour)

[11] Inoculation of cultures for induced gene expression

\* Tuesday

[12] Analysis of induced gene expression by enzyme activity measurements

[13] Analysis of PCR and restriction fragments plasmids prepared in [10].

## SAFETY

### General Points

All experiments involving recombinant DNA work must be approved by a local safety committee who are responsible for quantifying the biological risk and designating the level of containment that is required for the work to proceed. The committee has a legal obligation to submit annual reports on all experiments carried out in College to the Health and Safety Executive. This experiment has been approved subject to the work being carried out in accordance with 'good microbiological practice'.

### Specific Points

In addition to the safety procedures that are applicable to any laboratory work the following points should be noted.

- [1] Experiments must be carried out using good microbiological practice. This means that the normal safety precautions applicable to the handling of microorganisms apply to the experiments described in this manual.
- [2] Compounds that interact with DNA, e.g. ethidium bromide, are mutagens and carcinogens. Do not allow these compounds to come into contact with yourself or other people. Wear disposable gloves at all times.
- [3] The transilluminator is a powerful source of ultra violet radiation and will cause serious damage to unprotected skin and eyes. **Always** ensure that there is a uv-opaque material between you and the light source, i.e. wear gloves and use suitable eye/face protection.
- [4] Phenol-containing solutions can cause severe burns. Make sure that you are wearing gloves when handling these solutions.

- [5] If you have recently been prescribed medicines by your doctor, especially antibiotics, anti-inflammatory drugs or immunosuppressants, please inform Dr P Kille **before** starting the practical.

## PRACTICAL CONSIDERATIONS

Essentially, the basic techniques of gene cloning are straightforward providing that certain protocols are followed. These practical points are important if you are to obtain successful results.

- [1] Keep solutions on ice **at all times**. Even short exposure of certain components, e.g. some restriction endonucleases, to room temperature will cause significant inactivation.
- [2] Nucleases are ubiquitous and some are extremely stable enzymes. It is vital that these enzymes are not allowed to come into contact with your apparatus or solutions. **Always** wear disposable gloves - the skin is an excellent source of nucleases and there is enough enzyme in a fingerprint to ruin your experiments. Also, most of your solutions and apparatus have been autoclaved to destroy nucleases so avoid unnecessary handling.
- [3] The key to many of the techniques described in this manual is the ability to pipette small volumes carefully and accurately. It is essential when pipetting small volumes to ensure that the tip of the pipette is touching the surface of the container before expelling the solution. Always use clean pipette tips to avoid cross contamination. Pulse spin microfuge tubes to ensure that all of the added components of reaction mixtures are mixed at the bottom of the tube.
- [4] The transilluminator should be used for the minimum time possible as prolonged exposure of DNA samples to uv radiation causes random strand breakage - also the filter has a finite life. Please avoid scratching the surface of the transilluminator as this will destroy the effectiveness of the filter - a new transilluminator costs £1000.

A general point about recombinant DNA work is that reagents are extremely expensive.

Please use reagents/enzymes as sparingly as possible. In general, expensive components such as enzymes will have been pre-dispensed into tubes. The other components of the reaction

mixture should be added to these tubes (see protocols for further details).

## BACKGROUND TO THE EXPERIMENTS

Alginates are polysaccharides comprised of (1-4)-linked  $\beta$ -D-mannuronate and  $\alpha$ -L-guluronate. This polysaccharide is produced by marine macroalgae and certain bacteria, and the algal alginate is widely used by the food and pharmaceutical industries (approx. 22000 tonnes/annum).

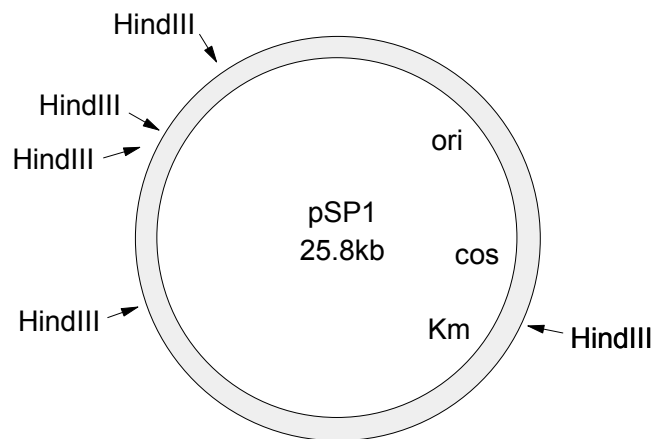
Certain bacteria produce enzymes (alginate lyases or alginases) which degrade alginates. A gene encoding an alginate lyase has been cloned from the bacterium *Klebsiella pneumoniae* into a cosmid vector to produce the construct pSP1. In this practical the bacterium *E. coli* strain LE392 will be used as a source of plasmid pSP1. This plasmid contains the *aly* gene which encodes the alginate lyase (Fig. 1).

### Objectives of the Practical

- [1] Both the plasmid vector (pHG327) and the 'foreign DNA' (pSP1) will be isolated and purified from cultures of *E. coli*.
- [2] Both pHG327 (Fig. 2) and pSP1 will be cut with an appropriate restriction endonuclease (*Hind*III).
- [3] Fragments of pSP1 will be added to the cut pHG327 and recombinant molecules will be allowed to form. The recombinant molecules will be treated with DNA ligase to reform the phosphodiester linkages.
- [4] Bacterial colonies will be screened on MacConkey agar to detect transformants.
- [5] Recombinants containing the *aly* gene will be isolated by detecting expression of the alginate lyase enzyme.

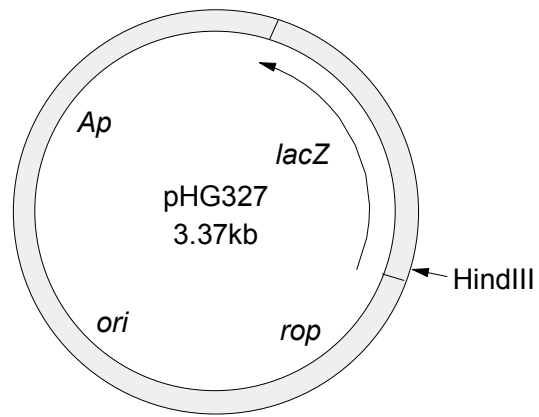
- [6] DNA will be isolated from recombinants, cut with restriction endonucleases and the fragments analysed on agarose gel electrophoresis. This will allow the *aly* gene to be mapped onto pSP1.
- [7] The polymerase chain reaction (PCR) will be used to identify the *HindIII* fragment of the pSP1 that contains the *aly* gene from crude bacterial extracts.
- [8] The expression of alginate lyase by recombinants will be quantified by measurement of specific enzyme activities.

Fig. 1 Map of pSP1 (Low copy number)  
(Positions of restriction sites are approximate)



(High copy number)

Fig. 2 Map of pHG327



## BACTERIA, ENZYMES AND ANTIBIOTICS

### Bacterial strains

You will use one or more of these strains of *Escherichia coli* which have the following genotypes:

*Escherichia coli* JM109  $\Delta(lacpro AB)$ , *endA1*, *rec A1*, *thi*, *sbcB15*, *hsdR4*, *supE44*, *relA1* (F', *traD36*, *proAB*<sup>+</sup>, *lacI*<sup>q</sup> $\Delta$ M15)

*Escherichia coli* JM107  $\Delta(lacpro AB)$ , *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, (F', *traD36*, *proAB*<sup>+</sup>, *lacI*<sup>q</sup> $\Delta$ M15) (pHG327, Ap<sup>r</sup>)

*Escherichia coli* LE392 *hsdR514* (*rk*<sup>-</sup>, *mk*<sup>+</sup>), *supE44*, *supF58*, *lacY1* (pSP1, Km<sup>r</sup>)

### Some genetic markers in frequently used *E. coli* strains (Data from Promega)

Symbol	Description	Effect
<i>endA</i>	Endonuclease mutation	Improves quality of plasmid DNA isolations
F'	Host contains F' episome	Provides essential functions to host cell
<i>gyr</i>	DNA gyrase mutation	Confers resistance to nalidixic acid
<i>hsdR</i>	Restriction system mutations	Allows cloning without cleavage of plasmid DNA
<i>lacI</i> <sup>q</sup>	Overproduces the <i>lac</i> repressor protein	Inhibits transcription from <i>lac</i> promoter
<i>lacY</i>	Galactose permease mutation	Blocks lactose utilization
<i>lacZ</i>	$\beta$ -D-Galactosidase mutation	Unable to produce $\beta$ -D-galactosidase. Strains suitable for use with <i>lacZ</i> -containing vectors
<i>proAB</i>	Mutations in proline metabolism	Requires proline for growth in minimal medium
<i>relA</i>	Mutation eliminating stringent factor	RNA synthesis in the absence of protein synthesis
<i>sbcB15</i>	Exonuclease I mutation	Allows general recombination in <i>recBC</i> mutants
<i>supE</i>	Suppressor mutation	Suppresses amber (UAG) mutations
<i>supF</i>	Suppressor mutation	Suppresses amber (UAG) mutations
<i>traD</i>	Transfer factor mutation	Prevents transfer of F' episome

*rec AI* Mutation in recombination Prevents recombination of introduced DNA with host DNA.

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### Restriction enzymes

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Enzyme	Microorganism	Specificity
<i>Bam</i> HI	<i>Bacillus amyloliquifaciens</i> H	G\GATCC
<i>Eco</i> RI	<i>Escherichia coli</i> RY 13	G\AATTC
<i>Hind</i> III	<i>Haemophilus influenzae</i> Rd	A\AGCTT
<i>Pst</i> I	<i>Providencia stuartii</i> 164	CTGCA\G
<i>Xho</i> I	<i>Xanthomonas holcicola</i>	C\TCGAG

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### Antibiotics

Ap Ampicillin

Km Kanamycin

Ap<sup>r</sup> Ampicillin-resistant phenotype

Ap<sup>s</sup> Ampicillin-sensitive phenotype

## MINI-PREPARATION OF DNA

Extraction of Plasmid DNA from bacterial cells.

### Materials/Solutions Required

5ml culture of *E. coli* JM 107 containing pHG327

5ml culture of *E. coli* LE392 containing pSP1

Collection Tubes.

Sterile water warmed to 70°C

SV Mini prep Kit™ (The SV Miniprep Kit is manufactured by Promega)

Cell resuspension solution (50mM-Tris, pH 7.5; 10mM-EDTA; 100µg/ml RNase A)

Cell lysis solution (0.2M-NaOH, 1% SDS)

Alkaline Protease solution

Neutralisation solution (4.09M Guanidine hydrochloride, 0.759M-Potassium acetate, 2.12M Glacial acetic acid)

SV spin columns

SV Column wash solution (162.8mM Potassium Acetate, 27.1mM Tris-HCl, pH7.5 - EDTA - Add 1.75 volumes of 95% ethanol before use i.e. 35mls of ethanol to 20mls Wash concentrate).

### Method

You will be provided with bacterial cultures that have been grown overnight at 37°C in medium containing appropriate antibiotics.

- [1] Pellet bacterial cells by centrifuging 5ml of each bacterial culture for 10mins at 3K in a bench top centrifuge.

- [2] Remove the clear supernatant. Add 250µl of Cell Resuspension Solution and resuspend the cell pellet by brief vortexing.
- [3] Transfer each cell suspension to separate sterile eppendofs.
- [4] To each add 250µl of Cell Lysis Solution and mix by inverting the tube four times (do not vortex). Incubate for 1-5 minutes at room temperature until solution clears.
- [5] Add 10µl of Alkaline Protease solution and mix by inverting the tube 4 times. Incubate for a further 5 minutes at room temperature. **Do not incubate for longer than 5 minutes**
- [6] Add 350µl of Neutralisation Solution and immediately mix by inverting the tube several times.
- [7] Spin in the microcentrifuge for 10 min.

#### **A. Purification with Centrifugation.**

- [8A] Transfer the cleared lysate to a Spin Column by decanting. Avoid disturbing of transferring any of the white precipitate with the supernatant. Insert the Spin Column into a collection tube.
- [9A] Spin in the microcentrifuge for 1 min. Remove the spin column from the tube and discard the flowthrough from the Collection tube. Reinsert the Spin Column into the Collection Tube.

[10B] Add 750µl of Column Wash solution (previously diluted with ethanol) to the Spin Column.

[11A] Spin in the microcentrifuge for 1 min. Remove the spin column from the tube and discard the flowthrough from the Collection tube. Reinsert the Spin Column into the Collection Tube.

[12A] Add 250µl of Column Wash solution (previously diluted with ethanol) to the Spin Column.

[13A] Spin in the microcentrifuge for 2 min.

[14A] Transfer Spin Column to a new sterile eppendoff.

### **B. Purification with Vacuum.**

[8B] Insert the Spin Column into vacuum manifold. Transfer the cleared lysate to a Spin Column by decanting. Avoid disturbing or transferring any of the white precipitate with the supernatant.

[9B] Apply vacuum to pull liquid through the column. When all liquid has been pulled through column, release vacuum.

[10B] Add 750µl of Column Wash solution (previously diluted with ethanol) to the Spin Column.

[11B] Apply vacuum to pull liquid through the column. When all liquid has been pulled through column, release vacuum.

[12B] Add 250µl of Column Wash solution (previously diluted with ethanol) to the Spin Column.

[13B] Apply vacuum to pull liquid through the column. When all liquid has been pulled through column, release vacuum. Transfer to Collection tube and spin in the microcentrifuge for 2 min to remove any residual wash solution.

[14B] Transfer Spin Column to a new sterile eppendoff (with the lid cut off).

### **DNA Elution**

[15] Elute the plasmid DNA by adding 100µl of nuclease-free water (at 65-70°C) to the Minicolumn.

[17] Spin the microfuge tube and column for 1 min in a microcentrifuge.

[18] Remove and discard the Minicolumn but keep the liquid. The plasmid DNA, which is contained in the remaining liquid should be transferred with a pipette to a fresh microfuge tube (with a lid) and may be stored at 4°C or -20°C until required.

**The rationale behind the various steps in the experimental protocol is as follows:**

- Step 3            Cell resuspension solution contains EDTA which makes the bacterial outer membrane permeable and RNase which degrades RNA.
- Step 4            The NaOH denatures the proteins and disrupts the chromosome. The detergent, SDS, lyses the bacteria allowing the plasmids to leak out of the cells. Alkaline Protease breaks down bacterial proteases.
- Step 5            The guanidine hydrochloride and potassium acetate forms a precipitate with the proteins and chromosomal DNA while the plasmid remains in solution. The acetic acid neutralises the alkali used in the previous step.
- Step 6-7          The plasmid (in solution) is separated from the precipitated proteins, chromosome and cell debris.
- Step 8-           Under high salt conditions Nucleic acids binds (plasmid DNA) to Spin Column.
- Step 9-13        Contaminating proteins and salts are washed away. The washing solution contains salt ethanol and a small amount of salt so that mono-nucleotides are removed but plasmid DNA remains bound.
- Steps 14         Remaining wash solution is removed.
- Steps 15-18     Water at 65°C causes the DNA to be released from the resin. The plasmid DNA solution is then collected in a fresh eppendoff when the column is spun in the microcentrifuge.

## RESTRICTION ENDONUCLEASE DIGESTION OF DNA

pSP1 is digested into a number of fragments using the restriction enzyme Hind III. The expression vector pHG 327 is also cut with Hind III, in a single location, causing it to linearise. The aim is to ligate the pSP1 fragments into the cut pHG 327. However, when the ligation is performed there is also the possibility that pHG 327 will reform without incorporating any fragments of pSP1. To prevent this we treat the linearised pHG 327 with a Shimp dephosphorylase enzyme which removes the 5' phosphate groups making it only possible for the pHG 327 to reform if a fragment from pSP 1 is incorporated.

### Materials/Solutions Required

10X Digest buffer

*Hind*III enzyme

DNA sample

Sterile water

### Method

[1] Decide a suitable volume of each plasmid DNA and the final volume of digestion.

	Analytical digest	Preparative digest	
		pHG327	pSP1
DNA volume	10µl	15µl	25µl
Final volume	20µl	30µl	50µl

[2] You will be provided with tubes containing 2µl of restriction enzyme. To each tube add 1/10 final volume of 10X Buffer, sample DNA and sufficient sterile water to give the appropriate final volume.

- [3] Flick the bottom of the tubes to mix the contents (remember that it is easy to denature the enzyme) and then microfuge for 2-3 seconds so that all of the contents are at the bottom of the tube.
- [4] Incubate the tubes at 37°C for 2 hours (see “DEPHOSPHOYLATION OF VECTOR DNA” for instructions of what to do with pHG327 digestion) and then transfer the tubes to a 65°C waterbath for 15 min to inactivate the endonuclease and dephosphoylation enzyme.
- [5] Use a portion (e.g. 7-8µl) of the digest for analysis by gel electrophoresis and store the remainder for use in the ligation reaction.

## **DEPHOSPHOYLATION OF VECTOR DNA**

### **Materials/Solutions Required**

Shrimp Phosphatase

### **Method**

pHG327 digestion only.

- [4a] After the pHG327 Vector DNA has been incubating with *Hind*III for 1 hour remove it from the water bath and add 1µl of Shrimp Phosphatase. Flick the bottom of the tubes to mix the contents and then microfuge for 2-3 seconds so that all of the contents are at the bottom of the tube.
- [4b] Place the digestion mixture in the water bath to incubate at 37°C for an additional 1 hour and then continue as for normal digestion's at step [5].



## FORMATION OF RECOMBINANT DNA MOLECULES

The digested pSP1 fragments are ligated into the linearised dephosphorylated pHG 327. Before the ligation is performed the digested DNA will be purified with chloroform/isoamyl alcohol and concentrated by precipitation with ethanol.

### Materials/Solutions Required

*Hind*III digested DNA samples

Sterile water

Chloroform/*iso*amyl alcohol

TE buffer

3M sodium acetate

100% ethanol (at -20°C)

10X Ligation buffer

T4 DNA ligase

### Method

[1] Add the following components in a microfuge tube placed on ice:

<i>Dephosphorylated Hind</i> III digested pHG327 (vector) DNA	20µl
<i>Hind</i> III digested pSP1 (foreign) DNA	40µl
Sterile diH <sub>2</sub> O	40µl
Total	100µl

[2] Add 100µl of chloroform/*iso*amyl alcohol to the tube, vortex and microfuge for 2 min.

- [3] Transfer the aqueous (upper) phase to a fresh tube. It is essential that none of the chloroform phase is transferred.
- [4] Add 10 $\mu$ l of 3M sodium acetate and 250 $\mu$ l of cold ethanol to the tube. Leave in an ethanol/dry ice bath (or at -70°C) for about 30 min.
- [5] Microfuge for 15 min, carefully pour off the supernatant and dry the pellet (may not be visible). **Be careful not to lose the pellet.**
- [6] Resuspend the pellet in 14 $\mu$ l of sterile water
- [7] You will be provided with a tube containing 2 $\mu$ l of T4 DNA ligase. To this, add 2 $\mu$ l of 10X ligation buffer, the resuspended pellet (14 $\mu$ l) and gently mix.
- [8] Briefly microfuge to bring contents to the bottom of the tube and incubate at 16°C overnight.

## TRANSFORMATION OF HOST BACTERIA WITH RECOMBINANT DNA

The recombinant DNA molecules formed during the ligation are inserted into *E. coli* cells and the bacteria containing the plasmids are selected by their resistance to Ampicillin.

### Materials/Solutions Required

50µl competent *E. coli* DH5α on ice.

1 ml Sterile S.O.C. Media.

Ligated DNA sample

LB Agar capsules.

100 mg/ml Amp.

### Method

#### (a) Preparation of Agar plates

- [1] Put 8 capsules of LB Agar into the provided 500 ml Duran Bottle and add 200 mls of Sterile water. Screw cap on **LEAVING IT LOOSE** and cover top with tin foil. Add a strip of autoclave tape and place into autoclave for 20 minutes at 120 p.s.i..
- [2] When autoclave cycle is finished **USE THERMAL RESISTANT GLOVES** to remove molten agar from autoclave. Tighten lid and swirl to insure capsules are fully dissolved and place in 55°C water bath.
- [3] Check molten L Agar is at 50°C remove and pour one agar plate ~20 mls (make sure this plate is labeled "LB-AGAR NO ANTIBIOTIC") then to the remainder add Amp to a final concentration of 100 µg/ml.

- [4] Mix by swirling and place back into 50°C waterbath for 2-3 minutes until majority of bubbles disappear.
- [5] Carefully pour media evenly into 6 sterile petri dishes using good aseptic technique. Any excess LB-agar should be washed down the sink immediately with lots of warm water.
- [6] Wait for plates to solidify (~30 minutes) and dry by opening slightly and placing upside down in the 37°C incubator.
- [7] Once dry close plate and leave on bench ready for transformation.

**(b) Transformation of *E. coli***

- [8] Add the DNA sample (maximum volume 5µl) to 50µl of competent cells and gently tap the tube to mix.
- [9] Incubate the tubes on ice for 30 minutes. Then at 37°C for 20 seconds and then replace them on ice for an additional 2 minutes.
- [10] Add 0.95ml of SOC media and incubate at 37°C for 1 hour.
- [11] \*Label the plates before adding bacteria.
- [12] Spread 150 µl portions of the transformed bacteria onto 6 of your LB<sub>Amp</sub> plates (prepared in Section a) and the last 100 µl portion on the LB plate containing no

antibiotic. \*Label remaining LB<sub>Amp</sub> plate and store in fridge for use in following week.

- [13] Incubate upright until the agar surface is dry before inverting the plates and leaving overnight at 37°C.

## SCREENING CLONES

Recombinant clones (i.e. those colonies which contain pHG 327 with a pSP1 insert) will be identified by insertional activation (described in rational section). The recombinant clones identified may contain any of the five fragments produced by digestion of pSP1 with Hind III. In order to determine which of these recombinant clones contains the aly gene (encoding alginate lyase), the ability of the colonies to degrade alginate is assessed.

### Materials/Solutions Required

MacConkey plate (contain 100µl Ap/ml)

Alginate plate (contain 100µl Ap/ml)

Sterile applicator sticks

Culture dish containing 24 wells each holding 2mls of LBamp media.

### Method : Monday Evening

- [1] Examine the plates that you have prepared previously. The LB plate with no antibiotic should show lawn growth whilst you should be able to identify single colonies on the LB<sub>Amp</sub> plates. Only bacteria containing pHG327 or pHG327 plus insert DNA should grow on LB<sub>Amp</sub> plates.
- [2] On back of MacConkey plate and Alginate plate draw a 6 x 4 grid and label each squares A1-A6, B1-B6, C1-C6 and D1-D6. Wells in culture dishes are already labelled in the same manner, check dish orientation.
- [3] Pick selected individual colony from LB<sub>Amp</sub> plates with Sterile applicator stick. Place gently onto square A1 of alginate plate, then A1 of MacConkey plate and finally into well A1 of culture dish. Repeat this procedure with new collonies until all squares all filled (total 24 collonies).

[4] Incubate the plates overnight at 37°C.

### **Tuesday Morning**

[5] Count the number of red colonies which are Lac<sup>+</sup> bacteria, and the white colonies which are Lac<sup>-</sup> on the MacConkey agar plate. Note location of white colonies.

[6] Overlay the alginate plates with 10%(w/v) cetyl pyridinium chloride (CPC) solution and leave for 5-10 min. Undegraded alginate will appear opaque white and colonies producing alginate lyase will be surrounded by a clear zone. Placing the plates on a black background will help you to visualise the clearing zones. CPC is bacteriocidal, therefore you will need to utilise the corresponding colonies on the MacConkey agar plates for further study. Note location of colonies that give clearing zones.

[7] Identify i) Colony that is white on MacConkey agar plate and gives clearing zone. ii) Colony that is red with no clearing zone. Take spare LB<sub>AMP</sub> plate prepared in previous week, divide in half and using sterile applicator sticks streak out these two colonies. Give plate to demonstrators at end of session.

[8] Locate the wells in culture dish that contain the liquid replicate cultures of the those colonies identified in [7]. Prepare bacterial DNA from these cultures and analyse by performing restriction digests (analytical, as for pHG 327) and PCR.

**The rationale behind each step of the experimental protocol is as follows:**

- Step 1 Lawn growth on L agar indicates that the competent cells were viable. No growth on MacConkey agar (containing ampicillin) indicates that the host cells were sensitive to ampicillin and cannot grow on this medium.
- Step 2 pHG327 confers antibiotic resistance on the host cells, therefore the transformants should be able to grow on medium containing ampicillin.
- Step 3 The vector pHG327 contains the *lacZ* gene encoding  $\beta$ -galactosidase, a gene which has been partially deleted in the particular host cells that we have used. Therefore, it is only the bacteria containing intact pHG327 that are able to metabolise lactose (present in MacConkey agar) to produce acid which turns the indicator phenol red (also present in MacConkey agar) from colourless to red. Those colonies that remain white should contain genuine recombinant DNA molecules. The cloning site in pHG327 is contained within the *lacZ* gene. Therefore, if a piece of foreign DNA has been inserted into this site the *lacZ* gene will be disrupted and no  $\beta$ -galactosidase will be produced. This phenomenon is called 'insertional inactivation' of a marker gene.
- Step 4 Duplicate plates are required of each colony because the alginate lyase detection procedure (step 6) kills the bacterial cells.
- Step 6 CPC is a cationic detergent which binds to high molecular weight alginates to form an opaque white precipitate. Alginate lyase reduces the molecular weight of the alginate sufficiently to prevent a significant precipitate from forming, hence the appearance of 'clearing zones' around alginate lyase producing colonies.

## **AGAROSE GEL ELECTROPHORESIS**

DNA may be fractionated according to molecular size by electrophoresis through agarose gels. This method will be used to analyse intact plasmids and also DNA fragments produced by restriction endonuclease digestion.

### **Materials/Solutions Required**

Agarose (0.7%) in 1 X TBE

Electrophoresis buffer - 5 X TBE (0.445M Tris, 0.445M boric acid and 0.01M EDTA, pH8.0)

Electrophoresis apparatus

Ethidium bromide solution (2µg/ml)

Masking tape

### **Method**

- [1] Seal the ends of the gel holder with tape.
- [2] Place comb in the correct orientation across the end of the gel holder.
- [3] Melt agarose in the autoclave.
- [4] Check that agarose is no hotter than 55°C and pour into the holder taking care to avoid air bubbles. Allow to set (approx. 20 min). Do this with the gel holder on the bench - not in the electrophoresis apparatus.

- [5] Carefully remove the comb and tape and place the gel holder into the electrophoresis apparatus. Ensure that the wells are placed over the red band. **Take care that the agarose gel does not slip off the plastic tray!**
- [6] Dilute the 5 X TBE (i.e. 1 vol of 5 X TBE and 4 vol of distilled water) and pour into the apparatus until the gel is just covered. Ensure that no air remains in the wells by washing with 1 X TBE.
- [7] Prepare samples or standards ( $\lambda$  phage DNA digested with a restriction endonuclease - see next page) by the addition of 1-5 $\mu$ l of loading dye. Heat samples at 65°C for 2 min, cool and load samples into the wells. Replace the lid and connect the power supply.
- [8] Run the gels at 100-120V until the dye has migrated approx. half the length of the gel.
- [9] **Switch off and disconnect the power supply.**
- [10] Place the gel in ethidium bromide solution and allow to stain for 15 min. **Ethidium bromide is carcinogenic - wear gloves.**
- [11] Rinse the gel with tap water.
- [12] Place the holder containing the gel on the transilluminator. The holder is uv transparent.
- [13] Ensure that your eyes and skin are adequately protected (gloves and visor or viewing shield) and switch on the transilluminator. The gel will be photographed for you.

- [14] Switch off the transilluminator, remove your gel and leave the equipment clean and dry.
- [15] If the gel is insufficiently stained then return it to the ethidium bromide solution for a little longer (step 10) and repeat the subsequent steps.

### Molecular size markers

Lambda phage DNA digested with either *EcoRI*, *HindIII* or a double digest with both enzymes.

<i>EcoRI</i> Fragments	<i>HindIII</i> Fragments	<i>EcoRI/HindIII</i> Fragments
21226	23130	21226
7421	9416	5148
5804	6557	4973
5643	4361	4277
4878	2322	3530
3530	2027	2027
	564	1904
	125	1584
		1330
		983
		831
		564
		125

To obtain a calibration curve for the estimation of molecular size, plot log (molecular size of markers) against mobility.

## AMPLIFICATION OF SPECIFIC DNA SEQUENCES BY PCR

Primers flanking the single Hind III cut site of pHG 327 are used in a amplification reaction. This allows us to determine the size of any fragment ligated into this vector.

### Materials/Reagents Required

Agar plates containing bacterial cultures

PCR Mix (Containing: Deoxyribonucleotide triphosphates (dNTPs)  
Oligonucleotide primers  
Sterile Water)

*Taq* DNA polymerase

*Taq* DNA polymerase buffer

25 mM MgCl<sub>2</sub>

Sterile H<sub>2</sub>O

0.2 ml thin wall PCR tubes

### Method

[1] Into a 0.2 ml thin wall PCR tubes add 10 µl PCR Mix, 2µl of *Taq* buffer, 2µl of 25 mM MgCl<sub>2</sub>, 5µl of the sterile H<sub>2</sub>O. Then add 1 µl of a 1/10 dilution of purified plasmid DNA. Flick the bottom of the tubes to mix the contents and then microfuge for 2-3 seconds so that all of the contents are at the bottom of the tube.

[2] Finally add 0.5µl of *Taq* DNA polymerase.

- [3] Place tubes in heating block with heated lib. The block will cycle hold the temperature at 95°C for 20 sec (denaturation of dsDNA), 50°C for 45 sec (annealing of primers to template DNA) and 74°C for 3.5 min (primer extension). This temperature cycle will be set to occur 30 times.
- [4] At the end of the cycling period, remove samples and analyse by electrophoresis on agarose gels.

### **Rationale behind the experiment**

The two primers that are used in this reaction bind to either side of the cloning linker (containing the Hind III site used for cloning) of pMG327. Amplification with an empty plasmid gives a small product of ~150bp. However, when a fragment is successfully cloned into the polylinker then the size of the amplified product is 150bp in addition to that of the inserted fragment.

## MEASUREMENT OF ALGINATE LYASE ACTIVITY

The aim of this part of the practical is to compare the specific activities of preparations of alginate lyase derived from the original and the recombinant organisms.

### Materials

Bacterial cultures

Substrate solution (0.66g of alginate and 1.75g of NaCl dissolved in 100ml of 50mM sodium phosphate buffer, pH 7.0)

Dinitrosalicylic acid reagent - DNS (5g of 3,5-dinitrosalicylic acid, 150g of potassium sodium tartrate and 100ml of 2M NaOH in a total volume of 500ml)

Coomassie Protein Reagent

2M NaOH

Bovine serum albumin (1mg/ml stock)

Chloroform

Glucose standard solution (1mg/ml)

1 M IPTG

### Method : Monday Evening

- [1] Set up duplicate 5 ml bacterial cultures of pSP1 with 50 µg/ml Kan, pHG327 with 100 µg/ml Amp (MacConkey red transformant giving no clearing zone on alginate plates) and an alginate positive transformant with 100 µg/ml Amp (MacConkey white transformant giving clearing zone on alginate plates).

- [2] To one of the cultures containing each construct add IPTG to a final concentration of 1 mM (the *lac* promoter will be activated in cultures where IPTG is added). Grow overnight at 37°C.

### **Tuesday Morning**

- [3] Pellet cells by centrifugation at 3000rpm for 15 min in a bench centrifuge.
- [4] Retain 1 ml of supernatant and place in a clean  $\mu$ fuge tube to measure the amount of enzyme and protein excreted from the cell. Discard remaining supernatant and resuspend pellet in 1 ml of fresh media and transfer to a clean  $\mu$ fuge tube.
- [5] Add 50  $\mu$ l of chloroform to each resuspended pellet, mix well (using vortex) and leave at room temperature for 5 min.
- [6] Pellet cell debris by centrifugation in microfuge for 10 mins and full speed. Retain the supernatant to measure enzyme and protein levels in cell cytosol.

### **Lyase Assay**

- [7] Set up duplicate eppendofs containing 0.5ml of substrate solution (pipette this viscous solution slowly), an appropriate amount of sample (e.g. 50-100 $\mu$ l) and sufficient distilled water to give a final volume of 0.75ml.
- [8] Incubate at 37°C for 1 hour.

- [9] Set up a series of dilutions of the glucose standard (approx. 0-0.3mg/tube final concentration) placing 0.75 ml of each into fresh microfuge tube.
- [10] Add 0.5 ml of DNS reagent to each reaction (enzyme assays or standards), mix well and heat at 100°C for 5 min.
- [11] Place 200 µl of each reaction into micro titre plate and read absorbance at 540nm. Using the glucose standard curve and the protein concentration (see below), calculate specific activities for the different enzyme preparations. Satisfy yourself that you are measuring initial reaction rates.

### **Protein estimation**

- [11] Dilute BSA solution so that you have range of standards between 0 and 1 mg/ml.
- [12] Pipette 20 µl portions in duplicate of each standard and each sample to be assayed into a known pattern of wells on a multiwell plate.
- [13] Pipette 230 µl of the Coomassie reagent into each well. Mix samples by sucking up and down with pipette, use a clean tip for each well.
- [14] After no more than 10 minutes you can read absorbance in the multiplate reader at 595 nm.
- [15] Calculate the specific activities of your preparations.

- [16] Compare the specific activities of the various preparations and comment on your results.

## USEFUL BOOKS

The following books contain plenty of useful practical advice on gene cloning and associated techniques. This is just a sample of the many practical manual type of books that are currently available.

Guide to Molecular Cloning Techniques (1987), Methods in Enzymology, Vol 152, (S L Berger & A R Kimmel, eds) 812pp., Academic Press, London.

DNA Cloning - A Practical Approach (Vols I and II) (1985), (D M Glover, ed.) IRL Press, Oxford.

Molecular Cloning - A Laboratory Manual (1989), J Sambrook, E F Fritsch & T Maniatis (2nd edition) Cold Spring Harbor Laboratory, Cold Spring Harbor.

PCR Protocols, M A Innis, D H Gelfand, J J Sninsky & T J White (eds) (1990) 482pp., Academic Press, London

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