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Repressor titration: a novel system for selection and stable maintenance of recombinant plasmids

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ABSTRACT

The propagation of recombinant plasmids in bacterial hosts, particularly in *Escherichia coli*, is essential for the amplification and manipulation of cloned DNA and the production of recombinant proteins. The isolation of bacterial transformants and subsequent stable plasmid maintenance have traditionally been accomplished using plasmid-borne selectable marker genes. Here we describe a novel system that employs plasmid-mediated repressor titration to activate a chromosomal selectable marker, removing the requirement for a plasmid-borne marker gene. A modified *E.coli* host strain containing a conditionally essential chromosomal gene (*kan*) under the control of the *lac* operator/promoter, *lac*O/P, has been constructed. In the absence of an inducer (allolactose or IPTG) this strain, DH1*lackan*, cannot grow on kanamycin-containing media due to the repression of *kan* expression by LacI protein binding to *lac*O/P. Transformation with a high copy-number plasmid containing the *lac* operator, *lac*O, effectively induces *kan* expression by titrating LacI from the operator. This strain thus allows the selection of plasmids without antibiotic resistance genes (they need only contain *lac*O and an origin of replication) which have clear advantages for use as gene therapy vectors. Regulation in the same way of an essential, endogenous bacterial gene will allow the production of recombinant therapeutics devoid of residual antibiotic contamination.

INTRODUCTION

The isolation of bacterial transformants containing recombinant plasmids and subsequent plasmid maintenance are keystones of recombinant DNA technology. Most commonly this is achieved by incorporation of a gene into the backbone of the plasmid that permits selective growth in medium containing antibiotics. For the production of recombinant therapeutics, however, where the goal is to generate a pure biological product in high yield for administration to patients, the use of antibiotics presents three main problems. The first is a loss of selective pressure under intensive culture conditions (e.g. high biomass or continuous culture) due to antibiotic degradation or inactivation leading to product yield reduction. The second is that the inevitable contamination of the product with residual antibiotic is highly undesirable, especially in the case of β-lactam antibiotics, carrying the risk of immune sensitisation and even anaphylaxis in recipients. Finally there is the possible impact of spread of drug resistance after chance gene transfer to environmental organisms and, in particular, pathogens.

A number of antibiotic-free selection systems are available in which the plasmid encodes a gene complementing a host auxotrophy. For example, a mutant host which is unable to synthesise an essential amino acid can be complemented with a plasmid carrying the gene which provides for its synthesis (1). However, this approach seriously limits the composition of the growth medium since the amino acid must be omitted, thereby limiting improvements to productivity which can be achieved through the manipulation of rich complex media. A variation on this approach uses a mutant *Escherichia coli* strain with a thermosensitive aminoacyl-tRNA synthetase gene (*valS*), with the wild-type *valS* on a pBR322-derived plasmid, thus allowing plasmid maintenance at temperatures non-permissive for the chromosomal mutation (2). This has the advantage of allowing selection in rich complex culture media. Some systems require only the expression of a plasmid-borne tRNA gene to effect a selection pressure. For example, the complementation of nonsense mutations in essential chromosomal genes by expression of a mutant suppressor tRNA which will restore faithful transcription (3). Alternatively, the survival (or growth advantage) of plasmid-free segregants can be prevented by placing a lethal gene (or gene which confers a metabolic burden) in the host chromosome and including a corresponding repressor system in the plasmid (4). Nevertheless, in some of these methods, plasmid-free segregants may continue to grow due to leakage of the selective gene product into the media from plasmid-bearing cells. In recombination-proficient hosts ($RecA^+$) there is also the possibility of homologous recombination between the gene used as a selectable marker and the chromosome, resulting in a loss of selection pressure.

Despite the wide choice of selection mechanisms, *all* current systems suffer from the disadvantage that they require plasmid-borne gene transcription and, in most cases, subsequent translation

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into protein. This has two consequences. The first, of general significance, is that expression of a marker gene on a high copy number plasmid will impose a metabolic burden on the host and could reduce product or biomass yield. The second, which is relevant to gene therapy, is that selectable marker genes may be cryptically expressed in recipient cells, reducing the efficiency of the therapy either as a result of alteration of gene expression (5) or through the induction of an immune response (6). Even in the absence of marker gene expression, there are short immunostimulatory DNA sequences (ISS) present on plasmid DNA backbones which contain CpG dinucleotides (7). There is, therefore, a need for a method of plasmid selection which does not require the presence of plasmid-borne bacterial genes.

We reasoned that it may be possible to achieve this by using the molar excess of plasmid over chromosomal genomes to competitively titrate a repressor from a *host* selectable gene, i.e. to use the plasmid molecule itself to activate selection. This system would require (i) that the host strain contains a chromosomal gene encoding a product essential to cell survival or growth (under the conditions used for culture of that host strain in the laboratory), (ii) that the gene is negatively regulated by a repressor protein such as LacI, (iii) an intracellular repressor concentration just sufficient to achieve repression of this gene, (iv) that the plasmid contains a binding site for the repressor and (v) that the plasmid copy number per cell was sufficient to achieve repressor titration.

Here we demonstrate the derepression of the lactose operon and plasmid maintenance by repressor titration, and report the construction of a model system to allow plasmid selection by repressor titration. The model system uses a kanamycin resistance gene integrated into the *E.coli* chromosome under the control of the lactose operator (O_1 and O_3) and promoter, *lac*O/P, as the gene essential for growth in kanamycin-containing medium. All plasmids used to demonstrate repressor titration contain the O_1 and O_3 sequences.

MATERIALS AND METHODS

Derepression of the *lac* operon by repressor titration

Escherichia coli strain DH1 was transformed with pUC18Tet, which has the *tet* gene removed from pBR322 as an *Eco*RI–*Pvu*II fragment and cloned into pUC18 (which possesses the *amp* gene, encoding β-lactamase). Single colonies from both transformed and untransformed plate cultures were inoculated into M9 minimal salts medium supplemented with thiamine (0.5 µg/ml) and either lactose (10 mM) or glucose (10 mM) as carbon sources, and ampicillin (100 µg/ml) where appropriate. Cells were grown to mid-log phase, harvested and assayed for β-galactosidase activity (8).

Plasmid maintenance by repressor titration

Escherichia coli strain Hfr 3000 YA694 (*lacI694*, *relA1*, *spoT1*, *thi-1*, λ) has the $lacI^s$ genotype, expressing a mutant repressor protein which is inducer insensitive (9). Therefore the *lac* operator of a $lacI^s$ mutant can only be derepressed by repressor titration. YA694 was transformed with pUC18 and inoculated into M9 minimal medium supplemented with thiamine, glucose and ampicillin. It was grown at 37°C for 14 h, then 0.5 ml was inoculated into 100 ml of M9 medium supplemented with thiamine and (i) lactose and ampicillin; (ii) lactose; (iii) glucose. These cultures were grown for 8 h and at the end of this period 2 OD_{600} units were harvested and frozen, and 0.5 ml of each culture was re-inoculated into 100 ml of fresh respective medium and grown at 37°C for a further 14 h. This procedure was repeated, resulting in sampling at ~15, 36, 55 and 72 cell generations. Plasmid DNA was then extracted from the harvested cells, restricted with *Eco*RI and analysed by agarose gel electrophoresis (Fig. 2).

Construction of repressor titration model strain

To develop the model system for plasmid selection by repressor titration, the *kan* gene derived from pUC4K (Pharmacia) was placed under the control of the pUC18 *lac*O/P. The *Xho*I–*Pst*I fragment containing *kan* was digested from pUC4K. *Xho*I restriction removed the promoter and the sequence coding for the first 10 amino acids of *kan*. pUC18 was restricted with *Sal*I and *Pst*I, and *kan* was ligated into this construct, creating an in-frame fusion between the sequence coding for the first 17 amino acids of *lacZ* and the truncated *kan*. The expression of *kan* was now under the control of the *lac*O/P. The *lackan* fusion was excised on a *Hae*II fragment, blunted and cloned into *Sty*I-linearised, blunted pN1 (10) such that it was flanked on both sides by *dif* locus chromosomal DNA homology forming the plasmid pN1*lackan* (Fig. 3). pN1*lackan* was linearised with *Sal*I, and used to transform calcium-competent JC7623 cells [*rec*B21, *sbc*C201, *rec*C22, *sbc*B15 (11,12)] by linear transformation (13,14). P1 phage transduction was used to move this chromosomal construct into the *dif* locus of *E.coli* DH1 (*F*$^-$, *supE44*, *recA1*, *endA1*, *gyrA96*, *thi*$^{-1}$, *hsdr17*, *relA1*). To allow this, DH1 was first rendered transiently RecA$^+$ by transformation with the unstable, *recA*-containing plasmid pPE13 (15).

Plasmid selection in the repressor titration model strain

Electrocompetent *E.coli* DH1*lackan* cells were prepared and transformed by electroporation (16) with the plasmids pTX0160 and pTX0160ΔAmp. pTX0160 (7.2 kb) was constructed by insertion of the *E.coli* B/r *ntr* gene (nitroreductase) into a CMV-based expression vector so it was under the control of the CMV immediate-early promoter and thus not expressed in *E.coli* (17), and recloning of the resultant 4.3 kb expression cassette into pBluescript KS+ (Stratagene). pTX0160ΔAmp was then generated by removal of the ampicillin resistance gene by cleavage of pTX0160 with *Bsp*HI and recircularisation of the larger of the two fragments generated. Transformants were selected from single colonies on LB kanamycin agar plates and grown in LB broth cultures with kanamycin, and cryopreserved in 20% glycerol. Untransformed and plasmid-containing DH1*lackan* were streaked directly from the cryopreserved cultures onto LB agar plates using an inoculating loop. The following were added to the media where appropriate: kanamycin sulphate (30 µg/ml), ampicillin (100 µg/ml) and isopropylthio-β-D-galactoside (IPTG; 23.1 µg/ml). Plates were incubated at 37°C for 16 h and photographed (Fig. 4). Rapid plasmid DNA extraction and agarose gel electrophoresis (18) confirmed that the transformed clones tested had maintained plasmids of the correct size, while untransformed DH1*lackan* contained no plasmid (data not shown).

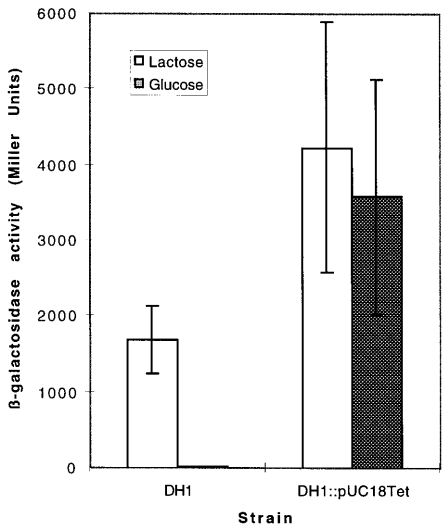

Figure 1. Derepression of the *lac* operon by repressor titration. Expression of β-galactosidase in *E.coli* DH1 in the presence and absence of pUC18Tet when grown under conditions of induction (supplemented with lactose) and repression (supplemented with glucose). Results are the mean of three independent experiments with the standard error displayed on error bars, and are expressed as activity units.

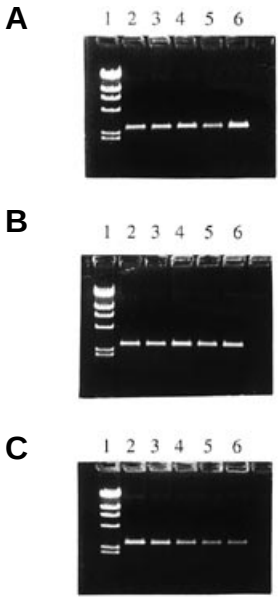

Figure 2. Stable plasmid maintenance by repressor titration. *Escherichia coli* YA694::pUC18 was grown on minimal medium supplemented with glucose and ampicillin, then inoculated into minimal media containing (**A**) lactose and ampicillin, (**B**) lactose and (**C**) glucose. Plasmid was extracted at intervals over 72 cell generations and subjected to agarose gel electrophoresis. Lane 1 contains *Hin*dIII-cut λ DNA size markers; lanes 2–6 are *Eco*RI-linearised pUC18 isolated after growth for ~0 (inoculum), 15, 36, 55 and 72 generations, respectively.

RESULTS

De-repression of the *lac* operon and plasmid maintenance by repressor titration

The ability of plasmid-borne sequences to titrate repressor away from a chromosomal gene *in trans* was first tested in DH1 (19,20). DH1 possess an intact lactose operon which is negatively regulated by the lactose repressor protein, LacI, which is constitutively synthesised by the cell at the relatively low level of 10–20 molecules per cell (21). LacI binds to the lactose operon operator, *lac*O, with high affinity ($K_d = 1 \times 10^{-14}$) under conditions of repression and prevents transcription of the β-galactosidase (*lacZ*), lactose permease (*lacY*) and transacetylase (*lacA*) genes. Upon de-repression with lactose or a non-metabolisable lactose analogue such as IPTG, or by the presence of multicopy *lac*O (22,23), LacI binds to the chromosomal *lac*O less frequently, permitting transcription. The expression of the operon is easily detected by assaying for β-galactosidase enzyme activity (8).

Untransformed DH1 and DH1 transformed with pUC18Tet (present at ~200 copies per cell) were grown in minimal salts medium as described. Comparable β-galactosidase activities are observed with DH1::pUC18Tet grown on glucose and lactose, whereas very much lower activities are seen with DH1 grown on glucose compared to lactose (Fig. 1). Also, the β-galactosidase activity is significantly greater in DH1::pUC18Tet compared to DH1 with the same sugar. This supports the hypothesis that repressor titration can regulate chromosomal gene expression.

The ability of repressor titration to allow stable plasmid maintenance was demonstrated using the endogenous genes of the lactose operon of *E.coli* YA694 (*lacI*^{s}) as the 'essential' chromosomal genes in minimal media with lactose as the sole carbon source. Plasmid (pUC18) concentrations remain constant over 72 generations with antibiotic selection (Fig. 2A; lactose and ampicillin), and with repressor titration alone (Fig. 2B; lactose). However, plasmid copy number decreased in the absence of selection pressure (Fig. 2C; glucose).

Developing a model system for plasmid selection by repressor titration

A model 'essential' gene under *lac*O control was introduced into the chromosome of *E.coli* DH1 to demonstrate plasmid maintenance in complex medium by repressor titration. DH1 is *recA*$^-$ and a suitable molecular cloning host commonly used for the efficient propagation of recombinant plasmids (20). For the purposes of this example the aminoglycoside 3′-phosphotransferase gene *kan*, conferring kanamycin resistance (24), was cloned such that its expression was under the control of *lac*O/P. This construct was then inserted into a plasmid with *dif* locus homology (Fig. 3), and then into the chromosomal *dif* locus of DH1 by P1 transduction (25). Transformation of this modified strain, DH1*lackan*, with plasmids containing *lac*O titrated the repressor from the antibiotic resistance gene, and allowed expression and growth in the presence of kanamycin (30 μg/ml). Untransformed cells could only grow in medium containing kanamycin in the presence of IPTG.

Demonstrating repressor titration in the model strain

DH1*lackan*, untransformed and containing the plasmids pTX0160 and pTX0160ΔAmp, was streaked onto LB agar plates supplemented with antibiotics and IPTG where required (Fig. 4). All cell lines are able to grow on LB media (Fig. 4A, i and B, i). Untransformed DH1*lackan* cannot grow on media supplemented with kanamycin (Fig. 4A, ii) unless IPTG is added (Fig. 4A, iii). When transformed with the plasmids pTX0160 or

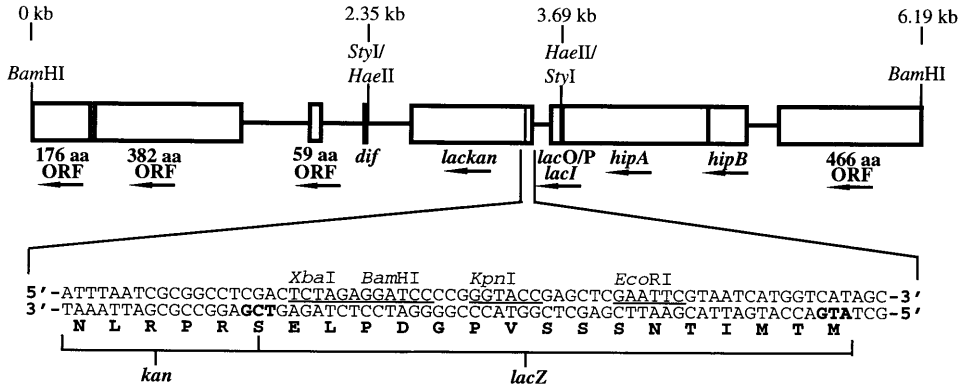

Figure 3. The construct which is cloned into the single *Bam*HI site of pUC18, forming the plasmid pN1*lackan*. The *lackan* fusion is located adjacent to the *dif* sequence and disrupts the C-terminus of the *hipA* ORF. The nucleotide sequence flanking the fusion site between *lacZ* and *kan* is illustrated, with selected restriction endonuclease sites underlined. The fusion protein start codon and the common serine residue are displayed in bold, and the corresponding amino acid sequence is displayed. When linearised, pN1*lackan* was used to introduce the *lackan* fusion into the *dif* locus of the *E.coli* chromosome.

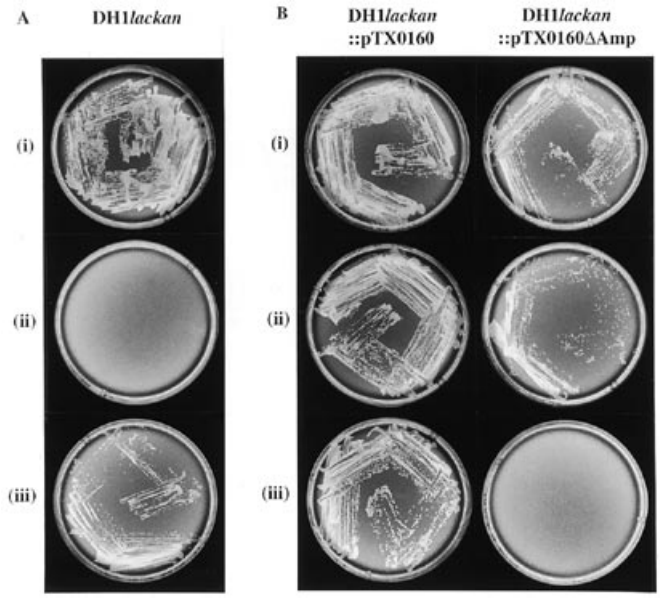

Figure 4. Growth of *E.coli* DH1*lackan* on media containing kanamycin. (**A**) Untransformed DH1*lackan* plated on (i) LB alone (control), (ii) kanamycin and (iii) kanamycin and IPTG. (**B**) DH1*lackan*::pTX0160 and DH1*lackan*::pTX0160ΔAmp, plated on (i) LB alone (control), (ii) kanamycin and (iii) ampicillin.

pTX0160ΔAmp, growth is possible in the presence of kanamycin (Fig. 4B, ii), but only pTX0160, containing the β-lactamase gene, could also be propagated on ampicillin (Fig. 4B, iii). This demonstrates the ability to select DH1*lackan* transformants containing antibiotic resistance gene-free plasmids.

DISCUSSION

The ability of plasmid-borne operator sequences to influence the expression of chromosomally-encoded genes by repressor titration has clearly been demonstrated (Fig. 1). In media supplemented with glucose, β-galactosidase expression occurred at a very low level in DH1 but was significantly increased in DH1::pUC18Tet. Repressor titration was also able to further derepress *lac*O under inductive conditions, as the level of β-galactosidase activity was also greater in DH1::pUC18Tet compared to untransformed DH1 in the presence of lactose. Transformation of DH1 with pUC18Tet thus resulted in the derepression of the lactose operon by LacI titration and demonstrated the possible utility of the repressor titration system.

Subsequently, the ability of such a system to maintain a plasmid in antibiotic-free minimal medium by controlling expression of genes required for sugar utilisation with *lac*O has also been demonstrated. Control of β-galactosidase expression from the lactose operon in LacI^s mutant *E.coli* grown in minimal medium, containing lactose as the sole carbon source, was utilised to stably maintain pUC18 (Fig. 2). Transformation resulted in the ability to grow on lactose and after 72 generations the plasmid yield was higher in cells grown with lactose than in cells grown with glucose. A minimum copy number was maintained permitting production of β-galactosidase, facilitated by repressor titration.

To construct the model repressor titration system, the in-frame *lackan* fusion (1.34 kb) was cloned into pN1 adjacent to the *dif* sequence (26) as shown in Figure 3. A 369 bp *Sty*I fragment, lost when the *lackan* fusion was inserted, contained the C-terminus of the *hipA* gene, which is believed to confer resistance to inhibition of peptidoglycan or DNA synthesis, but its precise physiological role has not been determined (27). This region was chosen as a convenient chromosomal insertion site away from essential genes. The *kan* gene, under the control of *lac*O/P, once inserted into the DH1 chromosome, is made 'essential' when the media is supplemented with kanamycin, as demonstrated by the inability of DH*lackan* to grow in the presence of kanamycin unless induced with IPTG (Fig. 4A). Transformation with a high copy number *lac*O-containing plasmid allows expression of *kan* and growth in presence of kanamycin (Fig. 4B), therefore allowing selection of a plasmid with no antibiotic resistance gene on antibiotic-containing media. In this way we have demonstrated maintenance and manipulation of constructs in the absence of plasmid-borne selectable marker gene expression.

We are currently designing a system where a host cell has been engineered so that a naturally-occurring essential chromosomal gene is placed under the control of *lac*O/P. Growth of such cells occurs only when induced or transformed with *lac*O-containing

plasmids. In this way, simply by successful transformation, antibiotic free plasmid selection and maintenance in rich complex media can be achieved by a transformed cell's ability to survive and grow.

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