EXPRESSION OF C. FIMI GENES FROM TRANSPOSON Tn10 TET PROMOTERS.

by

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ABSTRACT

In this study, the promoterless genes *cenA* and *cex* (both obtained from E. coli expressing recombinant DNA of C. fimi) were placed under the control of the divergent *tet* promoters from Tn10 and cloned into a broad host range plasmid, pJRD215. This system allowed us to investigate the expression and interaction of the gene products of these two genes (CenA and Cex) in four different Gram-negative host organisms: E. coli C600, an E. coli K12 derivative which is able to survive on cellobiose, R. capsulatus B10, and K. pneumoniae M5a1. The objective was to eventually find a Gram-negative host organism which would secrete the CenA and Cex proteins so that we could investigate whether it was now possible for that host organism to survive on cellulose.

All four host organisms used in this study expressed the *cenA* and the *cex* genes with different efficiencies, however, none of the host organisms secreted CenA and Cex to the culture medium.

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INTRODUCTION

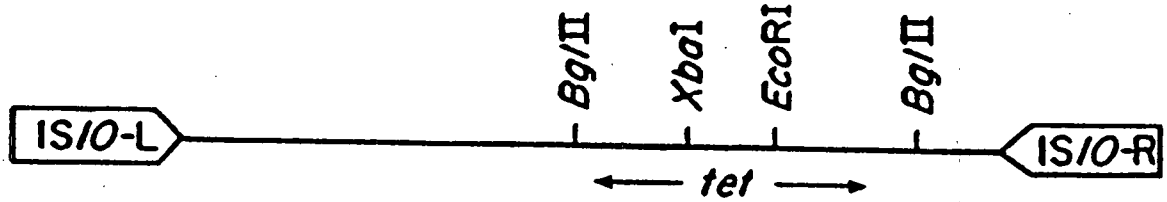
DIVERGENT CONTROL REGIONS

Sequences which allow divergent transcription from closely spaced sites are widespread in Prokaryotes, Eukaryotes and their viruses. More than 60 examples have been found to date (1). The first sets of divergent promoters were identified in the genome of bacteriophage λ (2,3) and in the *bio* operon on the E. coli genome (4). Subsequently, more than 20 cases of such divergent control regions have been identified on the E. coli chromosome. The widespread occurence of these divergent control regions implies that they play an important role in the regulation of gene expression.

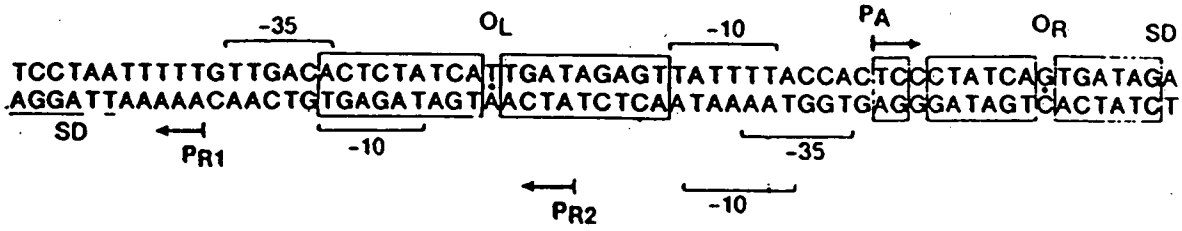
Divergent control regions have also been identified on transposable elements like Tn10 (5,6,7,8). Transposon Tn10 was originally isolated from nature as part of the conjugative plasmid R100, also known as R222 (9,10,11). The intact transposon is 9300bp in length and has 1400bp inverted repeats at its ends. The 6500bp of non-repeated material include the 2500bp tetracycline resistance determinant (see Figure 1). The 1400bp repeat sequences are closely related but non-identical IS elements, IS10-Right and IS10-Left, which encode the functions responsible for Tn10 transposition.

The tetracycline resistance determinant in the transposon Tn10 consists of two genes; the *tetA* resistance gene and the *tetR* repressor gene that are transcribed from divergent overlapping promoters (see Figure 1). The *tetA* gene encodes a 43.2 KDa membrane protein that appears to be both necessary and sufficient for resistance to tetracycline (12,13,14,15). The *tetR* gene encodes a 23.3 KDa protein that negatively regulates both its own synthesis and the synthesis of the TetA resistance protein (16,17). Both the resistance gene and the repressor are synthesized in the presence of tetracycline. Studies with purified Tet repressor have shown that in the absence of tetracycline the repressor binds to two adjacent operator sites, O_L and O_R, and that tetracycline is able to induce transcription of both *tetA* and *tetR* by binding to this

FIGURE 1: THE TRANSPOSON Tn10.



THE INTACT TRANSPOSON Tn10.



THE DIVERGENT CONTROL REGION OF Tn10

repressor and hence reducing its affinity for the operators (17,18,19). The operators overlap the promoters for *tetA* and *tetR* to different extents. Each operator is recognized and bound by a dimer of the *tetR* gene product (12,19).

The tetracycline resistance genes of Tn10 are readily distinguishable in both DNA sequence and phenotype from other types of tetracycline resistance genes, including those found on the plasmid pSC101 (and its derivatives pMB9 and pBR322) or the broad host range plasmids RP1 and RP4 (20,21).

Binding sites for regulatory proteins have often been observed within the divergent control regions and these proteins may regulate transcription in both directions. This characteristic makes these divergent control regions very attractive when systems are required for the simultaneous expression of more than one gene. For example, it is possible to clone two genes of interest, one on either side of a divergent control region, and to then tightly regulate the expression of both genes in vivo.

The fusion of divergent control regions to promoterless genes has also allowed the regulatory characteristics of such divergent promoters to be analysed (22,23,24,25). Promoter-probe vectors containing the gene combinations *lacZ/galK* and *lacZ/phoA* were used to study the divergent control regions of the pBR322 *tet* gene and the *tet* genes of transposon Tn10 (24). Levels of expression of the indicator genes allowed determination of promoter strengths. The induction kinetics of the divergent *tet* regulatory region from Tn10 were also studied. Expression of the indicator genes was repressed if the *tetR* regulatory gene of Tn10 was provided in trans by a second plasmid within the host strain. Addition of an inducer (the non-inhibiting Tc derivative, 2-acetyl-2-decarboxyamide Tc.) resulted in induction of expression from promoters $tetP_A$ and $tetP_R$. In other words, upon induction, transcription started simultaneously in both directions from the overlapping promoters of the *tet* control region.

CELLULASES

Cellulose is a linear polymer composed of glucose subunits linked by ß-1,4-glucosidic bonds. In the native state, cellulose molecules form fibers which are composed of compact crystalline domains separated by more amorphous regions. Cellulose degradation by bacteria and fungi is carried out by complex multienzyme systems. Work in our lab is concerned with the enzyme system in one of these organisms, the Gram-positive bacterium Cellumonas fimi.

The conversion of cellulose to glucose requires the activity of three types of enzymes. The cellulose is first co-operatively attacked by two types of extracellular cellulases; the endoglucanases (1,4,ß-D-glucan glucanohydrolase, EC 3.2.1.4) and the exoglucanases (1,4,ß-D-glucan cellobiohydrolase, EC 3.2.1.91). The product of these enzymes, cellobiose, is further hydrolysed by cellobiase (ß-D-glucoside glucohydrolase, EC 3.2.1.21) to glucose (see Figure 2).

Two C. fimi cellulase genes, *cenA* and *cex*, have been studied in detail (26,27). The *cenA* gene encodes a secreted glycosylated endoglucanase of 53.0 KDa and the *cex* gene encodes a secreted glycosylated exoglucanase of 49.3 KDa. The two structural genes have been cloned independently in E. coli on the vector pBR322 (26,27). The genes were expressed in E. coli to give non-glycosylated proteins retaining the specificities of the native enzymes from C. fimi. Both genes have been sequenced. Both *cenA* and *cex* encode proenzymes in which the mature polypeptide is preceded by a leader peptide. Although both activities are found in the periplasm of E. coli cells, neither enzyme is secreted into the culture medium (26,27).

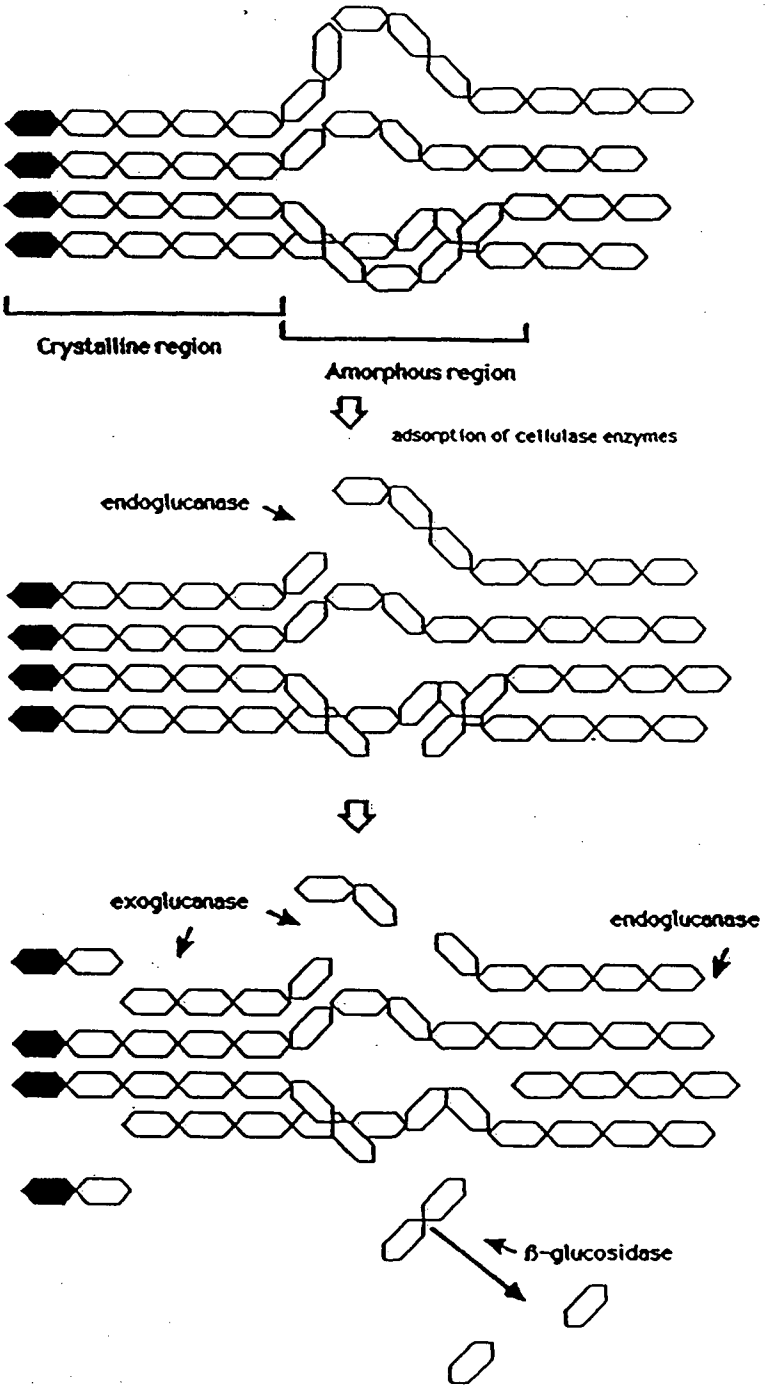


FIGURE 2: THE ENZYMATIC DEGRADATION OF CELLULOSE.

THE BROAD HOST RANGE PLASMID PJRD215

The plasmid RSF1010 belongs to the incompatibility group Q (Inc Q) and has the very useful property of replicating in most, if not all, species of Gram-negative bacteria. For this reason, it (and related or identical plasmids) is attractive as a cloning vector in Gram-negative organisms other than E. coli. RSF1010 is also of special interest due to its ability to be transferred between species by conjugation in the presence of a variety of self-transmissible plasmids including RK2 (28). However the size of RSF1010 (8.7kbp) and the fact that it contains few unique sites for cloning, makes it a far from ideal cloning vector. The cloning versatility of RSF1010 has been improved by changing the selective markers, inserting a *cos* site for in vitro packaging and by introducing a restriction site bank. This plasmid has been named pJRD215 (29). It retains the broad host range replicon and mobilization functions of RSF1010, which enables it to be used to carry novel genetic information to those bacteria that cannot be transformed readily.

OBJECTIVES

An understanding of the mechanisms of cellulose degradation by an organism requires the isolation and characterization of the individual components of the system. Once the relevant enzymes are known and purified, it should be possible to study further their role, for example by reconstituting mixtures and testing for synergy or by characterizing mutants unable to synthesise defined components. Hence a more detailed understanding of the mode of action of the CenA and the Cex proteins from C. fimi, whether they act individually or in concert, may be obtained if the *cenA* and the *cex* genes are cloned into non-cellolytic organisms and the expression of these genes studied in these hosts.

In this study, where *cenA* and *cex* (both obtained from E. coli expressing recombinant DNA of C. fimi) are placed under the control of the divergent *tet* promoters from Tn10, the interaction and

synergism of the gene products of these two genes (CenA and Cex) can be investigated in E. coli. Placing the two cellulase genes on either side of the divergent promoter region allows the simultaneous transcription of both genes. In addition, cloning these genes into a broad host range plasmid (pJRD215 (29)) allows the transfer of these genes to other Gram-negative organisms by conjugation or by transformation. In this way it is possible to study the interaction of the endoglucanase and the exoglucanase in other host organisms. The two Gram-negative organisms used in this study, other than E. coli were Rhodobacter capsulatus and Klebsiella pneumoniae . The photosynthetic bacterium R. capsulatus is of interest in this study because it possesses a ß-glucosidase and K. pneumoniae , a nitrogen-fixing bacterium, has the ability to grow on cellobiose (30). Cellulose degradation by microorganisms is usually accomplished more efficiently if a supplementary source of nitrogen is available (31,32). For these reasons various approaches have been suggested as ways to combine nitrogen fixation and cellulolysis and hence one strategy would be to clone cellulase genes into host organisms which are able to fix nitrogen. A second E. coli strain was also used which is a derivative of K12 and can survive on cellobiose.

The main objective of constructing a broad host range plasmid which carries the *cenA* and *cex* genes (under the control of a divergent promoter region) is to eventually find a Gram-negative host organism which will secrete the CenA and the Cex proteins. In this way we can investigate whether it is now possible for this host organism to survive on cellulose.

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

E. coli C600/pEC1 and C600/pcEC2 were used as sources of C. fimi exoglucanase (*cex*) and endoglucanase (*cenA*) genes, respectively. The Tn10 *tet* promoter genes were provided on plasmid pCB168. This plasmid was present in E. coli strain CB874 (thi, galK, rpsL, phoA8, rec A56::Tn10 site unknown (24).
The broad host range plasmid pJRD215 (27) was carried in E. coli strain MM294. The two E. coli strains which can survive on cellobiose (Cel^+), were derivatives of the K12 strain W4860 (33) The bacterial strains used in conjugative studies (see this section) were R. capsulatus strain B10 and K. pneumoniae strain M5a1. The 'helper' plasmid, pRK2013, (used to mobilize pJRD215) was carried in E. coli strain HB101 (34). For repression and induction studies, the E. coli strains M8820/pR751::Tn10 and JC10240 (which has Tn10 integrated into the chromosome) were used.

MEDIA

All E. coli strains were grown in Luria broth (LB; 10g tryptone, 5g YE, 10g NaCl per litre). Appropriate antibiotics were added to a final concentration as listed below:

	µg/ml	Abbreviations
Ampicillin	50	Amp
Kanamycin	40	Kan
Streptomycin	25	Sm
Trimethoprim	25	Tm

R. capsulatus was grown on RCV medium:

Solution	ml/litre of medium
10% $(NH_4)_2SO_4$	10
10% DL-Malate	40
1% EDTA	2.0
20% $MgSO_4 .7H_2O$	1.0
Trace elements	1.0
7.5% $CaCl_2.2H_2O$	1.0
0.5% $FeSO_4.7H_2O$	2.4
0.1% Thiamine. HCl	1.0
0.64 M KPO_4 (add last)	15
Deionised water	to 1000mls

K. pneumoniae was grown on M9 medium (35): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl. This solution was autoclaved, cooled and then 2ml 1M $MgSO_4$ and 0.1ml 1M $CaCl_2$ were added.

Agar plates contained 15g agar/litre. In case of carboxymethylcellulose plates, 11g agar were added.

DNA MANIPULATIONS

PREPARATION AND PURIFICATION OF PLASMID DNA

For small scale isolation of plasmid DNA, 5 ml of LB, containing the appropriate antibiotic, were inoculated with a single colony from a plate and grown to stationary phase (overnight, 37°C). Cells were collected by centrifugation and plasmid DNA was isolated by the alkaline lysis method (36). Plasmid DNA was further purified on a NACS-Prepac column, as described by the supplier, BRL.
For large scale isolation of plasmid DNA, 500 ml of LB plus the appropriate antibiotic, were inoculated with a single colony from a

plate and incubated overnight at 37°C. Plasmid DNA was isolated by alkaline lysis and purified by ultracentrifugation in a CsCl-ethidium bromide gradient (36).

RESTRICTION ENDONUCLEASE DIGESTIONS.

Restriction enzyme reactions were carried out as described by Maniatis et al (36). All enzymes were used under the conditions suggested by the manufacturer.

AGAROSE GEL ELECTROPHORESIS OF DNA.

Restriction fragments were separated on 0.8-1% agarose gels using TBE (89mM Tris Borate, 89mM Boric acid, 8mM EDTA, pH 8.0) buffer. Gels contained 1μg ethidium bromide/ml of buffer. DNA bands were visualized by fluorescence using a UV transilluminator. Desired DNA fragments were recovered from agarose gels using NA-45 DEAE membrane (Schleicher and Schuell). A strip of NA-45 was placed in an incision just ahead of the desired DNA band. Elecrophoresis was continued (150V, 2-3 mins) until binding was complete, as judged by ethidium bromide fluorescence using long wave UV. The strip was freed of residual agarose by shaking in 500μl NET buffer (0.15M NaCl, 0.1mM EDTA, 200mM Tris pH 8.0). DNA was eluted off the NA-45 strip by addition of 250μl high salt NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20mM Tris pH 8.0) and incubating at 65°C for 45 mins with occasional mixing. Residual ethidium bromide was removed by washing with water saturated n-butanol. The DNA was precipitated with 2.5 volumes of 95% ethanol.

ATTACHMENT OF LINKERS TO DNA

Blunt ended EcoR1 linkers were attached to DNA fragments using the following procedure: (i) DNA was blunt-ended using the Klenow fragment of DNA polymerase. The reaction was carried out as described by Maniatis et al (36). (ii) A fraction of the linkers (1μg out of 16μg) to be used in the ligation to the DNA, was labelled

using γ-^{32}P ATP: 2µl of 0.5µg/µl blunt-ended EcoR1 linkers were mixed with 0.1µl of γ-^{32}P ATP (specific activity = 7000Ci/mMole), 1µl 10x linker kinase buffer (0.7M Tris. Cl, pH 7.6, 0.1M $MgCl_2$, 50 mM DTT), 9µl sterile distilled water (sdH_2O) and 10 polynucleotide kinase (Pharmacia). This mixture was incubated at 37°C for 15 mins, then 1µl 10x linker-kinase buffer, 1µl 10mM ATP, 7µl sdH_2O and 10 U polynucleotide kinase were added and the reaction incubated for a further 30 mins at 37°C. (iii) 1µg of the radioactively labelled linkers, 15µg of unlabeled linkers and 5µg of DNA (to which the linkers were to be attached) were precipitated together and resuspended in 5µl 10x Apa1 assay buffer (60mM NaCl, 60mM 2-mercaptoethanol, 1mg/ml BSA), 5µl 4mM ATP, 5µl 10mM spermidine, 5µl 20mM DTT, 28µl sdH_2O and 20 U T4 DNA ligase. This mixture was incubated overnight on ice. After extraction with phenol/chloroform, the DNA was precipitated with 2 volumes 95% ethanol before resuspension in 50µl TE (pH 8.0) .

AUTORADIOGRAPHY.

Attachment of linkers to DNA was monitored by running an aliquot of the DNA on an agarose gel before and after digestion with EcoR1, transferring the gel to 3M Whatman filter paper and drying on a Biorad gel drier. The dried gel was placed in a Kodak X-Omatic film cassete with Kodak XRP-1 film and the film was exposed overnight at -70°C.

LIGATION

DNA fragments, at a molar ratio of insert to vector of 3:1, were precipitated together with ethanol and resuspended in 9µl sdH_2O. One µl of ligation buffer (50mM Tris pH 7.4, 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP, 100µg/ml BSA) and T4 DNA ligase were added. For sticky ended ligations, 10 U of ligase were added, whereas for blunt ended ligations, 400 U were added. The ligation

mixture was incubated overnight at 16°C.

TRANSFORMATION

Competent cells were prepared as described by Maniatis (36). Up to 100ng of DNA were used to transform cells. Suitable volumes of transformation mixtures (50µl, 100µl, 150µl) were spread onto appropriate media to select for transformants. Plates were incubated overnight at the appropriate temperature.

CONJUGATIVE CROSSES

The broad host range plasmid, pJRD215, used in the construction of pND_3 (see results section) is transferable between species by conjugation in the presence of a mobilising plasmid (28). Hence, triparental matings were carried out using two donors (E. coli C600 /pND_3 and E. coli HB101/pRK2013) and the recipient cells.

Donor and recipient cells were grown in liquid media to log phase, mixed in a 1.5ml Eppendorf tube (ratio 1:1:1) and pelleted by 30 sec. centrifugation. The cells were carefully resuspended in 1ml minimal medium (RCV in the case of R. capsulatus as recipient cells, and M9 in the case of K. pneumoniae as recipient cells), and 10µl spotted onto a nitrocellulose filter (0.7µm pore size) on a prewarmed minimal medium plate. The plates were incubated at 30°C overnight. The filters were placed into 1.5ml Eppendorf tubes with 1ml minimal medium and the cells resuspended on a vortex mixer (5 mins). Samples of the cell suspensions (50µl, 100µl, 150µl) were then plated onto selective medium. In the case of R. capsulatus as recipient cells, the selective media was RCV Sm and in the case of K. pneumoniae being the recipient cells, M9 Sm was used to select for transcongugants. The plates were incubated at 30°C for 24-48 hours.

ENZYMATIC STUDIES

PLATE ASSAYS FOR THE DETECTION OF ENDOGLUCANASE AND EXOGLUCANASE ACTIVITIES

Endoglucanase activity of recombinant clones was detected as follows: cells were grown overnight, at 37°C, on LB plates supplemented with 11g CMC (Sigma low viscosity grade)/litre and the appropriate antibiotic. Colonies were washed off the agar surface and the plates were flooded with 0.2% congo red solution. After 15 minutes gentle agitation on a rotary shaker (Labline), the congo red solution was poured off and 1M NaCl was used to wash off excess dye (15-30 mins gentle agitation on the rotary shaker). Zones of clearing of the dye appeared at the sites of endoglucanase positive colonies.

Exoglucanase activity of recombinant clones was detected as follows: Methylumbelliferyl cellobioside (MUC) was used to detect any colonies expressing exoglucanase activity. Cells were grown overnight at 37°C, on LB plates containing 100μm MUC and the appropriate antibiotic. The exoglucanase cleaves the substrate to release methylumbelliferone, which fluoresces under long wave U.V. irradiation.

PREPARATION OF CRUDE CELL EXTRACTS

Single colonies of recombinant clones were inoculated into 25ml of medium and incubated overnight at 37°C. Cells were harvested by centrifugation (10 mins at 8K in a JA20 Beckman rotor) and resuspended in 5ml 50mM KPO_4 (pH 7.0), 0.02% NaN_3. The suspensions were sonicated using a Bronson sonifier with a microprobe (Intensity setting of two, 3x30secs.). The suspensions were then centrifuged again (12K, 40 mins) to give a clear supernatant which was used in enzyme assays.

pNPCase ASSAY

The p-nitrophenylcellobioside assay (as described by Gilkes et al (37)) gives a measure of exoglucanase activity in cell extracts. The assay measures the hydrolysis of the agluconic bond of pNPC by following the release of p-nitrophenol (pNP). The reaction conditions were as follows: 500µl of prewarmed cell extract were mixed with 500µl of prewarmed pNPC (12.5mM in 100mM KPO_4 and 0.02% NaN_3) for 30 mins at 37°C, when 500µl of 1M Na_2CO_3 were added. Absorbance at 410nm was measured against a blank solution (carbonate added to the pNPC prior to enzyme). A standard curve using known dilutions of pNP was constructed. pNPCase activity was calculated using the following formula:

$$\frac{O.D.410 \times X}{Yt} = \text{Units (µmol/min/ml undiluted enzyme).}$$

where Y= µl undiluted enzyme in the reaction mix
t= incubation time (30mins)

1O.D. 410 = X nmoles pNP/1.5ml

CARBOXYMETHYLCELLULASE ASSAY

The CMCase assay measures reducing sugars released from CMC by reaction of the reducing groups with dinitrosalicyclic acid (DNS) (36). DNS reagent was prepared by dissolving 10g dinitrosalicylic acid, 2g phenol, 0.5g sodium sulfite and 200g sodium potassium tartrate in 500ml of 2% NaOH and then diluting this solution with 500ml water. The assay conditions were as follows: 250µl of prewarmed cell extract and 500µl of prewarmed 4% CMC (Sigma low viscosity; 50mM KPO_4, pH 7.0; 0.2mg/ml BSA) were mixed together and incubated at 37°C for 30mins. The reaction was stopped with 800µl DNS reagent. 50µl of standard glucose solution (1mg/ml) were added and the mixture steamed at 100°C for 15 minutes. It was then allowed to cool and absorbance at 550nm was measured against a blank solution (DNS reagent was added to the CMC before

the enzyme mixture).

A standard curve was constructed using dilutions of the standard glucose solution, to give a value for the µmoles of glucose/0.75ml which gives 1 O.D.550 (X). CMCase activity was then calculated using the formula:

$$\frac{\Delta O.D.\ 550 \times X \times 100}{Yt} = \text{Units (µmol/min/ml undiluted enzyme).}$$

where Y= µl undiluted enzyme in 0.75ml reaction mix
t= assay time.

ß-GALACTOSIDASE ACTIVITY

ß-galactosidase was used as a cytoplasmic marker in E. coli cells (see enzyme localization studies) and was measured according to Miller (38). One unit of ß-galactosidase is defined as that amount of enzyme that releases 1µmole of ONP/min at 37°C.

ALKALINE PHOSPHATASE ASSAY.

Alkaline phosphatase was used as a periplasmic marker in E. coli cells (see enzyme localization studies) and was measured according to Garen and Levinthal, (39) in which the rate of release of p-nitrophenol from p-nitrophenol phosphate is determined by following absorbency changes at 410nm. One unit is that activity liberating 1µmole p-nitrophenol/min at 25°C.

MALATE DEHYDROGENASE ASSAY

Malate dehydrogenase was used as a cytoplasmic marker in R. capsulatus cells in enzyme localization experiments (see this section). This enzyme catalyses the following reaction:

L-malate + NAD $\rightleftharpoons$ oxaloacetate + $NADH_2$

The activity was determined by a decrease in absorbency at 340nm owing to the oxidation of $NADH_2$. The following were placed into a 3ml cuvette : 2.6ml phosphate buffer (0.1M, pH 7.4), 0.2ml $NADH_2$, (3.75mM), 0.1ml cell extract and finally 0.1ml 6mM oxaloacetate. Readings were taken at 15 second intervals for 2 minutes against a blank solution (no substrate added).

Enzyme activity was calculated using the formula :

$$\text{Specific activity} = \frac{\Delta \text{ OD } 340/\text{min}}{6.2 \times \text{mg enzyme/ml reaction mix}}$$

One unit of activity causes $NADH_2$ to be oxidized at an initial rate of 1μmole/min. under specified conditions at 25°C .

PROTEIN CONCENTRATION

Protein concentrations were determined using the Bio-Rad assay (Bio-Rad labs) which is based on the differential color change of a dye in response to various concentrations of protein (40). Five μl of cell extract were diluted with 795 μl sdH_2O and 200μl of Bio-Rad reagent were added. This mixture was incubated at room temperature for no less than 15 minutes. Absorbancy measurements were taken at 595nm against a blank solution (5μl of 50mM KPO_4 substituted for cell extract). Standard curves were constructed, using bovine plasma albumin, to determine the μg of protein/0.5ml that give 1 O.D. at 595nm (X). The protein concentration was calculated using the following formula :

$$\frac{\text{O.D.595} \times X \times \text{dilution}}{1000} = \text{mg protein/ml (relative to bovine plasma albumin).}$$

LOCATION OF ENZYMATIC ACTIVITY

The osmotic shock procedure described by Nossal and Heppel (41) was used to obtain periplasmic and cytoplasmic cell fractions. Briefly, 30ml of medium and antibiotic were inoculated with a single colony and incubated overnight at 30°C. Cells were harvested by centrifugation at 8K for 5 mins and resuspended in 10ml of a cold solution of Tris.Cl (pH 7.1, 33mM), Sucrose (40%) and EDTA (1mM). The cells were sedimented and then resuspended in 10ml cold $MgCl_2$ solution (0.5mM). The cells were kept on ice (mixing gently) for 10 min, and then centrifuged again (8K, 5 min). The supernatant obtained was the periplasmic fraction. The cytoplasmic fraction was obtained by resuspending the sedimented material in 5ml KPO_4 buffer (0.1M, pH 7.0) and sonicating the suspension (see Preparation of crude cell extracts). After centrifugation (12K, 40min) the supernatant obtained was used as the cytoplasmic cell fraction.

VISCOMETRIC ASSAYS

The rate of hydrolysis of 4% CMC (Sigma low viscosity grade) by crude cell extracts was followed by determining ø (specific fluidity). ø is an index of polymer length. The method is as described by Gilkes et al (37) using a Cannon-Fenske viscometer (Fischer scientific-#13-616E).

RESULTS

SECTION ONE : CONSTRUCTION OF PLASMID pND_3- A BROAD HOST RANGE PLASMID IN WHICH AN ENDOGLUCANASE AND EXOGLUCANASE FROM C. FIMI ARE PLACED UNDER THE CONTROL OF TRANSPOSON Tn10 TET PROMOTERS

PART ONE : THE CONSTRUCTION OF pND_1. SEE FIGURE 3

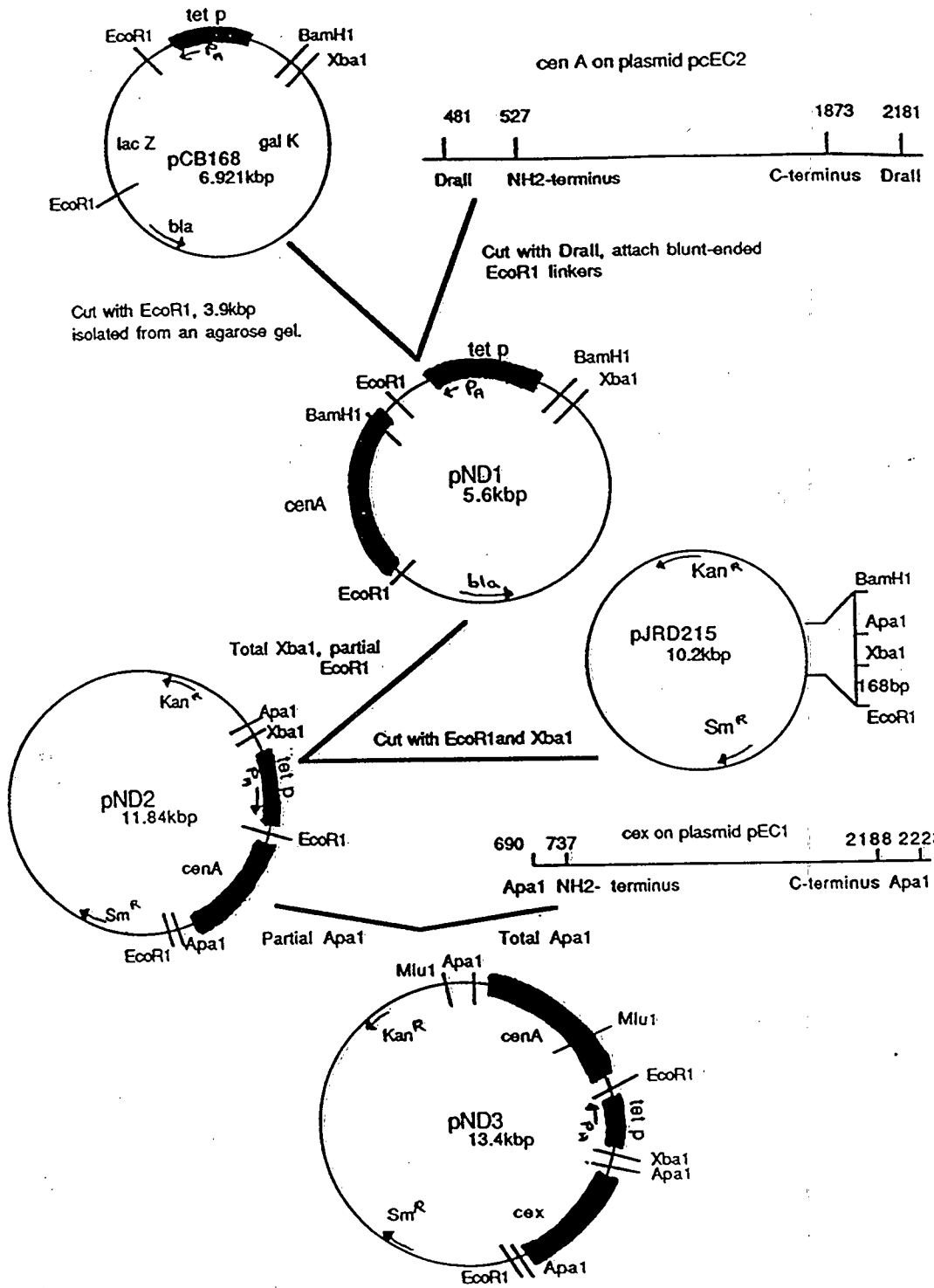
Plasmid pcEC2, carrying the endoglucanase gene (*cenA*) from C. fimi was cut with DraII enzyme. The DNA fragment carrying the endoglucanase gene (1.7kbp) was isolated from a 0.8% agarose gel using DEAE membrane. The DNA was blunt-ended using a Klenow reaction, and blunt-ended EcoR1 linkers were attached to the DNA. The DNA was then digested with an excess of EcoR1 (see Figure 4).

The plasmid pCB168, carrying the *tet* control region of transposon Tn10, was cut with EcoR1 and the 3.9kbp restriction fragment was isolated from a 0.8% agarose gel. This vector DNA and the DNA carrying the endoglucanase gene (insert DNA) were ligated together. The ligation mix was used to transform competent E. coli C600 cells. The cells were plated on LB Amp plates, and incubated overnight at 30°C.

Of the 1171 transformants obtained on the LB Amp plates, 120 colonies were selected at random to be screened for endoglucanase activity by replica plating on LB Amp CMC plates (see materials and methods). Of these 120 colonies, four gave strong halos on the congo red stained CMC plates and two gave weaker halos. Hence all six clones exhibited endoglucanase activity.

The plasmid DNA from the six endoglucanase positive transformants was extracted by the alkaline lysis method and digested with BamH1 to elucidate the orientation of the insert DNA in the constructs (see Figure 5). Two of the clones gave 0.38kbp and 5.22kbp sized BamH1 restriction fragments suggesting that the inserts were in the correct orientation. The plasmid that both these clones carried was named pND_1. Two clones gave BamH1 restriction fragments of sizes 1.4kbp and 4.2kbp which suggested that the insert DNA was in the wrong orientation. Expression of the *cenA*

FIGURE 3: THE CONSTRUCTION OF PLASMID pND_3.



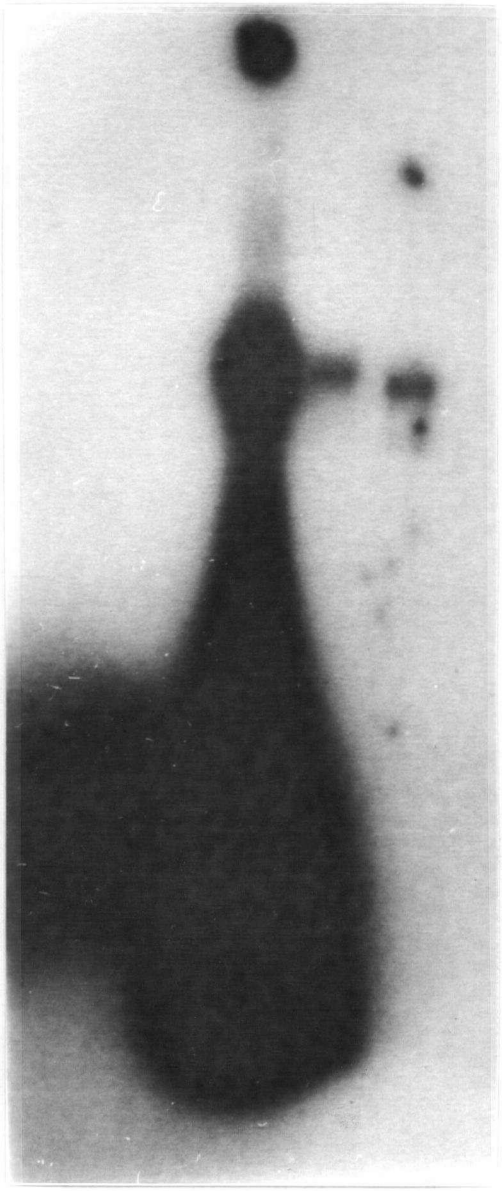


FIGURE 4: Autoradiogram following the attachment of $\gamma^{32}P$ labelled EcoR1 linkers onto pcEC2 Dra11 DNA fragment carrying the *cenA* gene. Lane 1:loaded 1µl out of 50µl of the pcEC2 DNA solution after the attachment of blunt ended EcoR1 linkers. Lane 2: 45µl of the pcEC2 DNA solution (after the ligation of linkers) was digested with EcoR1(40 units). After one hour, 1 µl was removed from this mix and loaded on the gel. Lane 3: one µl of the digestion mix, after overnight digestion, was loaded on the gel. Lanes 2 and 3 show how the excess linkers were digested off the pcEC2 DNA, the majority being removed after one hour's digestion.

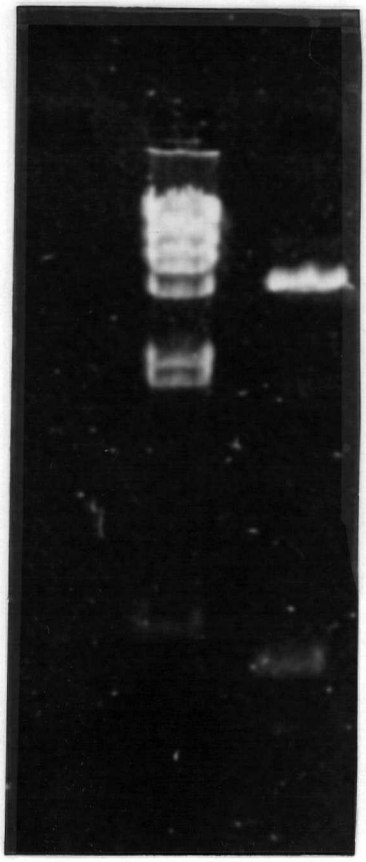


FIGURE 5: Plasmid pND_1 digested with BamH1. Lane 1:Lamda DNA digested with Hind111. Lane 2:BamH1 digested pND_1, showing the 5.22kbp and the 0.38kbp fragments.

gene in these two clones may have been from some unidentified vector promoter. The last two endoglucanase positive clones gave restriction fragments whose sizes suggested that two inserts had been cloned into the vector DNA.

One of the two clones (#5) in which the insert DNA was in the correct orientation, was selected for the second stage of the construction.

PART TWO:THE CONSTRUCTION OF pND2. SEE FIGURE 3.

The broad host range plasmid, pJRD215, was digested with the enzymes EcoR1 and Xba1. Plasmid pND_1, isolated from clone #5 (see last stage), was then totally digested with Xba1 and partially digested with EcoR1 (the DNA was digested 10-30 mins) to release a EcoR1-Xba1 fragment of 1.81kbp. This fragment was isolated from an agarose gel.

The 1.81kbp pND_1 DNA (insert DNA) was then ligated with the pJRD215 vector DNA and the ligation mix was used to transform competent E. coli C600 cells. The cells were plated on LB Kan plates and incubated overnight at 30°C.

The transformants obtained on the LB Kan plates were replica plated onto LB Kan CMC plates to screen for endoglucanase activity. Of the 400 colonies screened, two were found to be endoglucanase positive. The plasmid DNA from these two clones (designated pND_2) was digested with Apa1 (see Figure 6). Both clones gave Apa1 restriction fragments of sizes 10kbp and 1.84kbp.

One clone (#1), was selected for the final stage in the construction of pND_3.

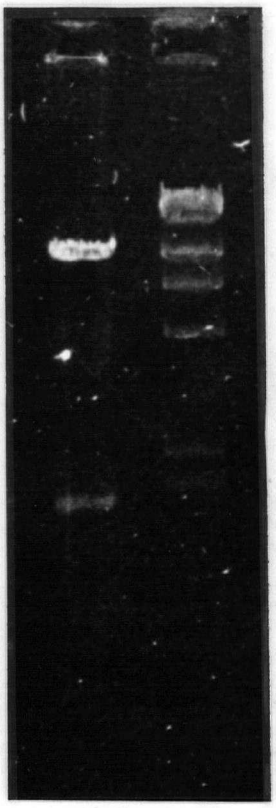


FIGURE 6: Plasmid pND_2 digested with Apa1. Lane1:pND_2 digested with Apa1, showing the 1.84kbp and the 10kbp fragments.
Lane 2:Lamda DNA digested with Hind111.

PART THREE- THE CONSTRUCTION OF pND_3. SEE FIGURE 3.

To make the final construct pND_3, pND_2 (vector DNA) was partially digested with Apa1 and ligated with the Apa1 fragment from pEC1 (insert DNA) carrying the exoglucanase gene. Both the vector DNA (11.84kbp) and the insert DNA (1.53kbp) were isolated from an agarose gel. The ligation mix was then used to transform competent E. coli C600 cells. The cells were plated on LB Kan plates and incubated overnight at 30°C. Any transformants obtained were then screened for endoglucanase and exoglucanase activities by replica plating on LB Kan CMC and LB Kan MUC plates.

This procedure was used twice but it failed to produce any endoglucanase/exoglucanase positive clones. So the protocol was modified. All the Apa1 restriction fragments from a total digestion of pEC1 and partial digestion of pND_2 were used in a ligation reaction in the hope that the correct restriction fragments would ligate together to form the desired construct. The ligation mix was then used to transform competent E. coli C600 cells and any transformants obtained were screened for endoglucanase and exoglucanase activities, as described earlier.

Of the 150 transformants screened for endoglucanase/ exoglucanase activities, 14 were found to be exoglucanase positive and of these two were endoglucanase positive. Of the rest, 80 clones were found to be endoglucanase positive but not exoglucanase positive, suggesting that all these clones had grown up from cells which had been transformed with religated vector DNA.

The plasmid DNA from the 14 exoglucanase positive clones was isolated and digested with Apa1 enzyme. Running the DNA from these clones on an agarose gel showed that two of these released a 1.84kbp and 1.53kbp Apa1 fragment (corresponding to the endoglucanase and the exoglucanase inserts respectively) as well as a 10kbp fragment, corresponding to the vector DNA. These two clones were identified as those which had been both endoglucanase and exoglucanase positive on indicator plates. One of the two endoglucanase/exoglucanase positive clones (#9) also released an extra Apa1 fragment which corresponded to an Apa1 fragment from Apa1 digested pEC1 (see Figure 7). Hence, the clone which released the correct number and size fragments when its DNA was digested

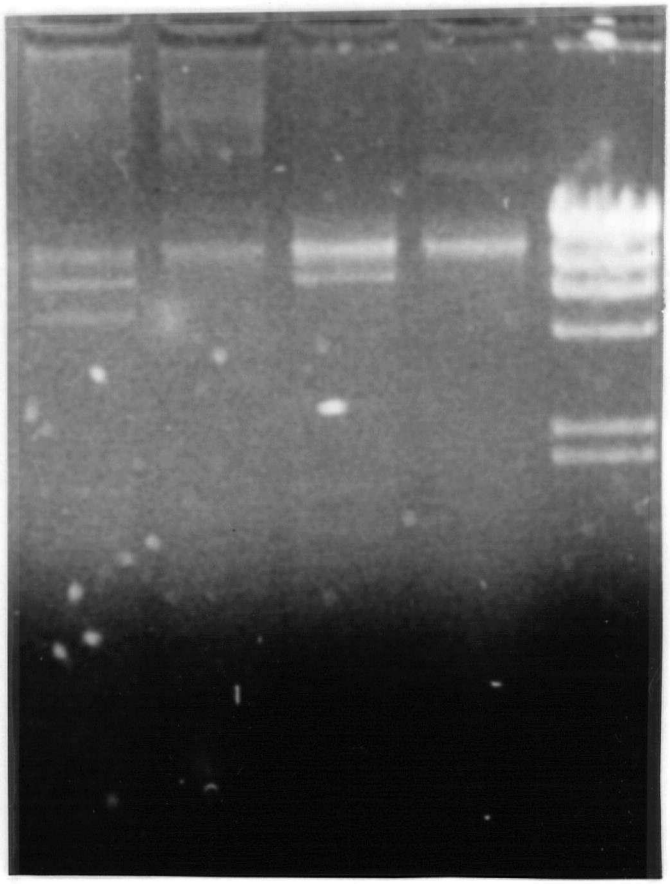


FIGURE 7: Apa1 digestion of clones #9 and #11. Lane 1: Clone #9 digested with Apa1, showing the 10kbp vector band, the 1.84kbp and the 1.53kbp bands. An extra band of about 6kbp is also seen in this clone. Lane 2: Undigested clone #9. Lane 3: Clone #11 digested with Apa1, showing the three correct fragments of sizes 10kbp, 1.84kbp and 1.53kbp. Lane 4: Undigested clone #11. Lane 5: Lamda DNA digested with Hind111. The DNA used in these digestions was isolated by a mini alkaline lysis procedure, and so the DNA is not being fully digested by the enzyme.

with Apa1.(#11) was used as a source of the constructed plasmid pND_3. The DNA from this clone was also digested with Hind111, Xba1 (Figures 8 and 9), Mlu1 (Figure 10) and Xba1 and Sca1 enzymes (Figure11).

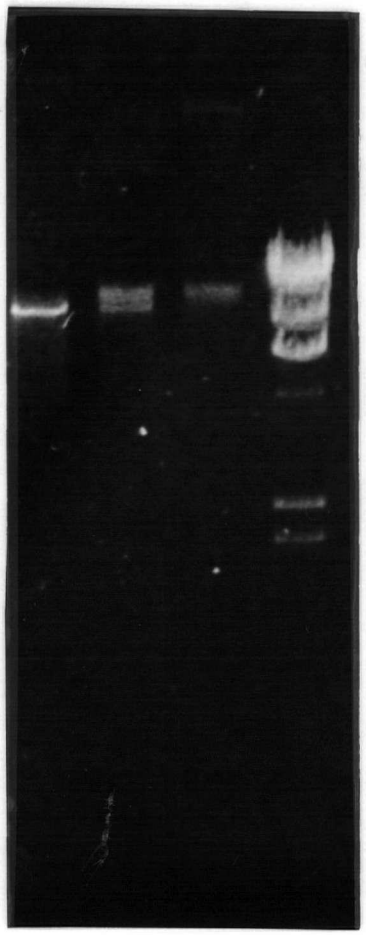


FIGURE 8: Plasmid pND_3 digestions. Lane 1: pND_3 digested with Hind111, showing the single band of approximately 13.4kbp. Lane 2: pND_3 digested with Xba1, again showing the single band. Lane 3: pND_3 undigested. Lane 4: Lamda DNA digested with Hind111.

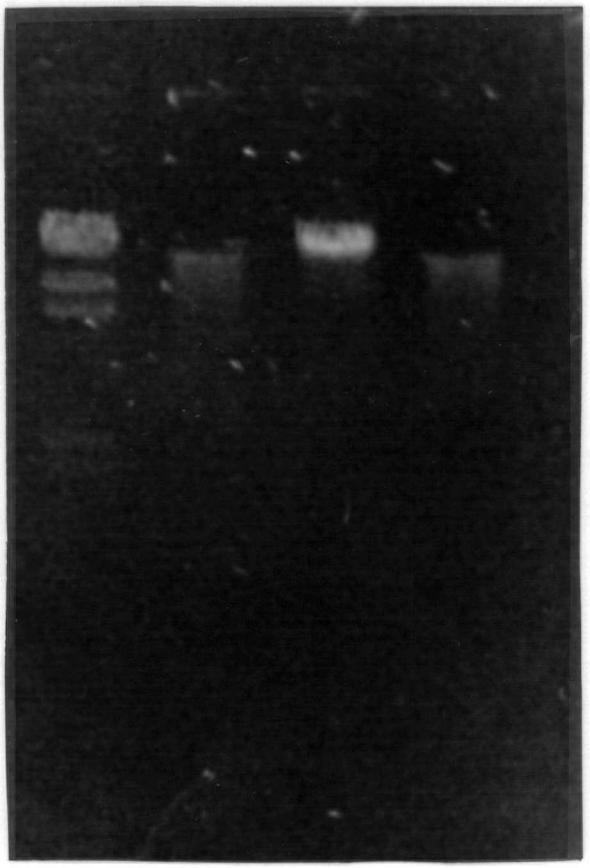


FIGURE 9: Plasmid pND_3 digestions. Lane 1: Lamda DNA digested with Hind111. Lane 2: pND_3 digested with Xba1, showing the single band of approximately 13.4kbp. Lane 3: pND_3 undigested. Lane 4: pND_3 digested with Hind111, again showing the single band.

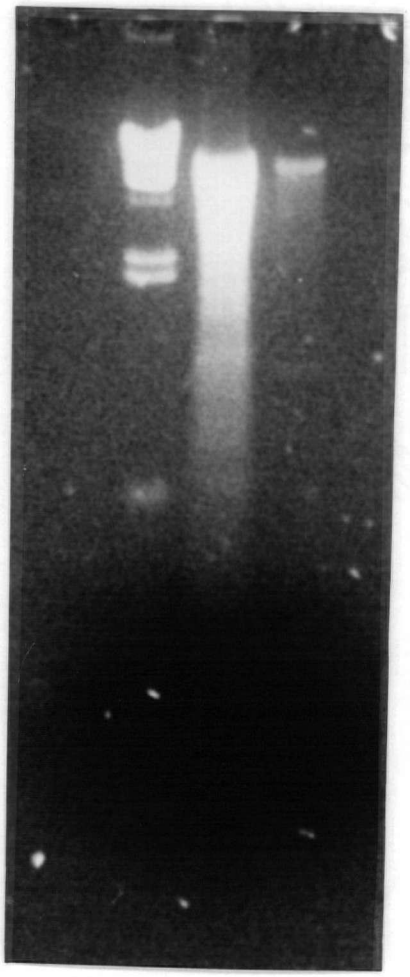


FIGURE 10: Lane 1: Lamda DNA digested with Hind111. Lane 2: E. coli clone #9 digested with Mlu1. Lane 3: E. coli clone #11, carrying the plasmid pND_3, digested with Mlu1 and showing the bands of sizes 1.1kbp and 12.2 kbp. From the size of these fragments released, the orientation of the *cenA* gene, in the plasmid pND_3, could be elucidated.

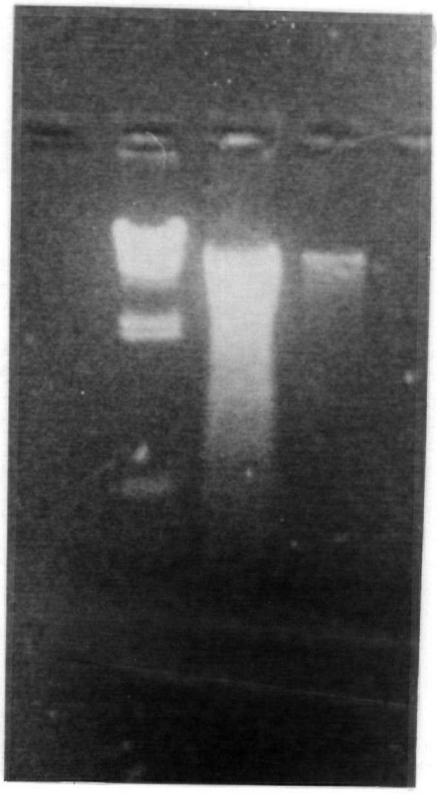


FIGURE 11: Lane 1: Lamda DNA digested with Hind111. Lane 2: E. coli clone #9 digested with Xba1 and Sca1 enzymes. Lane 3: E. coli clone #11, carrying the plasmid pND_3, digested with Xba1 and Sca1 enzymes and showing the bands of size 1.0kbp and 12.3 kbp. From the size of these fragments released, the orientation of the *cex* gene, in the plasmid pND_3, could be elucidated

SECTION TWO

QUANTITATION AND LOCATION OF ENDOGLUCANASE AND EXOGLUCANASE ACTIVITIES IN E. COLI RECOMBINANT CLONES.

DNS and pNPC assays were carried out to quantitate the endoglucanase and exoglucanase activities present in crude cell extracts of the two E. coli recombinant clones, #9 and #11 (see Table 1).

Results of assays carried out by previous workers on E. coli C600/pcEC2 and C600/pEC1 are also listed as sources of reference (26,27). E. coli C600 cells alone do not express any endoglucanase or exoglucanase activities. The *cenA* and the *cex* genes, in the plasmid pND_3, are lacking their promoter sequences. Presumably then, the expression of these two genes in the plasmid pND_3 relies on the Tn10 *tet* promoters.

Osmotic shock was used to localise the endoglucanase and exoglucanase activities in clone #11. The enzymes alkaline phosphatase and ß-galactosidase were used as periplasmic and cytoplasmic markers respectively (see Table 2).

The results show that both CenA and Cex are being exported to the periplasm in the E. coli cells, as was found by previous workers (26,27). This means that the leader sequences of the genes *cenA* and *cex* are being recognized and are functional in E. coli cells. No significant endoglucanase or exoglucanase activity was present in the culture medium.

SECTION THREE: MOBILISATION OF PND_3 FROM E. COLI C600 TO R. CAPSULATUS B10.

The broad host range plasmid, pJRD215 (29), which was used in the construction of pND_3, is transferable between gram-negative organisms by conjugation in the presence of a variety of self-transmissible plasmids, including RK2 (29). These so called helper plasmids provide trans-acting fertility functions. The kanamycin resistant helper plasmid, pRK2013, consists of the RK2 transfer genes cloned onto a colE1 replicon (34). This plasmid (present in E. coli HB101) was used in triparental matings (E. coli HB101 /pRK2013 x E. coli C600/ pND3 x recipient R. capsulatus B10).

TABLE 1:QUANTITATION OF ENZYME ACTIVITIES FROM CRUDE CELL EXTRACTS OF E. COLI CLONES #9 AND #11.

CLONE	DNS U/mg protein	pNPC U/mg protein
#9	7.0	0.38
#11	19.0	3.80
pcEC2	15.2	_
pEC1	_	1.43

U= units of activity: for the DNS assay, nmoles of glucose equivalents released/min. and for the pNPC assay, nmoles of p-nitrophenol released/min.

TABLE 2:LOCATION OF ENZYME ACTIVITIES IN E. COLI CLONE #11

ACTIVITY IN nMOLES PRODUCT/MIN/ML OF CULTURE				
ENZYME ASSAYED	IN PERIPLASMIC FRACTION	IN CYTOPLASMIC FRACTION	IN CELL EXTRACT	% RECOVERY
ENDO	5.70 (57)	2.92 (29)	10	86
EXO	0.57 (47.5)	0.44 (37)	1.2	85
ALK. PHOSPH.	0.64 (72)	0.27 (30)	0.89	102
ß-GAL.	0.93 (22)	3.49 (82)	4.26	104

Figures in brackets give % of total activity present in the fraction. Units of activity: for the endoglucanase activity, nmoles glucose equivalents released/min/ml culture; for the exoglucanase activity, nmoles p-nitrophenol released/min/ml culture; for the alkaline phosphatase activity, nmoles p-nitrophenol released/min/ml culture; and for the ß-galactosidase activity, nmoles o-nitrophenol released/min/ml culture.

Triparental matings were set up as described in the Materials and Methods section. R. capsulatus has a doubling time of 2 hours and so a ratio of five volumes of recipient culture to one volume of donor cultures was used. RCV minimal medium supported the growth of the prototrophic R. capsulatus recipient cells but selected against the growth of the auxotrophic E. coli donor cells. A second triparental mating experiment was set up where the number of recipient cells was increased (twice the volume used in the first mating experiment). The number of donor E. coli C600/pND$_3$ and recipient cells used in each experiment was obtained by plating dilutions of samples, taken from the cultures used in the mating experiments, on appropriate media plates and counting colonies growing up overnight at 30°C. These values, in addition to the number of transconjugants obtained from each experiment, were used to calculate the efficiency of the triparental matings (see Table 3). Using a greater number of recipient cells is shown to increase the number of transconjugants obtained.

One hundred transconjugants were picked onto RCV CMC Sm (15μg/ml) and RCV MUC Sm plates to screen for any endoglucanase and exoglucanase positive clones. All 100 clones were found to be positive for both activities, indicating that the plasmid pND$_3$ had been mobilized to these cells. R. capsulatus cells alone did not score positive for either endoglucanase or exoglucanase activities.

Sixteen endoglucanase/exoglucanase positive clones were selected at random to test if any E. coli cells were contaminating the colonies. This was done by restreaking the clones onto RCV Kan YE (0.1%) media. If any E. coli cells were present then they would grow up overnight on this media whereas R. capsulatus cells would not. E. coli and R. capsulatus cells are morphologically distinct (R. capsulatus colonies appear red with lighter coloring around them).

Two of the 16 clones showed contamination with E. coli colonies, however upon further testing on LB MUC Kan plates, these E. coli were found to be neither endoglucanase or exoglucanase positive. They were probably HB101/pRK2013 cells, which had either not been dissociated from the recipient R. capsulatus cell during the breaking of mating pairs, or had landed close to the recipient R. capsulatus cell during plating of the cells.

TABLE 3:TRIPARENTAL MATING EFFICIENCY WHEN USING R.CAPSULATUS B10 CELLS AS RECIPIENTS .

	TRANSCONJUGANTS/DONOR
CONJUGATION #1	$3x10^{-5}$
CONJUGATION #2	$13x10^{-5}$

Twice the volume of recipient cells used in conjugation #1 were used in conjugation #2.

DNS and pNPC assays were carried out on cell extracts of two randomly picked R. capsulatus clones, #10 and #12. The results of these assays are shown in Table 4. The exoglucanase activities obtained from these clones were comparable to those obtained from E. coli clone #11, however the endoglucanase activities were lower (see discussion). Further experiments to localise the enzyme activities (see Table 5), indicated that endoglucanase activity could not be detected in the periplasmic fractions whereas exoglucanase activity could. This could mean that the *cenA* leader sequence is not being recognized in the R. capsulatus cells and so is not functional in exporting the protein to the periplasm. Malate dehydrogenase was used as a cytoplasmic marker. A suitable periplasmic marker, which is easily assayed, is not available in R. capsulatus cells .

SECTION FOUR: MOBILIZATION OF pND_3 FROM E. COLI C600 TO KLEBSIELLA PNEUMONIAE M5A1.

Triparental mating experiments, similar to those used with R. capsulatus B10 as recipient cells, were set up to mobilize plasmid pND_3 into K. pneumoniae M5A1 cells. The plasmid pRK2013 (carried in E. coli HB101) was used again to help mobilize the plasmid pND_3 into the recipient cells. Both E. coli strains C600 and HB101 are auxotrophic, whereas the K. pneumoniae strain used is prototrophic. This meant that transconjugants from the triparental mating experiments could be selected on minimal-medium plates (M9 medium without addition of amino acids). Hence, the mating pair mixture was plated on M9 Sm (15µg/ml) plates. Two conjugation experiments were set up as before, where the second experiment used a larger volume of recipient cells. The efficiency of the conjugation experiments was calculated, as described for the R. capsulatus matings (see mobilization into R. capsulatus B10) and the results are shown in Table 6. Again the results show that using a larger number of recipient cells increases the efficiency of the conjugations.

Sixteen random transconjugants were picked onto M9 MUC Sm and

TABLE 4:QUANTITATION OF ENZYME ACTIVITIES FROM R. CAPSULATUS B10 CLONES #10 AND #12

CLONE	DNS U/mg protein	pNPC U/mg protein
#10	8.33	3.28
#12	6.24	2.37

U= units of activity. For the DNS assay, nmoles glucose equivalents released/min. and for the pNPC assay, nmoles p-nitrophenol released/min.

TABLE 5:LOCATION OF ENZYME ACTIVITIES IN R. CAPSULATUS B10 CLONE #10.

ENZYME ASSAYED	ACTIVITY IN nMOLES PRODUCT/MIN/ML OF CULTURE IN PERIPLASMIC FRACTION	IN CYTOPLASMIC FRACTION	IN CELL EXTRACT	% RECOVERY
ENDO	0	3.50 (79)	4.4	79
EXO	0.036 (22.5)	0.115 (72)	0.16	94.5
MALATE DEHY.	0.027 (1.3)	1.58 (76)	2.06	77

Figures in brackets give % of total activity present in the fraction. Units of activity: for the endoglucanase, nmoles of glucose equivalents released/min/ml culture; for the exoglucanase activity, nmoles p-nitrophenol released/min/ml culture and for the malate deydrogenase activity, nmoles NAD released/min/ml culture.

TABLE 6:TRIPARENTAL MATING EFFICIENCY WHEN USING K.PNEUMONIAE AS RECIPIENT CELLS.

	TRANSCONJUGANTS/DONOR
CONJUGATION #1	2.6x10-4
CONJUGATION #2	4.2 x10-4

Twice the volume of recipient cells used in conjugation #1 were used in conjugation #2.

M9 CMC Sm plates. All the clones were endoglucanase and exoglucanase positive. To test that the K. pneumoniae transconjugants were not contaminated with E. coli cells, samples of all 16 were examined microscopically after treating with Indian ink. K. pneumoniae cells possess a capsule and this is seen clearly using the indian ink. Three of the 16 clones seemed to be contaminated with E. coli cells, but the other 13 appeared E. coli free, even upon restreaking on LB Kan plates.

Two of the endoglucanase/exoglucanase positive clones were selected at random and pNPC and DNS assays were carried out on their cell extracts (see Table 7). The values obtained for both the assays were much lower than those obtained from the E. coli clone #11. Carrying out osmotic shock procedures on the two clones was not very successful (see Table 8) as the percentage recovery obtained was very low. This may have been because the osmotic shock procedure was not working well with these cells or due to the very low O.D. readings obtained from the assays, which may have been beyond the sensitive range of the spectrophotometer.

SECTION FIVE: TRANSFORMATION OF E. COLI CEL+ STRAINS.

The genes for cellobiose utilization are normally cryptic in E. coli, but recently the *cel* gene cluster for cellobiose utilization has been identified (33). The *cel* cluster is located at 37.8 min. on the E. coli map. It is not expressed in wild type strains of E. coli . Activation of the cel cluster by spontaneous mutation permits utilization of cellobiose, arbutin and salicin.

Two derivatives of the E. coli strain W4680 (33) were used in this study (TW34 and TW35).

The two E. coli strains were transformed with the plasmid pND_3 and the transformants obtained (selected on LB Kan plates) were screened for endoglucanase and exoglucanase activities on LB Kan CMC and LB Kan MUC plates. Of the 120 transformants screened, 60 from each transformation, all were positive for endoglucanase and exoglucanase activities.

TABLE 7:QUANTITATION OF ENZYME ACTIVITIES FROM K. PNEUMONIAE CLONES

CLONE	DNS U/mg protein	pNPC U/mg protein
#1	2.78	0.83
#2	3.70	0.74

Units for the DNS assay, nmoles glucose equivalents released/min. and for the pNPC assay, nmoles p-nitrophenol released/min.

TABLE 8:LOCATION OF ENZYME ACTIVITIES IN K. PNEUMONIAE CLONES

ENZYME ASSAYED	ACTIVITY IN nMOLES PRODUCT/MIN/ML OF CULTURE IN PERIPLASMIC FRACTION	IN CYTOPLASMIC FRACTION	IN CELL EXTRACT	% RECOVERY
CLONE #1				
ENDO	0.03 (5.6)	0.144 (27)	0.53	33
EXO	0.003 (3.6)	0.03 (36)	0.083	40
CLONE #2				
ENDO	0	0.04 (29)	0.14	29
EXO	0.003 (3.6)	0.03 (50)	0.063	51

Figures in brackets give % of total activity present in the fraction. Units for the endoglucanase, nmoles glucose equivalents released/ min/ml and for the exoglucanase, nmoles p-nitrophenol released/min/ml.

Two clones were selected at random, one from each transformation and the CenA and Cex activities were quantitated (see Table 9). The activities were also localized, (see Table 10) and again no CenA or Cex activity could be detected in the culture supernatants.

Ten clones were selected at random to test if they could still grow up on medium containing cellobiose as the carbon source. The clones were grown in 5ml M9 media (where the 0.2% glucose had been replaced with 1% cellobiose solution) overnight at 30°C. All ten clones grew up in this medium. In the next stage these clones were tested for growth on CMC containing medium (M9 medium, with 0.1% CMC as the carbon source replacing glucose). The original host strain, E. coli TW34, was also tested for growth on this medium. All eleven cultures were streaked heavily onto the M9 0.1% CMC Kan plates and incubated overnight at 30°C . None of the eleven strains grew up on the CMC medium, even after seven days incubation at 30°C. It was then decided to try growing the ten clones and the control strain on M9 media containing 0.1% CMC and 0.05% cellobiose to help start the growth of the E. coli cells. However, after a week's incubation on this medium at 30°C, no significant growth of any of the strains was observed.

It is known that secretion of the cellulases is essential for their degradative action on cellulose. The fact that CenA and Cex are not being exported to the cells' exterior in these E. coli Cel^+/pND_3 strains probably helps explain why these cells are unable to grow on medium containing CMC (see discussion).

TABLE 9:QUANTITATION OF ENZYME ACTIVITIES FROM E. COLI CEL+/PND3 CLONES

CLONE	DNS U/mg protein	pNPC U/mg protein
# 1	9.30	1.2
# 2	10.5	1.3

Units for the DNS assay, nmoles glucose equivalents released/min. and for the pNPC assay, nmoles p-nitrophenol released/min.

TABLE 10:LOCATION OF ENZYME ACTIVITIES IN E. COLI CEL+/PND3 CLONES

	ACTIVITY IN nMOLES PRODUCT/MIN/ML OF CULTURE			
ENZYME ASSAYED	IN PERIPLASMIC FRACTION	IN CYTOPLASMIC FRACTION	IN CELL EXTRACT	% RECOVERY
ENDO.CLONE #1	2.90 (47)	2.48 (40)	6.20	87
CLONE #2	2.90 (44)	2.78 (41)	6.80	85
EXO.CLONE #1	0.47 (52)	0.32 (36)	0.90	88
CLONE #2	0.45 (48)	0.36 (37)	0.98	85
ALK. PHOSPH.#1	0.55 (77)	0.14 (20)	0.71	97
CLONE #2	0.61 (73)	0.18 (22)	0.83	95
ß-GAL.CLONE #1	0.47 (15)	2.30 (74)	3.10	89
CLONE#2	0.51 (19)	2.14 (74)	2.90	93

Figures in brackets give % of total activity present in the fraction. Units of activity:the endoglucanase activity, nmoles glucose equivalents released/min/ml culture; for the exoglucanase activity, nmoles p-nitrophenol released/min/ml culture; for the alkaline phosphatase activity, nmoles p-nitrophenol released/min/ml culture; and for the ß-galactosidase activity, nmoles o-nitrophenol released/min/ml culture.

SECTION SIX: VISCOMETRIC STUDIES ON E. COLI C600 pND_3 CLONE #11.

Cell extracts of E. coli C600/pND_3 clone #11, C600/pcEC2 and C600/pEC1 were made from 30ml cultures grown overnight at 30°C. The rates of hydrolysis of 4ml 4% CMC by 1ml of cell extract was then assayed using a Cannon-Fenske viscometer (see Figure 12). Specific fluidity was calculated using the formula:

$$\frac{1}{S_p} = \frac{t}{t_o} - 1$$

where t = time taken for the liquid meniscus to pass mark B. The real incubation time = t_x (time at start of viscosity measurement + $\frac{t\ (\text{viscosity})}{2}$

t_o = time for the solvent, without any enzyme or CMC added, to pass through the markers.

$$\text{Specific fluidity , } \phi_{sp} = \frac{1}{\frac{1}{S_p}}$$

See Figures 13 and 14 for the results of the viscometric assays. The graphs show how the cell extract from the E. coli C600 clone #11 behaves more like the cell extract from the cells harboring the *cenA* gene alone (E. coli C600/pcEC2).

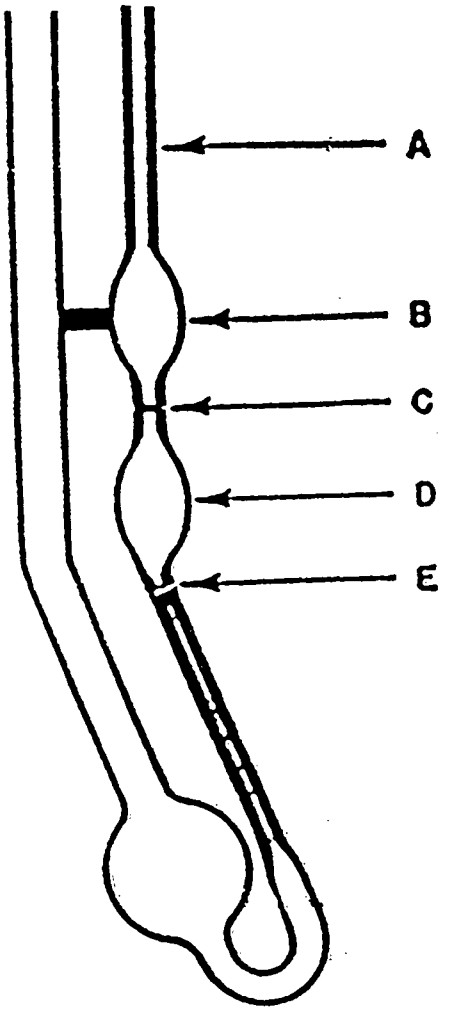
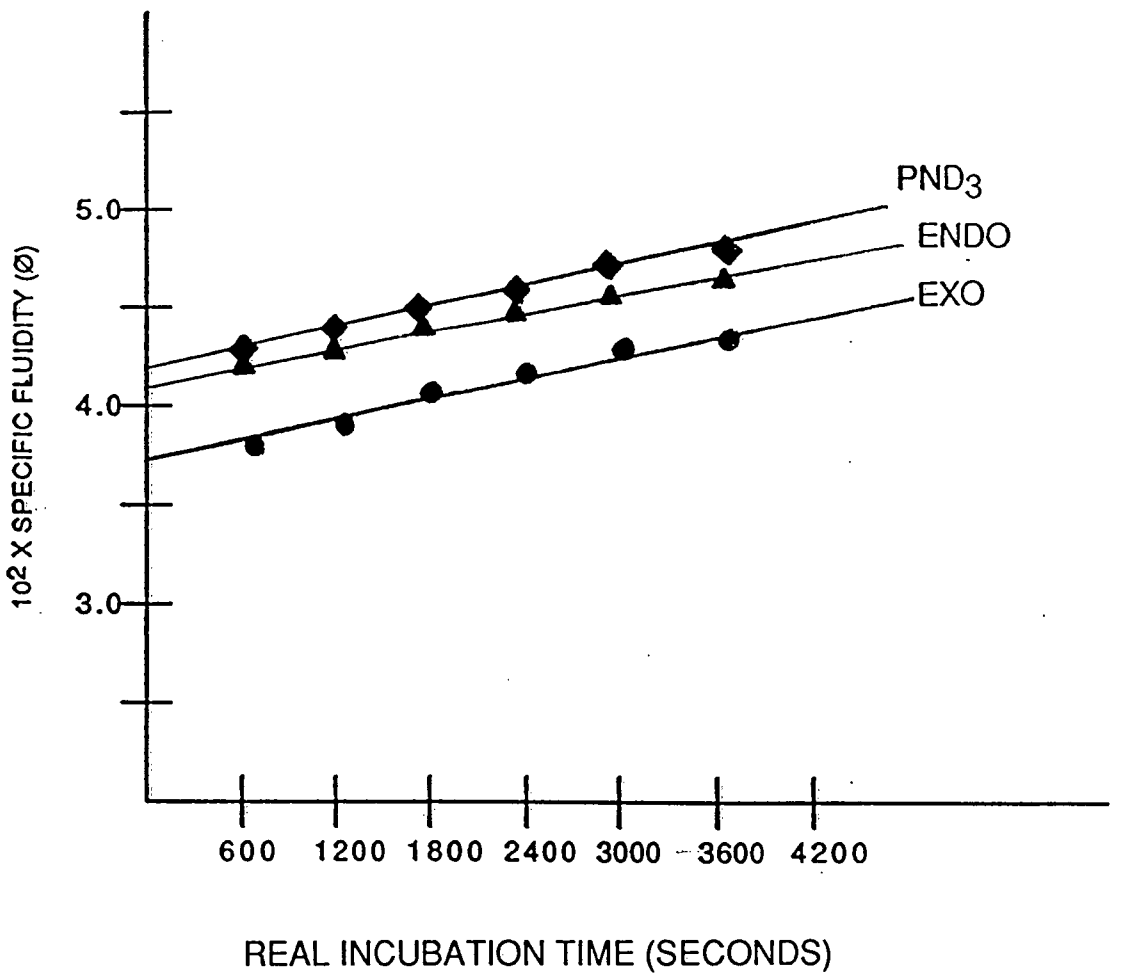


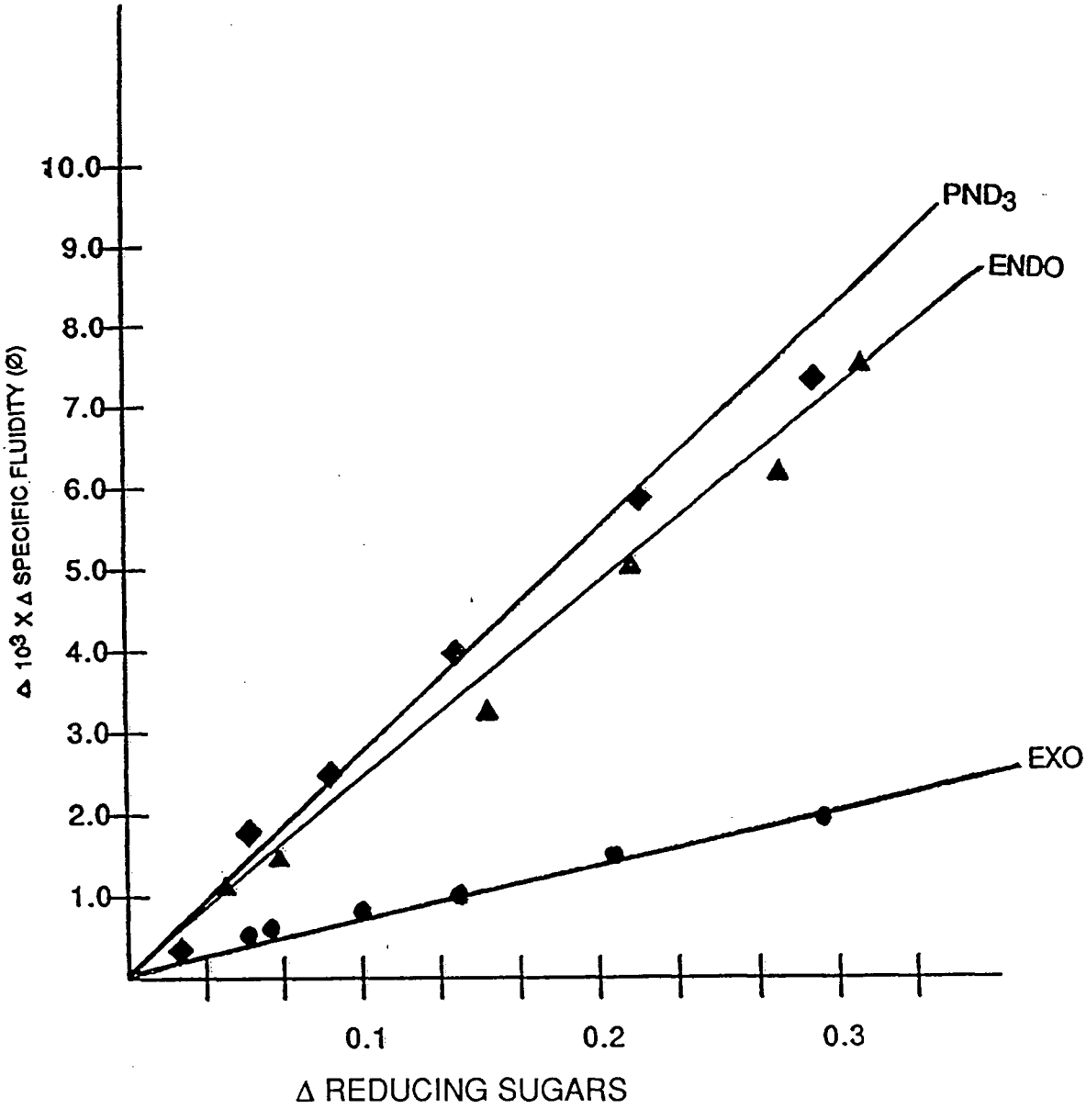
FIGURE 12: Cannon-Fenske viscometer. At time zero, 12ml 4% CMC (Sigma low viscosity grade) in 50mM K phosphate pH7.0 and 0.02% NaN_3 were mixed with 3ml of enzyme or crude cell extract. Five ml of this mix was then transferred immediately to the viscometer and the liquid was drawn up to past mark C (into the bulb B) by attaching a pipette pump onto A. At 10 minutes, the time (t) was taken for the liquid meniscus to pass mark E. Similar readings were taken every 10 minutes for 60 minutes. At each time point, 0.75ml were removed from the remaining mix to determine the reducing sugars present (using the DNS assay).

FIGURE 13: SPECIFIC FLUIDITY AGAINST REAL INCUBATION TIME.



The curves represent results of viscometric assays carried out on cell extracts of E. coli C600 cells containing the plasmid pND_3 ('pND_3') curve , the plasmid pcEC2 ('Endo' curve) or the plasmid pEC1 ('Exo' curve).

FIGURE 14: Δ SPECIFIC FLUIDITY AGAINST Δ REDUCING SUGARS



The curves represent results of viscometric assays carried out on cell extracts of E. coli C600 cells containing the plasmid pND_3 ('pND_3') curve , the plasmid pcEC2 ('Endo' curve) or the plasmid pEC1 ('Exo' curve).

SECTION SEVEN: REPRESSION AND INDUCTION OF ENDOGLUCANASE AND EXOGLUCANASE ACTIVITIES IN E. COLI STRAINS CARRYING THE PLASMID pND_3.

In order to test that the cenA and the cex genes are in fact under the control of the tet promoters from the transposon Tn10, the E. coli strain M8820/pR751::Tn10, which has Tn10 inserted into the plasmid pR751, was transformed with the plasmid pND_3. The trans-acting *tetR* gene product from the Tn10 would then, hopefully, recognize the operator sites, OL and OR, on the Tn10 tet region of plasmid pND_3 and hence repress the transcription of both the *cenA* and the *cex* genes. The addition of tetracycline or the non-inhibiting tetracycline derivative, 2-acetyl-2-decarboxyamide Tc, would then induce the transcription of these genes by binding to the repressor molecules and reducing their affinity for the operator sites.

The plasmid pR751 carries resistance to trimethoprim (Tm) and so the E. coli M8820/pR751 transformants were selected for on LB Kan Tm (25μg/ml) plates. Transformants were checked for endoglucanase and exoglucanase activities on LB Kan Tm CMC and LB Kan Tm MUC plates respectively. Of the 150 transformants screened, all were positive for both activities. When the levels of the endoglucanase and the exoglucanase activities were quantitated using DNS and pNPC assays, it was found that the activities were lower compared to those levels obtained from E. coli C600/pND_3. However it is probably not valid to compare the activities obtained from these two different host strains (see discussion).

One clone was selected at random and grown up in 5ml of LB Kan Tm overnight at 30°C. Two 1 litre flasks containing 500ml LB Kan Tm were inoculated with 0.1ml of the overnight culture and the time of addition and O.D.600 readings, after addition of the inoculum, were noted. The flasks were then placed in a shaking water bath at 30°C. At regular time intervals, 6 ml of culture were removed from each flask . One ml of this was used to take O.D.600 readings and the rest was used for making cell extracts. At a given

time point during the exponential phase of growth (see Figure 15) 2μg/ml of the inducer (2-acetyl 2-decarboxyamide Tc) was added to one of the flasks. Six ml samples, for both O.D.600 readings and cell extracts were removed as before but this time at shorter time intervals, up to 50 mins after the addition of the inducer.

DNS and pNPC assays were carried out on the cell extracts to measure the levels endoglucanase and exoglucanase activities at each time point (see Figures 16 and 17). The results obtained indicated that the addition of the inducer did not seem to have any effect on the levels of the enzyme activities compared to the levels obtained from the control culture. There were several possible reasons. The inducer itself may not have been functioning; it is a light-sensitive compound and so could easily have been inactivated before use. Another possibility is that there was no repression of the endoglucanase and exoglucanase activities in the original E. coli M8820/pR751::Tn10 transformants and that the expression of the cenA and the cex genes was simply low in this host strain compared to the levels obtained from the E. coli C600 clone #11. The lack of repression could be because Tn10 was carried on a low copy number plasmid (pR751) so that the levels of repressor molecules produced were insufficient to repress the transcription of the cenA and the cex genes on plasmid pND_3.

The E. coli strain JC10240 (42), with Tn10 on its chromosome, was used in a second set of repression/induction experiments. The E. coli cells were transformed with pND3, and transformants were selected on LB Kan plates. One hundred transformants were screened for endoglucanase/exoglucanase activities, and of the 100 screened all were double positives. One clone (#2) was selected at random and 0.1ml of an overnight culture was used to inoculate two one litre flasks containing 500ml of LB Kan. The time of the inoculation and the O.D.600 readings after the addition of the inoculum were noted from both flasks. The flasks were then placed in a shaking water bath at 30°C. As in the first experiment, six ml samples were removed from each flask at regular time intervals for O.D.600 readings and for preparing cell extracts. At a given time point during the exponential phase (see Figure 18), 2μg/ml of tetracycline was added to one of the flasks. Six ml samples were removed as before from each flask at five minute intervals, for up to 40 minutes after the addition of the inducer. Carrying out pNPC

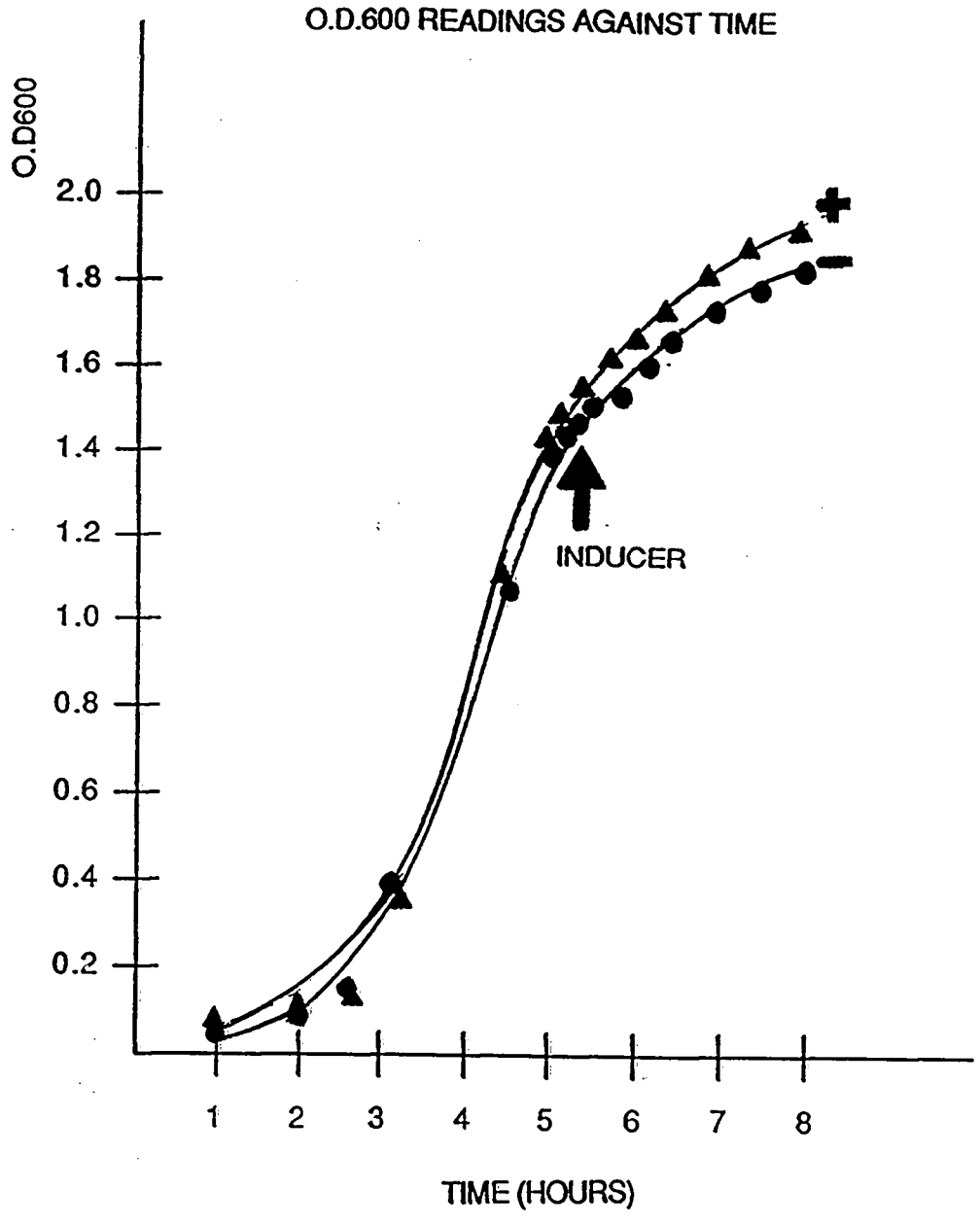


FIGURE 15: Growth curves of E. coli M8820/pR751::Tn10, pND_3 cultures. The plus (+) and minus (-) signs indicate whether the inducer (2-acetyl 2-decarboxyamide Tc) was added to the growing culture or not. The arrow indicates the time of addition of the inducer (2-acetyl 2 decarboxyamide Tc) to the plus (+) culture.

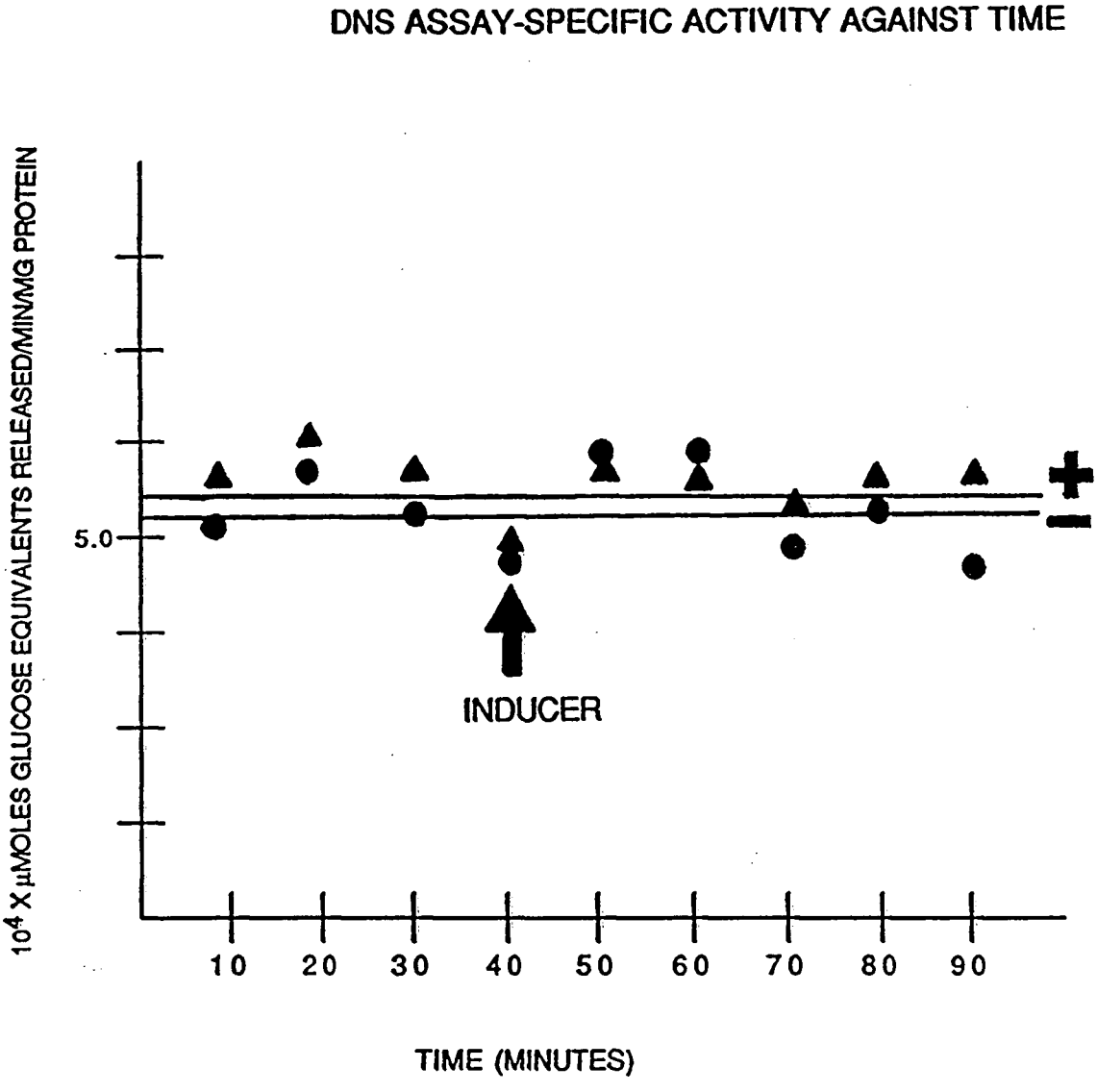


FIGURE 16: Results of DNS assays carried out on cell extracts of 5ml samples removed from the growing cultures of E. coli M8820/pR751::Tn10,pND$_3$. The arrow indicates the time of addition of the inducer (2-acetyl 2-decarboxyamide Tc) to the plus (+) culture.

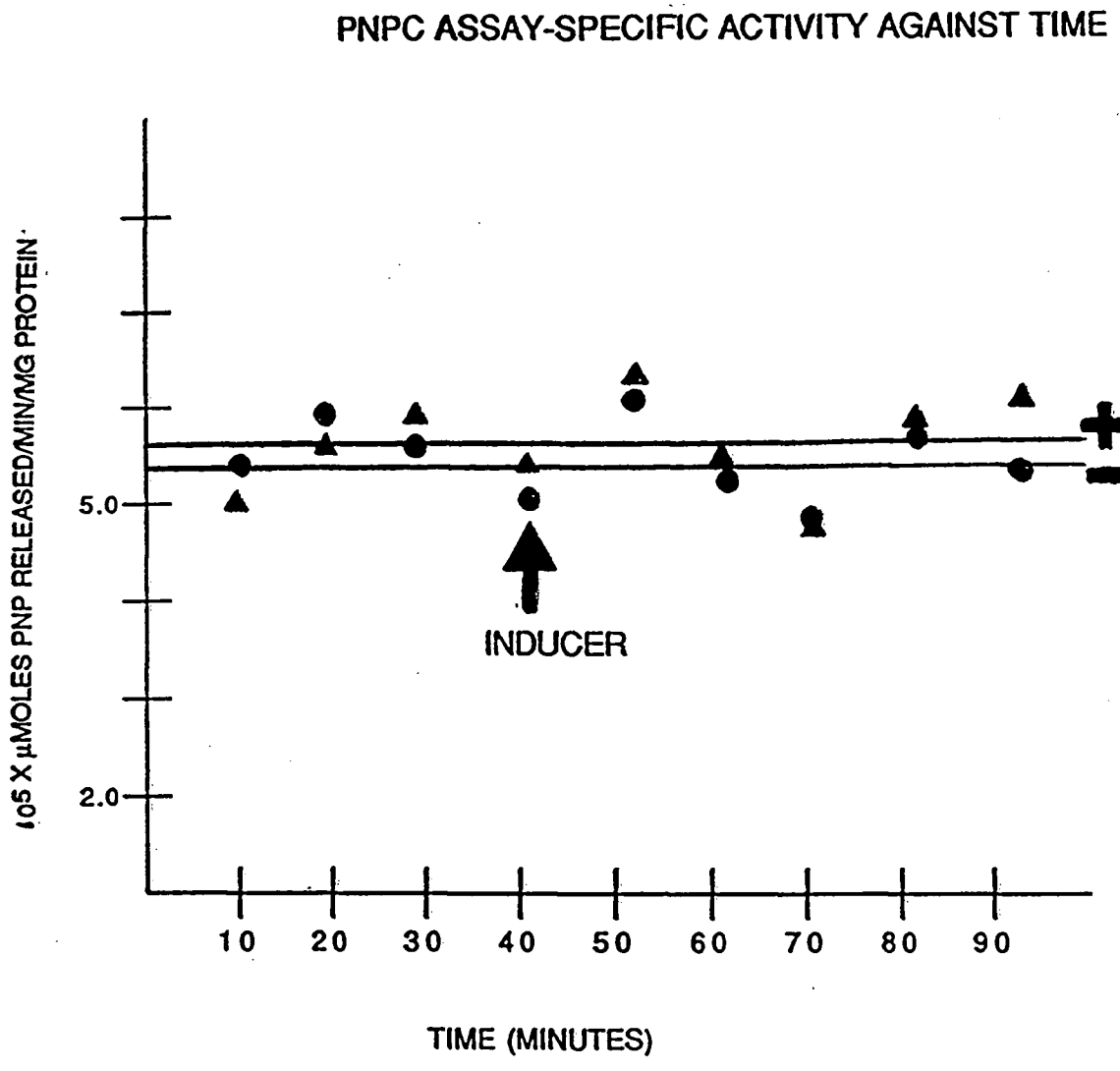


FIGURE 17: Results of pNPC assays carried out on cell extracts of 5ml samples removed from the growing cultures of E. coli M8820/pR751::Tn10,pND$_3$. The arrow indicates the time of addition of the inducer (2-acetyl 2 decarboxyamide Tc) to the plus (+) culture.

and DNS assays on the crude cell extracts showed that induction of both endoglucanase and exoglucanase activity occurred 5 minutes after the addition of the tetracycline (see Figures 19 and 20).

As additional evidence that induction of enzyme activities was occurring after the addition of the tetracycline, two flasks containing 100ml of LB Kan were inoculated with a single colony of clone #2, and 2μg/ml of tetracycline was added to one of the flasks. The cultures were then incubated overnight in a shaking water bath at 30°C and cell extracts of the two cultures were then prepared and used in DNS and pNPC assays. The results of these assays are given in Table 11.

The results showed that the *cenA* and the *cex* genes were indeed under the control of the *tet* promoters from Tn10 (see discussion).

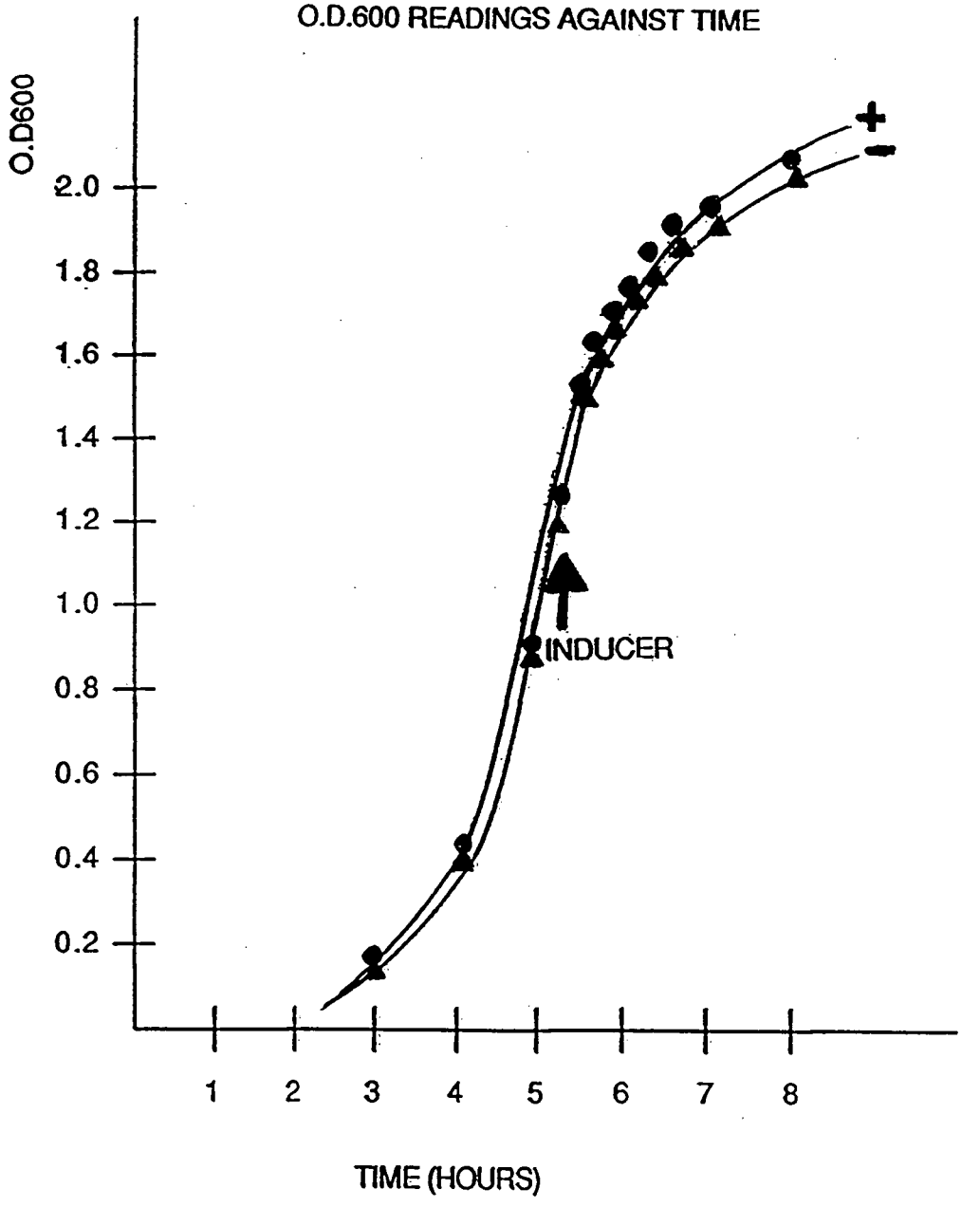


FIGURE 18: Growth curves of E. coli JC10240/pND$_3$ cultures. The plus (+) and minus (-) signs indicate whether the inducer (tetracycline) was added to the growing culture or not. The arrow indicates the time of addition of the inducer to the plus (+) culture.

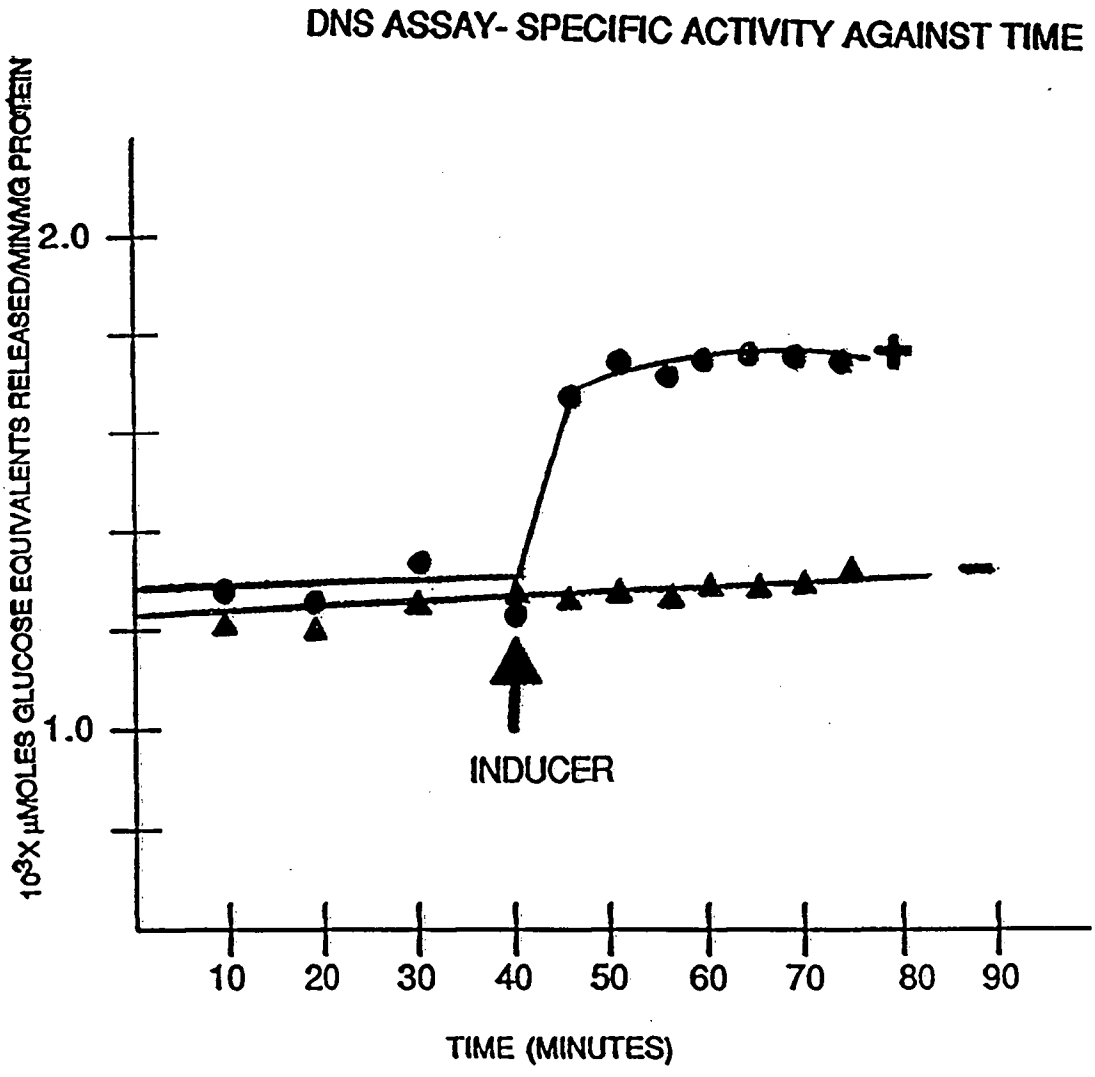


FIGURE 19: Results of DNS assays carried out on cell extracts of 5ml samples removed from the growing cultures of E. coli JC10240/pND$_3$. The arrow indicates the time of addition of the inducer (tetracycline) to the plus (+) culture.

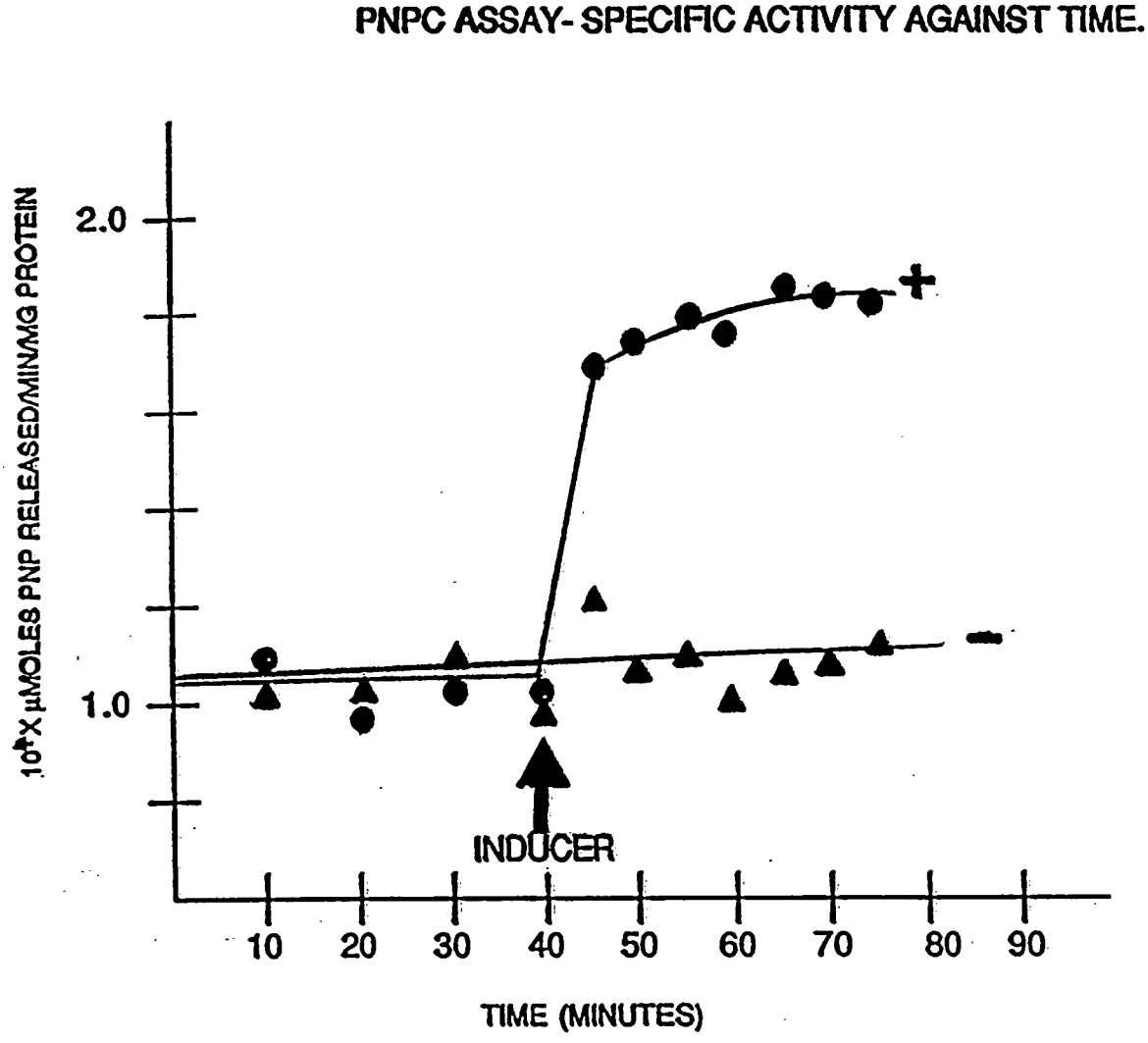


FIGURE 20: Results of pNPC assays carried out on cell extracts of 5ml samples removed from the growing cultures of E. coli JC10240/pND$_3$. The arrow indicates the time of addition of the inducer (tetracycline) to the plus (+) culture.

TABLE 11:QUANTITATION OF ENZYME ACTIVITIES FROM E. COLI JC10240/PND$_3$ CLONE #2 .

	DNS U/mg protein	pNPC U/mg protein
+ TETRACYCLINE	5.2	0.64
- TERACYCLINE	3.0	0.36

Units for the DNS assay, nmoles glucose equivalents released/min. and for the pNPC assay, nmoles p-nitrophenol released/min.

DISCUSSION

The broad host range plasmid pND_3 constructed in this project has been successfully maintained in four different Gram-negative organisms and has allowed the simultaneous expression of the *cenA* and the *cex* genes in these host organisms. Placing the C. fimi genes in non-cellulolytic organisms has also allowed us to examine the effects of heterologous expression on these genes.

The levels of expression of the *cenA* and the *cex* genes in these four organisms varies, as might be expected. Several factors could be influencing the accumulation of the CenA and Cex proteins. These include transcriptional and translational efficiency, growth rate of the cells, and the sensitivity of the products to proteases present in the different host organisms.

The levels of enzyme activity from E. coli C600 clone #11 were comparable to those obtained when *cenA* and *cex* were introduced separately into E. coli C600 cells on plasmid pBR322 (26,27). The levels of endoglucanase and exoglucanase activities obtained from the E. coli Cel^+/pND_3 clones were lower than those obtained for the E. coli C600 clone #11 by a factor of two, but approximately the same percentage of each activity was present in the periplasm of the E. coli Cel^+/pND_3 clones as was found in the periplasm of the E. coli C600 clone #11. The levels of CenA and Cex enzyme activities were much lower in the K. pneumoniae host strain used, suggesting that the expression of the *cenA* and the *cex* genes were more strongly affected by one or more of the factors discussed above.

The R. capsulatus recombinant clones gave exoglucanase activities which were comparable to those obtained from E. coli C600 clone #11, but the endoglucanase activity was lower. In addition CenA was not exported to the periplasm of the cells, implying that the *cenA* leader sequence was not recognized in the R. capsulatus cells, or that the protein was degraded by proteases present in the periplasm of the cells. The Cex leader peptide is 10 amino acids longer than that of CenA, and has more positive charge at its N-terminus. These properties may play a role in exporting Cex more efficiently in this organism.

R. capsulatus has been widely used in various biochemical and genetic studies but little is known about the factors that affect gene expression in this species. Broad host range plasmid vectors were constructed for expression of heterologous genes in R. capsulatus. These utilized an RK2 derived replicon and the *rxcA* promoter to obtain transcription of genes within appropriately positioned DNA fragments (43). The expression vectors were used to obtain the individual synthesis of CenA and Cex proteins. The cellulase genes were expressed either from their native translation signals or from the *rxcA B870ß* gene translation initiation signals to form a hybrid protein. R. capsulatus cultures containing the expressed cellulase genes could not be grown using CMC as a sole carbon source, though significant amounts of cellulase activity were found in extracts of such cells (43).

None of the four host organisms used in the present study secreted CenA and Cex to the culture medium. Presumably in these Gram-negative hosts, the outer membrane acts as a permeability barrier to the release of proteins to the external medium. The excretion of the cellulases is essential for their degradative action on cellulose, hence mutants of these organisms which leak the cellulases would be desirable. Leaky mutants have defects in the outer membrane, thereby allowing the diffusion of exported polypeptides from the periplasm into the surrounding medium. A leaky mutant strain of E. coli which leaked cellulases was previously isolated in this lab (44).

Before leaky mutants are isolated, it might be necessary to investigate whether the host organisms are in fact able to survive on cellulose which has been pretreated to break it down into smaller subunits. Pretreatment could include using acid swollen cellulose or even enzymatically cleaved cellulose (i.e. treated with CenA and Cex proteins).

The results of the viscometric assays indicate that the cell extracts from E. coli C600 clone #11 (carrying the plasmid pND_3), gave CMC hydrolysis rates which were similar, if not identical, to those obtained from extracts of cells carrying the *cenA* gene alone (on plasmid pcEC2). This is difficult to explain, but one possibility is that, in the construct pND_3, the *cenA* gene is under the control of the stronger promoter ($tetP_A$) in the *tet* control region,

whereas the *cex* gene is under the control of the weaker promoter (*tetP*$_R$). Under maximally inducing conditions, the *tet A* promoter (*tetP*$_A$), has been reported to be 7-11 times more active than the *tet R* promoter, whereas under repressing conditions, the basal level of transcription from *tet P*$_R$ is more nearly equal to that from *tet P*$_A$ (45). Further viscometric experiments on the effect of mixing CenA and Cex in varying ratios, are required before firm conclusions can be made from the viscometric assays carried out on the cells carrying the plasmid pND$_3$.

The results of the repression/induction experiments carried out on the E. coli JC10240/pND$_3$ clone #2 showed that the *cenA* and the *cex* genes are indeed under the control of the *tet* promoters from Tn10. The levels of induction of CenA and Cex activities were not very high, being about a 30% and 50% increase for the CenA and Cex activities respectively. There could be a number of reasons for this. A major one being that the intact Tn10 was present in the E. coli JC10240/pND$_3$ clone #2, thus the tetracycline resistance protein, TetA, would have been present and functioning in the host cells. Tn10 is thought to mediate resistance to tetracycline by causing active efflux of the antibiotic. Hence, once the resistance mechanism starts to function we would expect a decrease in the concentration of tetracycline inside the cells, which could explain why the induction of both CenA and Cex decreases 5 minutes after the addition of the tetracycline. The modest level of induction might also be a result of the low level of repression in E. coli JC10240 which carries *tetR* on the chromosome as a Tn10 insertion. We may have obtained greater levels of repression if the TetR was on a high copy number plasmid and would then perhaps have seen greater levels of induction of the two proteins on addition of the tetracycline. Therefore a better repression and induction system may be to have the *tetR* gene alone carried on a high copy number plasmid and to use the non-inhibitory tetracycline derivative 2-acetyl 2-decarboxyamide Tc.

In contrast to E. coli, yeast and certain Gram-positive bacteria are better host organisms for the expression and secretion of cellulases because they actively secrete a variety of proteins into the culture medium. For these reasons it would be of interest to transfer the *cenA* and the *cex* genes to these organisms. An added

advantage of the plasmid pND_3 is that the *cenA* and the *cex* genes, along with the *tet* promoter region, can be excised from the plasmid as a cartridge (using unique flanking restriction enzyme sites) and cloned into another system quite readily. Hence we could clone this cartridge into the plasmid pLS1(46), for example, which is a broad host range shuttle vector transferable between Gram-negative and Gram-positive organisms. This would then allow us to investigate the expression of the *cenA* and the *cex* genes in Gram-positive bacteria.

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