

***Escherichia coli* strains that allow antibiotic-free plasmid selection and maintenance by repressor titration**

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ABSTRACT

We report the construction of two novel *Escherichia coli* strains (DH1*lacdapD* and DH1*lacP2dapD*) that facilitate the antibiotic-free selection and stable maintenance of recombinant plasmids in complex media. They contain the essential chromosomal gene, *dapD*, under the control of the *lac* operator/promoter. Unless supplemented with IPTG (which induces expression of *dapD*) or DAP, these cells lyse. However, when the strains are transformed with a multicopy plasmid containing the *lac* operator, the operator competitively titrates the LacI repressor and allows expression of *dapD* from the *lac* promoter. Thus transformants can be isolated and propagated simply by their ability to grow on any medium by repressor titration selection. No antibiotic resistance genes or other protein expressing sequences are required on the plasmid, and antibiotics are not necessary for plasmid selection, making these strains a valuable tool for therapeutic DNA and recombinant protein production. We describe the construction of these strains and demonstrate plasmid selection and maintenance by repressor titration, using the new pORT plasmid vectors designed to facilitate recombinant DNA exploitation.

INTRODUCTION

Antibiotics and antibiotic resistance genes have traditionally been used for the selection and maintenance of recombinant plasmids in hosts such as *Escherichia coli*, but their continued use is undesirable in many areas of biotechnology, such as gene therapy protocols that involve the direct introduction of plasmid DNA into patients. In the USA, federal regulations specifically discourage the use of selection markers that confer resistance to antibiotics in significant clinical use in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes (1). Vectors should also be designed to minimise the amount of bacterial DNA they contain because short immunostimulatory sequences including CpG dinucleotides are present in plasmid backbones (2).

Widespread concern about antibiotic resistance genes in transgenic crops has recently helped to undermine public confi-

dence in genetically modified food. In the UK, the Advisory Committee on Novel Foods declared that the β -lactamase gene (*bla*, conferring resistance to ampicillin) when present in transgenic corn posed an 'unacceptable risk' of transfer to the gut bacteria of livestock eating the corn (3). Where *bla* is present in transgenic crops, it serves no purpose and is a relic from plasmid selection during the cloning process. In their review on horizontal gene transfer, Dröge *et al.* (4) conclude: 'effort should be put into the development of new strategies to remove antibiotic resistance determinants from transgenic constructs'.

In recombinant protein production, the antibiotic resistance gene present on the plasmid normally expresses the resistance enzyme constitutively at an unnecessarily high level. This invariably exerts a metabolic burden on the host cell, and can result in the resistance enzyme, as well as the antibiotic itself, being present as a contaminant in the required product. The removal of *bla* from a therapeutic plasmid has been shown to increase the efficiency of transgene expression 2-fold, and the removal of other vector sequences (including the *lacZ* α -complementation sequence) also resulted in enhanced transgene expression (5). Using a plasmid containing the kanamycin resistance gene *kan* and a recombinant gene of interest, Panayotatos (6) showed that a mutation causing a significant reduction of synthesis of kanamycin phosphotransferase resulted in a doubling of the recombinant protein production.

Existing antibiotic-free selection systems depend upon plasmid-borne gene expression of a protein or a tRNA that can be used in selection, and therefore they suffer from many of the same drawbacks as antibiotic resistance selections. The plasmid selection system that we have developed is based on a novel use of the phenomenon of repressor titration. Repressor titration has been used to identify and study natural operators (7), to isolate operator mutations (8) and to find ideal *lac* operator sequences from eukaryotic genomes (9).

To manipulate repressor titration and effect a plasmid selection pressure, the derepression of a negatively-regulated chromosomal operator/promoter system controlling a conditionally essential gene is achieved using a multicopy plasmid containing the same operator sequence. Under normal conditions, a repressor protein binds to the chromosomal operator and prevents transcription. The repressor is released when it binds to its inducer, which is often the substrate of the gene under control. However, when a molar excess of the operator sequence is present on a multicopy plasmid, it titrates the repressor from the chromosomal operator, allowing transcription to take place.

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We previously described an *E. coli* strain, DH1*lackan*, in which the *lac* operator/promoter (*lacOP*) was used to control expression of *kan* on the *E. coli* chromosome (10). This strain allowed the selection of plasmids that possessed the *lac* operator sequences *O*₁ and *O*₃; these titrated the *lac* repressor allowing *kan* expression. Although DH1*lackan* enabled the selection of plasmids that did not contain antibiotic resistance genes, it was still dependent on kanamycin for the selection of transformants. Here we describe the construction and characterisation of *E. coli* strains DH1*lacdapD* and DH1*lacP2dapD*, which allow antibiotic-free plasmid selection and maintenance in complex media. These strains contain an ectopic copy of a *dapD* gene whose expression is controlled by a *lac* promoter. We demonstrate antibiotic-free plasmid selection and maintenance in these strains using the pORT plasmid vectors that facilitate the exploitation of repressor titration.

MATERIALS AND METHODS

Reagents and media

Standard reagents and kits were obtained from reputable suppliers and suppliers' instructions were followed. Standard molecular biology techniques and microbiological media were as described in Ausubel *et al.* (11). Additions to bacterial growth media were: kanamycin (50 µg ml⁻¹), DAP (80 µg ml⁻¹), ampicillin (100 µg ml⁻¹) and IPTG (0.1 mM).

Genetic manipulations

Escherichia coli strain DH5α (Gibco BRL) was used as a routine cloning host and JC7623 (12) and DH1 (13) were used for transformation by linearised plasmid and P1 transduction, respectively. In the latter case a RecA⁺ plasmid was present in *recA*⁻ DH1 to allow homologous recombination, after which plasmid-free *recA*⁻ segregants of the appropriate genotype were isolated. In principle, the genetic manipulations of the *E. coli* chromosome involved manipulating the appropriate chromosome region inserted into a plasmid (for example, pUC18; 14), modifying it as desired, and then reintroducing the modified DNA by homologous recombination into the chromosome using linearised DNA and strain JC7623, which is *recBC sbcBC* and supports homologous recombination of linear DNA (15). P1 transduction was then used to transfer the required locus to the final strain. Details of all of the constructions are available on request.

DH1Δ*dapD* was deleted for the complete chromosomal *dapD* gene but had all bounding sequences intact. The *dapD* gene was replaced by the *kan* gene of pUC4K (16). The deletion replacement was first made in a recombinant plasmid, which was linearised and used to transform JC7623. P1 transduction was used to transfer the Δ*dapD*::*kan* locus to DH1 containing the RecA⁺ plasmid pPE13 (17). Loss of the plasmid led to RecA⁻ DH1Δ*dapD*.

The ectopic *lac-dapD* locus was inserted into the *dif* region (18,19) of the chromosome of DH1Δ*dapD* using homologous recombination with linear DNA to produce DH1*lacdapD*, containing a wild-type *lac* promoter, and DH1*lacP2dapD*, containing a mutant *lac* promoter that exhibits reduced gene expression. Both these strains were selected by their ability to grow on IPTG plates. However, upon inoculation into LB broth, DH1*lacdapD* was also able to grow without IPTG

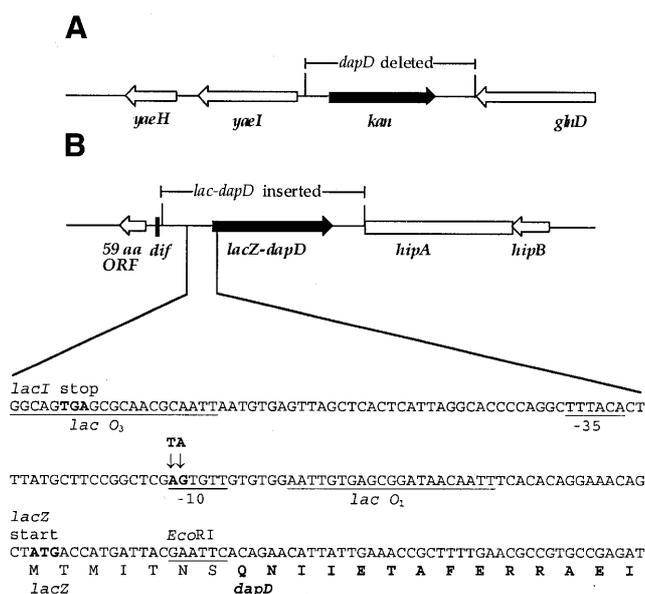


Figure 1. Modifications to the chromosome of DH1*lacP2dapD*. (A) In the *dapD* locus, the *dapD* gene is completely replaced by *kan*. (B) The *lacOP-dapD* fusion is inserted into the *dif* locus, and the nucleotide sequence of the promoter region is shown, including the mutations -11 A→G and -12 T→A that converted DH1*lacdapD* into DH1*lacP2dapD*. The first four amino acids of *dapD* are replaced by the first seven from *lacZ* in the fusion protein.

present. This was due to a very low level of leaky expression from the *lac* promoter providing sufficient *dapD* expression for DAP-independent growth. The genotype of DH1*lacdapD* and DH1*lacP2dapD* is: *recA endA1 gyrA96 thi1 hsdR17 supE44 relA1 Δ(dapD)::kan hipA::lac-dapD*. These two strains differ only in that the former contains the wild-type *lac* promoter and the latter, the 2 nt change in *lacP* to give *lacP2* (Fig. 1).

Construction of the pORT vectors

These were constructed from pBluescript SK(+) (Stratagene) by cleaving at all eight *MaeII* sites and self-ligating the largest fragment (1258 bp). This contained the pMB1 origin of replication, *lacOP* and truncated *lacZ* sequences, but no *bla* gene. This plasmid, pMAEII, was transformed into DH1*lacdapD* and selected by repressor titration on LB agar. Two primers were designed with the 3' ends homologous to regions flanking *bla*, and the 5' ends containing recognition sites for six restriction endonucleases: *AvrII*, *StyI*, *SfiI*, *BglII*, *FseI* and *NaeI* (underlined). Primer AMPSTOP (5'-GGCCGCGCCCGGCTAGGGTCTGACAGTTACCAATGCTTAATC-3') included the *bla* stop codon (bold), and primer AMPKPN1 (5'-GGGGTACCTAGGCCGGCCGGCCGCACTTTTCGGGGAAATGTGCGC-3') contained a *KpnI* site (bold) to determine insert orientation. These were used to amplify *bla* by PCR from pBluescript SK(+). *bla* was then cloned as a blunt fragment into *PvuII*-cleaved pMAEII. Plasmid vectors were produced (2293 bp each) with *bla* in both orientations (pORT1 and pORT2). The six flanking restriction sites were termed 'excision sites' (XSs), and cutting with any one of these enzymes and religating removed *bla* and generated a

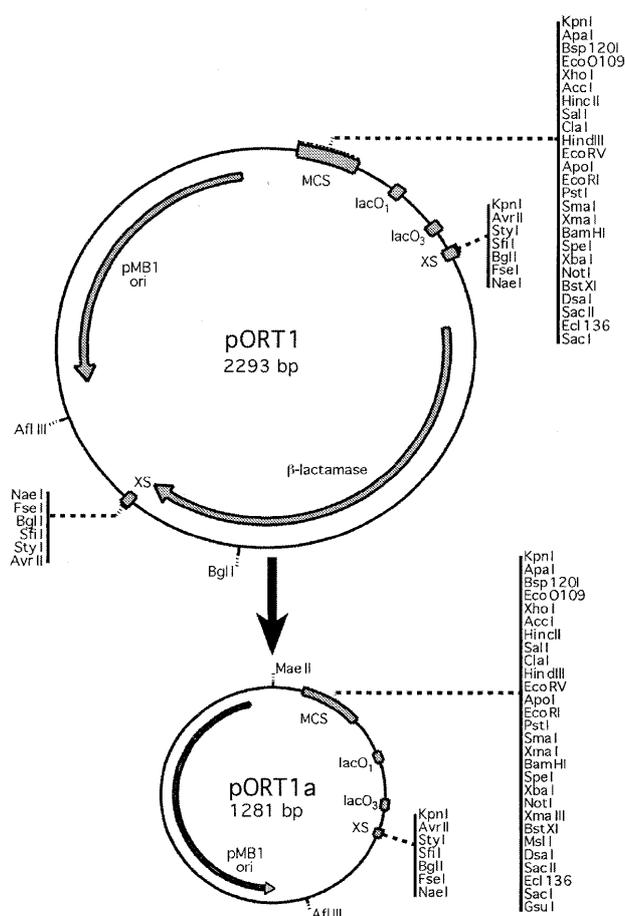


Figure 2. Cloning vectors pORT1 and pORT1a. They contain a large multi-cloning site (MCS) flanked by M13 forward and reverse sequencing primer sites, and a minimal backbone with a pMB1 ori and lacOP. The bla gene in pORT1 is flanked by two XSs, containing directly repeated recognition sites for six restriction endonucleases. Cleaving with any one of these, followed by religation produces the bla-free minimal vector pORT1a, which can be selected by repressor titration. The single XS generated can be used as an alternative polylinker in pORT1a. Indicated restriction sites shown are unique, with the exception of KpnI and those in the XS of pORT1.

single XS. This was done with FseI and the resulting DNA was transformed into DH1lacdapD to generate 1281 bp pORT1a and pORT2a (Fig. 2 shows this for pORT1).

RESULTS

Construction and characterisation of strains suitable for plasmid selection by antibiotic-free repressor titration

In the construction of the repressor titration strains, the first stage was the complete deletion of *dapD* from the chromosome of DH1 to generate DH1 Δ *dapD*. The conditionally essential gene *dapD* is normally located at 0.185 Mb on the *E.coli* chromosome (20). *dapD* encodes tetrahydrodipicolinate *N*-succinyltransferase, which catalyses the conversion of tetrahydrodipicolinate and succinyl-CoA to *N*-succinyl- α -amino- ϵ -keto-pimilate and CoA in the lysine/diaminopimelate

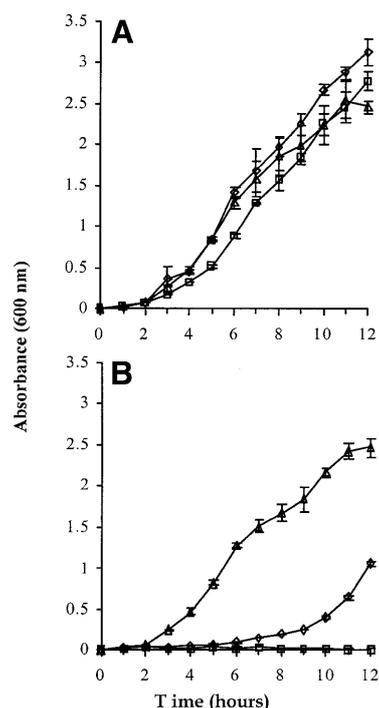


Figure 3. Growth of (A) DH1lacdapD and (B) DH1lacP2dapD. Inoculum cultures were grown overnight in LB broth (with DAP added to the cultures not transformed with pORT1a) and 200 μ l pelleted, washed three times to remove residual DAP and resuspended in 1.0 ml media. This was used to inoculate experimental cultures to an initial optical density of $A_{600} = 0.004$, and cultures were incubated at 37°C with constant agitation. Optical density was measured hourly by absorbance at 600 nm. Squares, no additives; diamonds, +IPTG; triangles, contain pORT1a. Data points are the means and error bars represent standard deviations of triplicate experiments.

pathway (21,22). DAP cross-links peptidoglycan in the bacterial cell wall (23) and is a precursor for lysine biosynthesis (24). Mutants with disrupted *dapD* are therefore DAP and lysine auxotrophs. As lysine is present in the rich media often used to grow *E.coli* (such as LB broth) and DAP is not, *dapD* mutants will undergo cell lysis unless DAP is added to the medium (25).

The first strain constructed (DH1lacdapD) had the endogenous *dapD* gene completely deleted and the ectopic *dapD* under the control of the wild-type *lac* promoter. The *dif* locus was chosen as a suitable ectopic location (1.59 Mb) (20) for the insertion of the *lacOP*-*dapD* fusion, as in the orientation shown there are no genes within 35 kb upstream which could cause transcriptional readthrough and thus *dapD* expression in the absence of induction or repressor titration (Fig. 1). DH1lacdapD grows in liquid culture lacking DAP when uninduced by either IPTG or repressor titration (Fig. 3A), suggesting that basal expression of tetrahydrodipicolinate *N*-succinyltransferase from the *lac* promoter was sufficient to synthesise enough DAP for growth. Nevertheless it was possible to use this strain for selection of plasmids after transformation by repressor titration (see below).

In order to make a strain whose growth in the absence of DAP was dependent on derepression of *lacP*, two mutations

were introduced to reduce *lac* promoter activity and basal expression of tetrahydrodipicolinate *N*-succinyltransferase, yet allowing sufficient expression for good growth after induction by IPTG or repressor titration. The first mutation, $-11 \text{ A} \rightarrow \text{G}$, virtually abolishes expression from *lacP*, but the second mutation, $-12 \text{ T} \rightarrow \text{A}$, shifts the transcription start site to -1 and restores β -galactosidase activity to $\sim 23\%$ that of wild-type in the presence of activated CAP and $2\text{--}3\%$ under conditions of catabolite repression during growth in glucose (26). The strain carrying this promoter-down mutation, *DH1lacP2dapD*, was dependent on IPTG or repressor titration for growth in liquid media lacking DAP (Fig. 3B). Although *DH1lacP2dapD* grew in liquid media in the presence of IPTG or DAP, its growth on solid media containing DAP and/or IPTG was restricted to areas of the plate containing a high inoculum: few isolated single colonies were present. In contrast, transformation with a *lacO*-containing plasmid led to single colonies that grew well on solid media, thereby allowing the use of this strain for plasmid selection by repressor titration.

Novel vectors for use in repressor titration

The pORT vectors (Fig. 2) were designed for use in repressor titration strains, but any other *lacO*-containing, high copy number plasmid can be selected by repressor titration and stably maintained. The use of plasmids pORT1 and pORT2 allows initial transformations and recombinant DNA manipulations to be carried out in any host strain using Ap^{R} as a selection (most of the *lacZ* α -peptide was removed in the construction of these plasmids to reduce non-essential DNA to a minimum, so blue/white selection is not possible). Then *bla* is removed as a final step prior to transformation into *DH1lacP2dapD* or *DH1lacdapD*. This is achieved by exploiting the six restriction endonuclease recognition sites that flank *bla* in direct repeat. Three of these (*AvrII*, *SfiI* and *FseI*) have rare cleavage sites, so the probability of all being present in an insert is very low.

Growth of *DH1lacdapD* and *DH1lacP2dapD*

The overall growth in liquid medium lacking DAP of uninduced *DH1lacdapD* was slightly less than when IPTG was present, apparently as a consequence of a lag in reaching full log growth, underlining the failure to repress expression from wild-type *lacOP* completely in this strain (Fig. 3A). *DH1lacdapD* containing pORT1a grew initially as fast as the IPTG-induced control although in late log phase its growth rate reduced and after 24 h A_{600} was ~ 3.2 as compared to ~ 4.0 for the plasmid-free control.

DH1lacP2dapD containing pORT1a showed an identical growth behaviour to *DH1lacdapD* containing pORT1a (Fig. 3B): perhaps this reduced growth during late log and stationary phase growth is a consequence of the burden of maintaining the high copy number pORT1a plasmid, despite it not expressing any protein products. *DH1lacP2dapD* did not grow at all without IPTG (Fig. 3B). Although the initial rate of growth of IPTG-induced *DH1lacP2dapD* was lower than that of *DH1lacP2dapD* containing pORT1a and IPTG-induced *DH1lacdapD*, at 24 h the densities of the plasmid-free cultures were identical ($A_{600} \sim 4.0$). It seems likely that the apparent lag in the growth of the IPTG-induced *DH1lacP2dapD* is a consequence of some lysis induced by DAP limitation during the short period between DAP removal by washing and the effective *in vivo* action of IPTG. As both the leaky and non-leaky IPTG-

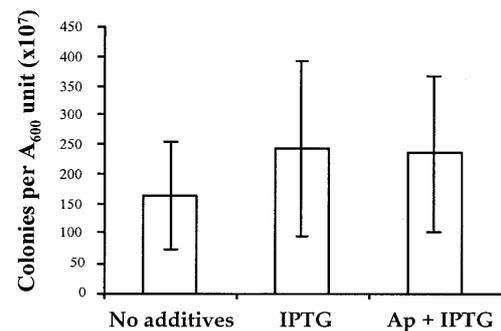


Figure 4. Propagation of pORT1 in *DH1lacP2dapD*. *DH1lacP2dapD*(pORT1) was grown in LB broth by repeated subculture (at 37°C with constant agitation) with no additives, with IPTG and with IPTG + Ap. Each culture was subcultured at intervals of 24 h (inoculum density $A_{600} = 0.01$) over 5 days. A dilution series was prepared from a volume of the final cultures equivalent to $0.1 A_{600}$ units and plated on LB agar + Ap. The results represent the mean number of colonies counted from triplicate experiments when plated at a 10^{-6} dilution (error bars represent standard deviations).

induced cultures reached similar final cell densities, we conclude that the concentration of IPTG used allowed sufficient *dapD* expression in *DH1lacP2dapD* for this not to be a growth-limiting factor in liquid medium.

Figure 4 shows the number of plasmid-containing colonies obtained by plating out equal numbers of cells of *DH1lacP2dapD* (pORT1) (as determined by A_{600} measurements) from cultures grown by daily subculture over 5 days (~ 47 generations). These cultures were grown under three conditions: repressor titration selection (no additives), no selection (IPTG) and antibiotic selection (Ap + IPTG). There is no significant difference in the number of colonies from these three cultures, indicating that the plasmid was not lost, even when grown with IPTG when repressor titration is no longer effective. Therefore, pORT1 is a stable plasmid suitable for use as a recombinant DNA vector.

Plasmid selection by repressor titration after plasmid transformation

The ability of *DH1lacdapD* to act as a host for plasmid transformation during selection by repressor titration was compared with the efficiency of transformation by antibiotic selection (Ap^{R}) using pUC18, and pUC18 Δ *lacO*, deleted for a *PvuII* fragment that removes 322 bp containing *lacO*₁ and *lacO*₃. pORT1a was also used to demonstrate selection of an antibiotic gene-free plasmid. The LB agar plates used contained Ap, Ap + IPTG and no additives, and the results of these experiments are shown in Table 1.

When *DH1lacdapD* was transformed with pUC18, transformants were obtained on all three plates at equivalent numbers, as pUC18 confers both Ap^{R} and repressor titration. To confirm that colonies growing with no Ap present were indeed pUC18 transformants, 100 colonies were streaked onto Ap plates, where they all re-grew. Transformants of pUC18 Δ *lacO* grew on Ap + IPTG plates at an equivalent number to those of pUC18, thus Ap + IPTG represents antibiotic selection alone. There was no growth on Ap plates, as without the *lacO* sequences, repressor titration could not function. The few colonies seen in the absence of Ap did not contain

plasmids since they failed to re-grow on Ap + IPTG plates. A negative control transformation was also performed (no DNA added) and similar colony numbers were seen as for pUC18 Δ lacO with no additives. These were due to leaky *dapD* expression in DH1*lacdapD*, caused by compensating mutations allowing cells to grow in the absence of IPTG, as negative control colonies did form single colonies when re-streaked onto plates with no additives.

Table 1. Plasmid selection by repressor titration

	Transformation efficiency: colonies/fmol DNA		
	No additives	Ap	Ap + IPTG
pUC18	618.02	587.36	575.73
pUC18 Δ lacO	9.90	0	632.39
pORT1a	589.69	0	0

Calcium-competent DH1*lacdapD* (100 μ l aliquots) were transformed with pUC18, pUC18 Δ lacO and pORT1a by adding 0.5 μ g plasmid DNA, heat shocking at 42°C for 45 s, and incubating at 37°C in 900 μ l LB broth containing IPTG for 1 h. Cells were spun down and washed to remove IPTG and resuspended in 1.0 ml LB broth, then plated on LB agar (100 μ l of a 1/100 dilution of the transformation cultures) containing no additives, Ap or Ap + IPTG. Results represent the means of three separate experiments in which transformations were plated in triplicate. Transformation efficiencies are displayed in colonies per femtomole of plasmid DNA. A negative control (no DNA) had the same number of colonies as those for pUC18 Δ lacO with no additives, but no colonies with Ap present.

pORT1a, being devoid of the *bla* gene, produced no transformants where Ap was present, but transformants were obtained in its absence at similar numbers to pUC18. It should be noted that transformation efficiencies are expressed in colonies per femtomole DNA, as smaller plasmids would be present at a higher concentration per unit mass. The values for pUC18 were in the range of 10⁵ transformants per μ g DNA.

DISCUSSION

We have created two strains, DH1*lacdapD* and DH1*lacP2dapD*, which allow the selection and propagation of plasmids free from the burden of plasmid gene expression that leads to antibiotic resistance, or indeed any other expression of protein encoding or RNA genes from the plasmid vector. This avoids the problems associated with the use of antibiotics and antibiotic resistance genes in therapeutic protein or DNA vaccine production, gene therapy or genetic engineering applications.

Deletion of chromosomal *dapD* was required rather than simple insertional inactivation, as there was concern that a partially deleted *dapD* may undergo homologous recombination with the *lacOP-dapD* cassette that had been integrated at an ectopic position, thereby restoring wild-type *dapD*. Note that during the genetic constructions, strains were proficient in homologous recombination until the last step when the RecA⁺ plasmid was removed, in order that the desired recombinational events could occur.

Transformation efficiencies in DH1*lacdapD* by repressor titration and antibiotic selection are equivalent, and calcium-competent cells were used as we have seen higher and more

consistent transformation efficiencies with these cells than with electro-competent DH1*lacdapD*.

The *lacP-dapD* fusion contains a 21 bp natural lactose operator, *O₁*, located between the *lacP* and the start codon of *lacZ*. An additional 21 bp 'pseudo-operator', *O₃*, is also present 92 bp upstream of *O₁* and includes the stop codon of *lacI*. *O₁* is the major target for *LacI* binding, although deletion of *O₃* reduces repression 50-fold (27). Plasmids that we have selected by repressor titration so far (e.g. pUC, pORT) are high copy number and contain both *O₁* and *O₃*. For gene therapy and the construction of transgenic organisms, the absence of non-essential expressed sequences and reduction in the size of the plasmid backbone of pORT1a is highly desirable for the reasons discussed earlier, as well as to facilitate the cloning and maintenance of large DNA fragments.

A low level of tetrahydrodipicolinate *N*-succinyltransferase expression from the wild-type *lac* promoter of DH1*lacdapD* occurs even when uninduced, which allows growth in liquid culture (but not single colony formation on solid media) without DAP. This was overcome by introducing mutations into the *lac* promoter that reduced expression, thereby generating DH1*lacP2dapD*, a strain that requires DAP, IPTG or repressor titration for its growth. DH1*lacP2dapD* differs from DH1*lacdapD* in only two nucleotides, which represent mutations to the *lac* promoter controlling *dapD*. On solid media, DH1*lacP2dapD* will only form single colonies if transformed with a *lacO*-containing plasmid. The inability of DH1*lacP2dapD* to grow well on solid media in the absence of repressor titration is a consequence of its *recA*⁻ status, since derivatives of this strain carrying either of two different RecA⁺, *lacO*⁻ plasmids grew well on plates, as did a plasmid-free derivative of DH1*lacP2dapD* that contained a chromosomal RecA⁺ allele. DH1 Δ *dapD* also did not grow well on plates supplemented with DAP unless the strain was RecA⁺. Previous studies of Δ *dapD* mutants have been restricted to RecA⁺ strains (28–30). We believe it likely that this inability to form single colonies on solid media is a consequence of increased DNA damage under these conditions, which is particularly detrimental to *recA*⁻ strains. Consistent with this view is the observation that *E.coli* mutants that lack superoxide dismutase (SOD) contained a compensatory mutation in *dapD*, thereby relieving their growth defects resulting from SOD deficiency (30). This report proposed that dipicolinate, which is the immediate precursor of tetrahydrodipicolinate and can also be produced by tetrahydrodipicolinate oxidation, chelates iron lost from superoxide-damaged iron–enzyme clusters and thus allows iron to be recycled. It may be that the accumulation of precursors in the *dapD* mutants leads to an effective increase in cytoplasmic iron concentrations in SOD⁺ strains, thereby increasing DNA damage as a consequence of the Fenton reaction. This could lead to the inviability of the *recA*⁻ strain.

DH1*lacP2dapD* has a significantly lower and more variable transformation efficiency compared to DH1*lacdapD*, perhaps because of its greater sensitivity to DAP depletion. Nevertheless, it is possible to select plasmid-containing cells by repressor titration using DH1*lacP2dapD* as Figure 3 demonstrates. We recommend that DH1*lacdapD* be used for the construction of antibiotic gene-free plasmids at the stage of antibiotic resistance gene removal, while DH1*lacP2dapD* is used as a final production strain. As DH1*lacP2dapD* is the first non-leaky, inducible DAP auxotroph, it may also be useful as

a model strain for assessing *dapD* as a potential antibiotic target.

Since these repressor titration strains retain the chromosomal *kan* gene used to replace the deleted *dapD* gene, the next step is to create a derivative deleted for *kan* as well as *dapD*, and which therefore contains no antibiotic resistance gene. We conclude that repressor titration is a highly effective means of selecting plasmid-containing cells after genetic transformation and we are confident that repressor titration will be an extremely effective mechanism for maintaining plasmids that may not otherwise be stably maintained.

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