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Effect of Plasmid Copy Number and *lac* Operator Sequence on Antibiotic-Free Plasmid Selection by Operator-Repressor Titration in *Escherichia coli*

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Key Words
Recombinant protein · Metabolic burden · DNA vaccine · *lac* repressor · DAP · DH1*lacdapD*

Abstract
The *Escherichia coli* strain DH1*lacdapD* enables plasmid selection and maintenance that is free from antibiotics and selectable marker genes. This is achieved by using only the *lac* operator sequence as a selectable element. This strain is currently used to generate high copy number plasmids with no antibiotic resistance genes for use as DNA vaccines and for expression of recombinant proteins. Until now these have been limited to pUC-based plasmids containing a high copy number pMB1-derived origin of replication, and the principle $lacO_1$ and auxiliary $lacO_3$ operators. In this study we have shown that this system can also be used to select and maintain pBR322-based plasmids with the lower copy number pMB1 origin of replication, and that $lacO_1$ alone or a palindromic version of $lacO_1$ can provide a sufficient level of repressor titration for plasmid selection. This is advantageous for recombinant protein production, where low copy number plasmids are often used and plasmid maintenance is important. The degree of repressor titration due to these plasmids was measured using the natural lactose operon in *E. coli* DH1 as a model.

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Introduction

The use of antibiotics and antibiotic resistance genes in the production of recombinant biotherapeutics is undesirable for a number of reasons. These include the risk of antibiotic gene transfer to pathogenic organisms [Davidson, 1999], plasmid loss due to plasmid size and metabolic stress [Smith and Bidochka, 1998] or antibiotic breakdown during culture and the risk of contamination of the product with antibiotics [Baneyx, 1999]. Strategies have been developed to avoid the use of antibiotics, including plasmids enabling complementation of a host auxotrophy, or possessing post-segregational killing (PSK) mechanisms [Hanak and Cranenburgh, 2001]. However, these share another major disadvantage with the antibiotic-selected plasmids: the metabolic burden associated with constitutively expressed plasmid-borne genes resulting in lower rates of growth [Bentley et al., 1990]. The solution to this problem is to use a plasmid with a non-expressed selectable marker. The Operator-Repressor Titration (ORT) system enables the selection and maintenance of plasmids that are free from expressed selectable marker genes and require only the short, non-expressed *lac* operator for selection and maintenance [Williams et al., 1998]. The principle *Escherichia coli* ORT strain, DH1*lacdapD* [Cranenburgh et al., 2001], has been used to produce several important DNA vaccine candidates such as the HIV-1 vaccine pTHr.HIVA [Hanke and McMichael, 2000].

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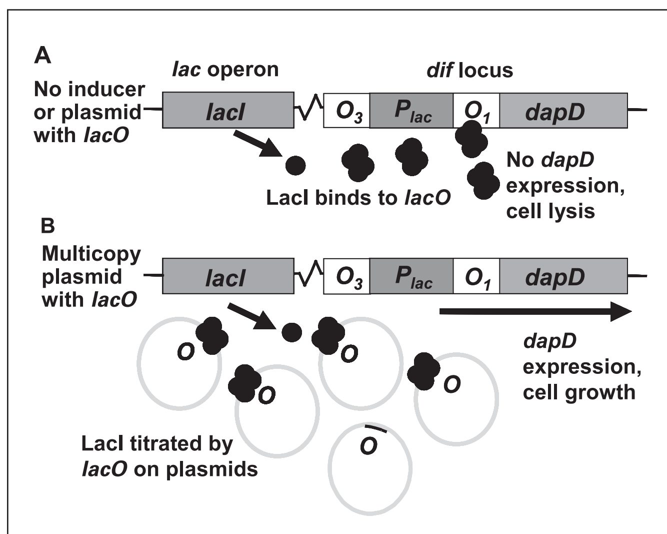

Fig. 1. The mechanism of repressor titration. A representation of the chromosome of DH1*lacdapD* in (**A**) the absence, and (**B**) presence of a *lacO*-containing multicopy plasmid.

Repressor titration occurs in a cell containing the following three components: (i) a chromosomal gene under negative regulation by a promoter that incorporates an operator, (ii) a multicopy plasmid that possesses an operator (with a similar, but not necessarily identical, sequence to the chromosomal operator) and (iii) a second chromosomal gene that expresses a repressor protein that is able to bind to both plasmid and chromosomal operators. In DH1*lacdapD* the chromosomal gene controlled by P_{lac} is *dapD* [Richaurd et al., 1984], which encodes the enzyme tetrahydrodipicolinate N-succinyl transferase that catalyses a step in the lysine/diaminopimelate (DAP) biosynthesis pathway. DAP cross-links peptidoglycan in the bacterial cell wall, so in the absence of *dapD* expression, DH1*lacdapD* cells will lyse. There is no DAP present even in rich media such as Luria-Bertani (LB) or Terrific Broth, as they do not contain components derived from bacteria. The LacI repressor protein binds to the $lacO_1$ and $lacO_3$ operators and blocks transcription of *dapD*. Therefore, an untransformed cell of DH1*lacdapD* can only grow to form a single colony on a nutrient agar plate if an inducer of the *lac* operon such as isopropyl-β-D-thiogalactopyraniside (IPTG) is present. However, when a plasmid possessing *lacO* sequences is introduced into the cell, the binding of the repressor protein to the plasmid-borne operator derepresses the chromosomal operator and allows *dapD* expression (fig. 1).

Although DNA vaccine and gene therapy applications require the highest possible plasmid copy number, recombinant protein expression can benefit from using low copy number plasmids instead [Jones et al., 2000], so it was important to determine if these could be selected and maintained by ORT. In addition to plasmid copy number, we investigated the effect of different sequences of *lac* operators on repressor titration. For those plasmids that do not possess the $lacO_1$ and $lacO_3$ operators with the optimum spacing found in the pUC series, we investigated $lacO_1$ alone and a palindromic version of *lacO* as potential selectable markers. This would make the conversion of conventionally selected plasmids to ORT plasmids easier, and reduce the size of the ORT plasmid. The results presented here test the versatility of the ORT system and determine its potential for recombinant protein production.

Results and Discussion

Plasmid Elements

Virtually all *E. coli* plasmids in common use as cloning or expression vectors were originally derived from the natural *E. coli* plasmid pMB1 [Yoshimori et al., 1972]. The pMB1 derivative pBR345 was used to construct pBR322 [Bolivar et al., 1977]. The pBR322 derivative pUR1 was the ancestor of the higher copy number pUC series [Vieira and Messing 1982], including pUC18 [Norrander et al., 1983]. Both the pUC18- and pBR322-derived plasmids constructed for these experiments have a degree of sequence identity including *bla* (retained to enable the identification of plasmid-containing cells by antibiotic selection) and the pMB1 origin of replication *(ori)*. The plasmids were designed to be small and to not contain any unnecessary expressed elements, so that metabolic burden would not be a differentiating factor. Therefore, the tetracycline resistance gene *tet* was removed from all the pBR322-based plasmids.

There are two important differences with respect to copy number between the pBR322 and pUC plasmids. Twigg and Sherratt [1980] deleted a section of the pMB1-derived plasmid pAT153 that resulted in up to a 3-fold increase in copy number. This was later found to encode the Rom protein, present in pBR322 but absent from pUC18. The pMB1 *ori* encodes two overlapping RNA transcripts: RNAII is the primer for plasmid DNA synthesis, and RNAI is the antisense inhibitor of RNAII [Tomizawa, 1984]. Rom stabilises the binding of RNAI to RNAII [Tomizawa and Som, 1984]; in the absence of

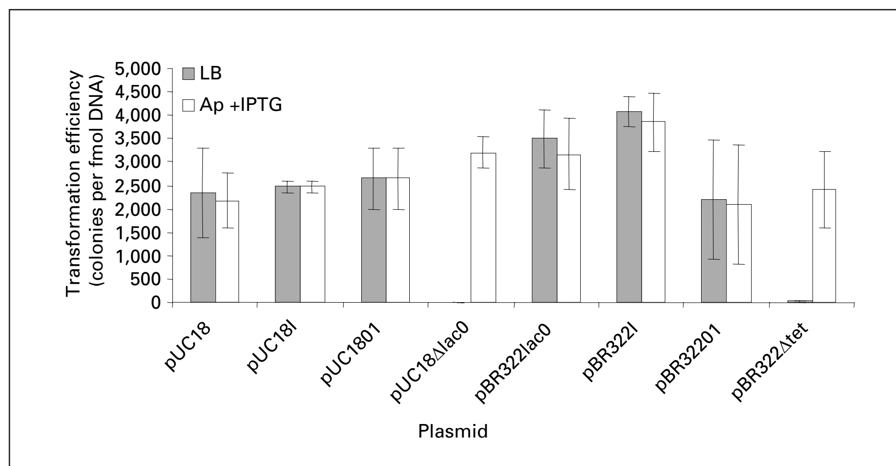

Fig. 2. Transformation efficiency of DH1*lacdapD*. Following transformation, cultures were plated onto LB agar with no additives (ORT selection) and with ampicillin (Ap) plus IPTG (antibiotic selection). Results represent the number transformant colonies per femtomole of plasmid DNA used in the transformation, and are the mean of three separate experiments with standard deviation displayed as error bars.

Rom, RNAII is able to dissociate from RNAI, and therefore to prime plasmid replication, at a greater rate. The second factor is a G→A mutation in the pMB1 *ori* from pBR322 to the pUC series [Minton et al., 1988] that causes a further copy number increase in *rom*$^-$ plasmids by altering RNAII conformation [Lin-Chao et al., 1992].

These mutations enable pUC plasmids to reach 500–700 copies per cell [Minton et al., 1988]. The single chromosomal copy of *lacI* expresses LacI repressor protein at ~20 copies per cell [Müller-Hill et al., 1968]. It was previously thought that this significant molar excess of plasmid was necessary to achieve sufficient levels of repressor titration in ORT *E. coli* strains. It was not known if lower copy number pBR322 plasmids with 39–55 copies per cell [Lin-Chao and Bremer, 1986] would achieve the same effect.

In the wild-type *E. coli lac* operon, the principle operator, $lacO_1$, is present between the promoter and *lacZ* cistron. Two auxiliary operators are also present: $lacO_3$ is 92 bp upstream and $lacO_2$ is 401 bp downstream of $lacO_1$ (in the *lacZ* cistron). The deletion of either auxiliary operator results in a 2- to 3-fold decrease in repression, but eliminating both reduces repression greater than 50-fold [Oehler et al., 1990]. pUC18 has the $lacO_1$ (AATTGTGAGCGGATAACAATT) and $lacO_3$ (GGCAGTGAGCGCAACGCAATT) sequences with the optimum 71 bp spacing, but often the low copy number expression plasmids possess only a single $lacO_1$, or $lacO_1$ and $lacO_3$ with suboptimal spacing, e.g. the pTrc series [Amann et al. 1988]. For our experiments we tested both high (pUC18-based) and low (pBR322-based) copy number plasmids with the following *lacO* configurations: (i) $lacO_1$ and $lacO_3$ with the optimal spacing (71 bp between the elements), (ii) a single $lacO_1$, (iii) a single 'ideal' *lacO*, and (iv) no *lacO* sequences, as described in table 1. The 'ideal' version of *lacO* is a 20 bp perfectly palindromic sequence (AATTGTGAGCGCTCACAATT), based on the sequence reported in Sadler et al. [1983]. This binds the repressor 10-fold more tightly than $lacO_1$, and was tested in case $lacO_1$ alone was not sufficient for repressor titration.

Table 1. Plasmids used in this study

Plasmid	Size, bp	Operator	Source or reference
pUC18	2,686	$lacO_1$, $lacO_3$	Norrander et al., 1983
pUC18O1	2,085	$lacO_1$	This study
pUC18I	2,084	Ideal *lacO*	This study
pUC18ΔlacO	2,364	None	Cranenburgh et al., 2001
pBR322lacO	3,188	$lacO_1$, $lacO_3$	This study
pBR322O1	3,104	$lacO_1$	This study
pBR322I	3,103	Ideal *lacO*	This study
pBR322Δtet	2,866	None	This study

Plasmid Transformation by Antibiotic and ORT Selection

The transformation efficiencies by antibiotic and ORT selection were equivalent for all plasmids possessing at least one *lac* operator sequence (fig. 2). This demonstrates that both high and lower copy number plasmids are able to achieve a sufficient degree of repressor titration to enable cell growth and survival following transformation. Although there is no difference in transformation efficiency between the three operator arrangements tested,

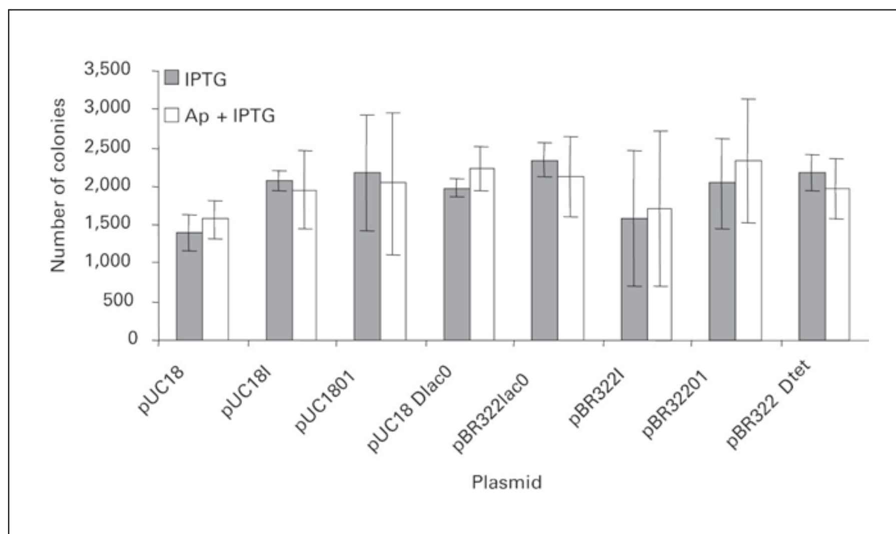

Fig. 3. Plasmid maintenance in DH1*lacdapD*. Strains were subcultured through approximately 53 generations, and standard cell numbers plated onto LB agar containing IPTG (enabling growth of all viable cells) and ampicillin plus IPTG (enabling growth of plasmid-containing cells). Results are the mean colony numbers from three separate experiments with standard deviation displayed as error bars.

the plasmids without a *lac* operator were not selected by ORT. These results demonstrate the possibility of using low copy number plasmids in an ORT strain, which is advantageous for applications such as recombinant protein production. To select plasmids that do not initially contain a *lacO* sequence in ORT strains, it is possible to simply clone the ideal *lacO* using overlapping oligonucleotides into a suitable restriction site.

Plasmid Maintenance during Serial Subculture

No plasmid loss was detected in the plasmid maintenance experiment (fig. 3), as the numbers of plasmid-containing cells (on ampicillin and IPTG) were equivalent to the total viable cell counts (on IPTG). Although DH1*lacdapD* requires a *lacO*-containing plasmid or IPTG for growth on agar plates, it does not need a plasmid or IPTG for growth in the equivalent liquid culture [Cranenburgh et al., 2001]. This may be because the very low level of leaky expression from P_{lac} driving *dapD* expression is able to produce sufficient DAP for survival in liquid culture, but not sufficient for the harsher environment of the surface of an agar plate. Therefore if plasmid loss had occurred during the subculture, there would be a difference in the relative colony numbers on the plates as any plasmid-free segregants would have continued to grow during the subculture. Plasmid maintenance studies on PSK mechanisms give an inaccurate measure of the rate of plasmid loss, as cells that lose a plasmid are killed. With ORT using DH1*lacdapD*, plasmid loss would be evident if it did occur, but has not been observed. This also confirms that it is not plasmid loss that is responsible for the absence of transformants of plasmids without *lacO* in the previous experiment (fig. 2), but the absence of repressor titration.

Measuring the Level of Repressor Titration Using β-Galactosidase Assays

E. coli DH1 possesses a natural lactose operon as does DH1*lacdapD*, but DH1 was chosen for measuring the effects of repressor titration (fig. 4) as it has a single copy of P_{lac} which is not involved in cell survival under the conditions used. Glucose was used as the sole carbon source as it reduces the basal level of expression from P_{lac} by catabolite repression [Matthews and Nichols, 1998], thus allowing low levels of *lacZ* expression to be detected. The values representing β-galactosidase activity are presented as absorbance at 580 nm rather than specific activity, as we are interested in comparing the relative levels of activity rather than determining absolute values within the context of this experiment. There was virtually no β-galactosidase activity with untransformed DH1, but when IPTG was added, an activity of A_{580} of approximately 2.0 was measured. A greater level of *lac* operon derepression was produced by repressor titration from pUC18, indicating that IPTG does not cause full induction. There was also very little β-galactosidase activity with the two plasmids lacking *lacO* sequences, as expected. Together with the transformation experiments, these results indicate that even the very low levels of repressor titration from pBR322O1 are sufficient for plasmid selection by ORT.

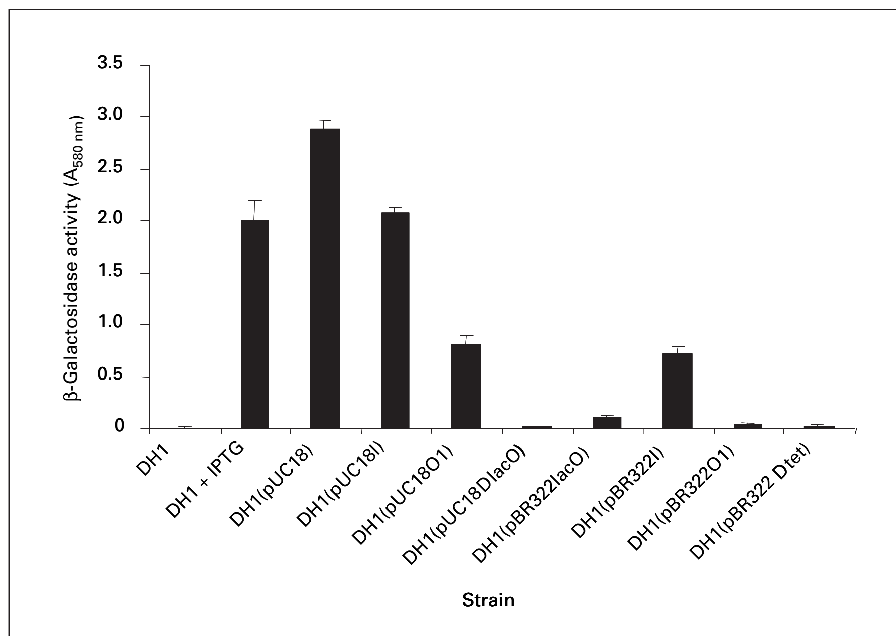

Fig. 4. Derepression of the chromosomal *lac* operon in DH1 by repressor titration. β-Galactosidase activities from cell lysates measured by absorbance at 580 nm. Results are the mean values from three separate experiments with standard deviation displayed as error bars.

For each *lacO* sequence type or operator arrangement, the level of repressor titration was higher in the pUC18 than in the corresponding pBR322-based plasmid, due to the difference in plasmid copy number. When the repressor dissociates from the chromosomal operator, it has a higher probability of binding to *lacO* on a high copy number rather than a low copy number plasmid. The repressor titration levels due to the ideal *lacO* were significantly higher than those for *lacO$_1$* for each plasmid type, due to the 10-fold tighter binding of the repressor by this operator sequence [Sadler et al., 1983].

Another difference between the high and low copy number plasmids is the relative difference between repressor titration due to *lacO$_1$* and *lacO$_3$* (pUC18 and pBR322lacO) and ideal *lacO* (pUC18I and pBR322I). With pUC18, the repressor titration levels are higher than those for pUC18I, which is expected as LacI binding to *lacO$_1$* and *lacO$_3$* should be tighter than binding to ideal *lacO*. However, for pBR322lacO the repressor titration levels are lower than for pBR322I, although the sequences of *lacO$_1$*, *lacO$_3$* and the inter-operator region are identical in pUC18 and pBR322lacO, and the same ideal *lacO* is present in pUC18I and pBR322I. Further experiments would be required to determine if this is due to differences in binding affinities and kinetics.

LacI has an affinity for non-specific DNA in addition to its tight and highly specific binding to *lacO* [Lin and Riggs, 1975]. LacI searches for *lacO* by binding to non-specific DNA and travelling along, combined with transfer to other chromosomal regions by dissociation and association or direct intersegment transfer, as illustrated in Matthews and Nichols [1998]. A high copy number plasmid without *lacO* contributes significantly to the amount of this non-specific DNA, yet there is no detectable increase in β-galactosidase expression between DH1 and DH1(pUC18ΔlacO). This indicates that the non-specific binding of LacI to plasmid DNA has no effect on repressor titration.

Experimental Procedures

Reagents and Techniques

Restriction and modification enzymes were obtained from commercial suppliers and used following the manufacturers' protocols. Standard molecular biology techniques were employed as described in Ausubel et al. [1994]. Strains were grown in LB medium [Miller, 1992] at 37 °C, supplemented with 0.1 mg/ml ampicillin or 0.1 m*M* IPTG where required.

Plasmid Construction

A new series of plasmids were constructed, based on high copy number pUC18 [Norrander et al., 1983] and low copy number pBR322 [Bolivar et al., 1977]. These all contained the *bla* gene for ampicillin resistance and one of the following: (i) *lacO$_1$* and *lacO$_3$* at the optimal spacing, (ii) a single *lacO$_1$*, (iii) ideal *lacO* [Sadler et al., 1983] and (iv) no operator. Plasmids were created in *E. coli* cloning strains DH5α and XL10-Gold (Invitrogen). To create the versions of pUC18 and pBR322 containing single operators (*lacO$_1$*

or ideal *lacO*), these plasmids were first cut with restriction enzymes *Aat*II and *Nar*I and the intervening DNA removed. Then the following oligonucleotides were synthesised, annealed (which created overhangs compatible with those generated by *Aat*II and *Nar*I) and ligated to the plasmids (operator sequences are underlined). For $lacO_1$: LACOA (5′-CAATTGTGAGCGGATAACAATT-3′) and LACOB (5′-CGAATTGTTATCCGCTCACAATTGACGT-3′); for the ideal *lacO*: IDEALA (5′-CAATTGTGAGCGCTCACAATT-3′) and IDEALB (5′-CGAATTGTGAGCGCTCACAATTGACGT-3′). pBR322 was cut with restriction enzyme *Eco47*III and the intervening DNA removed to create pBR322Δtet. The 322 bp *Pvu*II fragment containing $lacO_1$ and $lacO_3$ from pUC18 was ligated to *Eco47*III-cut pBR322 to create pBR322lacO. The regions containing the operators and all cloning junctions were verified by DNA sequencing.

Transformation Efficiency

Calcium-competent DH1*lacdapD* cells were prepared as 0.1-ml aliquots [Ausubel et al., 1994] and transformed with 0.5 μg of each plasmid. Following a heat shock at 42 °C for 45 s, 0.9 ml LB broth containing IPTG (to ensure cell survival prior to plating) was added and transformation cultures were incubated at 37 °C for 1 h. These were washed twice in fresh LB broth to remove IPTG and a 10^{-3} dilution plated onto LB agar (ORT selection) and LB agar containing ampicillin and IPTG (antibiotic selection control). Experiments were performed in triplicate for each strain and colonies counted to determine transformation efficiency. As the plasmid sizes vary, these were converted into transformants per femtomole of plasmid DNA (fig. 2).

Plasmid Maintenance

The eight DH1*lacdapD* transformant strains were inoculated into LB broth with ampicillin and IPTG, and these cultures were used to inoculate triplicate cultures of 5.0 ml LB broth to an inoculum density (determined by absorbance at 600 nm) of 0.01. These were grown for 24 h (37 °C, 200 rpm) and subcultured into fresh LB broth at A_{600} = 0.01. This subculturing was repeated in the absence of ampicillin for 5 days, after which the cultures had undergone approximately 53 generations. The final cultures were sampled to ensure equal cell numbers by extracting volumes equivalent to 1.0 ml of culture at A_{600} = 0.1. These were diluted to 10^{-6} and plated onto LB agar containing IPTG to allow all viable cells to grow, and IPTG with ampicillin to allow only plasmid-containing cells to grow. Colonies were counted to determine the degree of plasmid maintenance (fig. 3).

β-Galactosidase Assays

The eight plasmids were transformed into *E. coli* DH1 [Hanahan, 1983]. Along with untransformed DH1, these were inoculated into 5.0 ml M9 minimal medium [Miller, 1992] supplemented with 0.1 *M* glucose, 1.0 μg/ml thiamine, and 0.1 mg/ml ampicillin for the plasmid-containing strains, and grown at 37 °C and 200 rpm overnight. These were used to inoculate fresh cultures without ampicillin in triplicate at A_{600} = 0.05, with two sets of three cultures for DH1 with and without 0.1 *M* IPTG. These 30 cultures were grown to mid-log phase and sample volumes equivalent to 1 ml at A_{600} = 0.5 were taken to ensure equal cell numbers and centrifuged to obtain cell pellets. These were resuspended in 0.1 ml phosphate-buffered saline and 15 μl 0.1% sodium dodecyl sulphate and 30 μl chloroform was added, vortexed for 10 s, centrifuged for 1 min and 20 μl of each sample supernatant used in triplicate β-galactosidase assays. The High Sensitivity β-Galactosidase Assay Kit (Stratagene) was used following the micro β-Galactosidase Assay protocol in the instruction manual (revision #078001b). This uses the galactoside analogue chlorophenol red-β-*D*-galactopyranoside as a reporter, with absorbance at 580 nm measured using a microplate reader (fig. 4).

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