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pBR322 Vectors Having Tetracycline-Dependent Replication

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Abstract

Few *Escherichia coli* cloning vectors are available that can both be stably maintained and efficiently cured. One such vector is pAM34, a pBR322 derivative constructed by Gil and Bouché (1991). Replication of this plasmid is driven by the *lacZYA* promoter under control of a gratuitous inducer. However, *lac* operator-repressor interactions are also used to regulate many expression systems which limits the utility of pAM34. In this report pAM34 has been modified by replacement of the *lac* regulatory elements with those of the transposon Tn10 tetracycline resistance module. This resulted in medium copy number plasmids that are dependent on the presence of tetracycline (or less satisfactorily, anhydrotetracycline) for replication. The tetracycline-dependent plasmids are rapidly lost in the absence of tetracycline and plasmid loss is markedly accelerated when the host strain expresses a tetracycline efflux pump.

1. Introduction

Plasmids containing replication origins derived from plasmids pMB1 (e.g., pBR322 and pUC19) or p15A (e.g., pACYC184) are very often used for molecular cloning and expression studies in *Escherichia coli* and its close relatives (e.g., *Salmonella enterica*). However, some uses of these plasmids require that they be stably maintained but subsequently eliminated. An example would be in testing a gene thought essential for growth because straightforward attempts (e.g. recombineering) to delete the gene have been unsuccessful. The usual approach is to introduce a second copy of the gene on a plasmid and repeat the deletion protocol. If the deletion construct is obtained, it can be argued that the gene is essential for growth. However, a higher standard of proof would be to show that the deletion strain cannot survive upon elimination of the plasmid and its complementing copy of the gene. A problem is that the above cloning vectors are not readily eliminated and their loss often requires complex and markedly inefficient protocols. Other plasmids such as the pSC101-derived plasmids having a point mutation in an essential replication protein that renders replication temperature-sensitive (Hashimoto-Gotoh et al., 1981) have been widely used. Unfortunately, these plasmids tend to be large and of very low copy number which

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complicates cloning procedures. Moreover, reversion of the point mutation responsible for temperature-sensitivity is often a problem since the mutation compromises plasmid maintenance at the permissive temperature. Gil and Bouché (Gil and Bouche, 1991) took another approach. They substituted the *lacZYA* promoter for the pBR322 promoter that normally initiates transcription of the RNA primer required for plasmid replication in the pMB1, p15A and RSF1030 family of plasmids. Their final vector, pAM34, contained the LacI^Q allele which overproduces the lactose repressor. LacI overproduction presumably compensated for the fact that pAM34 lacks both of the secondary (*lacO2* and *lacO3*) operators (a secondary operator is required for full repression of the *lacZYA* promoter (Oehler et al., 1994; Oehler et al., 1990)). Although pAM34 is a valuable isopropyl-β-D-thiogalactopyranoside (IPTG)-dependent vector, the vector has two drawbacks. First, the high IPTG concentrations (500 μM) required for normal growth of strains carrying pAM34 precludes or complicates use of IPTG inducible expression vectors. Second, the *lacZYA* promoter requires binding of the CAP cyclic activator protein and binding occurs only when CAP is complexed with cyclic-AMP. Since growth in glucose results in extremely low intracellular cyclic-AMP levels, pAM34 cannot be used in media that contain glucose, the carbon source preferred in chemically defined media.

The transposon Tn10 tetracycline (Tet) regulatory system seemed a likely candidate for construction of an alternative to pAM34. This system is very well understood at the molecular level (Hillen and Berens, 1994) and has been used to control gene expression in diverse bacteria (Bertram and Hillen, 2008), archaea (Guss et al., 2008) and eukaryotes (Bertram and Hillen, 2008; Gossen and Bujard, 1992). The *tetA* gene encodes a TetA(B) tetracycline efflux pump whereas the *tetR* gene encodes the repressor which is inactivated upon binding of Tet or Tet analogues. The two genes are adjacent but their transcription is bidirectional. The promoters of the two genes overlap and contain tandem operators that bind TetR and repress transcription of both *tetA* and *tetR*.

This paper reports replacement of the *lac* regulatory system of pAM34 with the Tn10 Tet regulatory system results in highly useful plasmids. This allows the use of IPTG-inducible expression plasmids and glucose containing media. Moreover, in the absence of Tet these plasmids can be readily eliminated from virtually all the cells of a culture.

1.1 Construction of plasmids pCY1107, pCY1108 and pCY1109

The KpnI site located immediately downstream of the pAM34 *lac* promoter (Gil and Bouche, 1991) was used to insert the *tetA* promoter in place of the *lac* promoter (Fig. 1). A second KpnI site present in a pAM34 multiple cloning site (MCS) was first removed to simplify the replacement. Plasmid pAM34 was digested with BamHI and religated. This removed the two MCS sites that bracket the *aadA* spectinomycin resistance determinant inserted as a “stuffer” fragment designed to be replaced by the gene of interest (Gil and Bouche, 1991) (Fig. 1). However, this manipulation resulted in loss of the BamHI site presumably because it created an uninterrupted palindrome of 234 bp by juxtaposition of the omega elements (Frey and Krisch, 1985) that flank the *aadA* segment outside the pAM34 MCS sites (Gil and Bouche, 1991) (Fig. 1). Such uninterrupted palindromes are known to block plasmid propagation (Collins, 1980; Collins et al., 1982; Elhai and Wolk, 1988; Tear

et al., 2015) which exerts strong selection for plasmids having deletions of one or both of the inverted repeat sequences. Indeed, the recombinant plasmid isolated, pCY1103, contained a deletion of about 120 bases that included the BamHI site.

To replace the *lac* promoter and *lacI^Q* repressor sequences of the deleted version of pAM34 (called pAM34 BamHI) with the *tetA* promoter and *tetR* repressor sequences, pAM34

BamHI was digested with AatII and treated with T4 DNA polymerase plus the 4 dNTPs to obtain a blunt ended fragment. This was purified, digested with KpnI and the resulting 4570 bp fragment was purified by agarose gel electrophoresis. The Tet expression plasmid pWQ572, which contains the *tetA-tetR* segment of pWQ552 (King et al., 2014) was digested with NheI, blunt ended by treatment with T4 DNA polymerase plus the 4 dNTPs, gel purified and then digested with KpnI to give an 827 bp fragment containing the *tetA* promoter and *tetR* repressor gene. This fragment was gel purified and ligated to the pAM34-derived 4570 bp fragment to give pCY1103. Plasmid pCY1103 retained ampicillin resistance and the *rop* gene that controls plasmid copy number and was suitable to test the feasibility of driving replication using the Tn10 *tetA* promoter. However since the plasmid lacked useful cloning sites, the omega-MCS-*aadA*-MCS-omega segment of pAM34 was restored by replacement of the PciI-FspI segment of pCY1103 with that of pAM34 to give pCY1107.

Plasmid pCY1107 contained redundant sites for two useful enzymes KpnI and EcoRI, the second copies having been introduced with the *tetA* promoter. To make the MCS sites unique the pAM34 BaeI site that was fortuitously retained upon introduction of the *tetA* promoter was utilized. BaeI is a type II restriction enzyme that cleaves DNA substrates twice to excise its recognition site and generate a 28 bp fragment with five base 3' overhangs. Since the segment excised by BaeI included the EcoRI and KpnI sites (plus the intervening NcoI site), BaeI digestion provided a straightforward means to remove these sites. However, the segment could not simply be deleted since the excised fragment included part of the *OI* operator of the TetA-TetR control region. Hence, the excised segment was replaced with a synthetic 28 bp fragment that restored the *OI* operator but eliminated the sites for the NcoI, EcoRI, KpnI and BaeI sites (the KpnI site included much of the BaeI recognition site) to give pCY1108. Plasmid pCY1107 was digested with BaeI and the large fragment was purified and ligated to the cassette to give pCY1108. The cassette was assembled by annealing two synthetic oligonucleotides: 5'-

AGAGCAATTCACCATCGTACGCTGCTTGCAAAC-3' and 5'-

AGAGCAATTCACCATCGTACGCTGCTTGCAAAC-3' containing a BsiWI site

(underlined). Sequencing of the entire replication and regulatory regions of pCY1108 demonstrated that the constructions proceeded as expected. BsiWI ends are compatible with those generated by several useful enzymes including Acc65I, a neoschizomer of KpnI. To allow use of these vectors with compatible ampicillin-resistance plasmids the β -lactamase gene was replaced with the Tn903 kanamycin resistance gene by separate digestions of pCY1108 and p34s-Km3 (Dennis and Zylstra, 1998) with SspI plus AhdI and with SacI, respectively. The AhdI and SacI 3' ends were converted to blunt ends as above and the appropriate fragments were recovered from agarose electrophoresis gels and ligated to give pCY1109 in which all but the last 8 codons of the β -lactamase gene had been replaced with the 932 bp kanamycin cassette.

1.2 Replication of plasmid pCY1107 in *E. coli*

Plasmid preparations were made from either overnight cultures or cultures grown to late log phase (OD of 1) as previously described (Chakravarty and Cronan, 2015) and analyzed on agarose gels either as intact plasmids or following *Sma*I digestion (Fig. 2). The host strain carried a chromosomal *Tn10* insertion thereby allowing tetracycline (Tet) to be used as inducer. Although the original plan was to use AhTet as the inducer, use of this analog was problematic. The level of AhTet giving maximal induction of the *tetA* promoter (100 ng/ml) (King et al., 2014; Lutz and Bujard, 1997) slowed growth relative to cultures induced with Tet. A ten-fold decrease in AhTet level also slowed growth. Moreover, overnight cultures induced with AhTet reached only about half the cell density of Tet induced cultures and gave generally disappointing plasmid yields (Fig. 2A, lanes 7 & 8). In some cases the AhTet-grown overnight cultures appeared partially lysed and sometimes lysed while being stored on the bench at room temperature. In contrast, AhTet induced cultures grown to late log phase were much better behaved and in a few cases contained as much or more plasmid than cultures induced with Tet (Fig. 2B, lanes 7 & 8). Recourse to the literature indicated that AhTet is one of a series of tetracycline analogs that act in an unknown manner on the cell membrane (rather than the ribosome) and kill by cell lysis (Oliva et al., 1992; Rasmussen et al., 1991). Moreover, AhTet is bactericidal whereas Tet is bacteriostatic (Oliva et al., 1992) and AhTet is not a substrate for the Tet efflux pumps (Oliva et al., 1992; Rasmussen et al., 1991). Due to the erratic behavior of AhTet grown cultures plus its unknown mode of action, Tet was considered the inducer of choice in this work.

Determination of the copy number of pCY1107 relative to a plasmid having an intact pBR322 origin showed that the plasmid replicated much like pBR322 over a 100-fold range of Tet concentrations (0.1–10 µg/ml) (Fig 2A lane 1–6) although the plasmid contents varied only about 3-fold. Tet concentrations above and below this range gave decreased colony sizes and in the case of Tet concentrations lower than 0.1 µg/ml Tet-independent colonies arose which are presumably due to mutations inactivating TetR (strains carrying pAM34 similarly accumulate IPTG-independent colonies at suboptimal IPTG concentrations). The frequency of Tet-independent colonies in cultures grown with (0.1–10 µg/ml) was roughly 10^{-7} . If the plasmid carries a toxic gene, this could provide a selection for Tet-independent cells. However, these could be eliminated by comparing growth in the presence or absence of Tet. Moreover, *tetR* is a small gene (689 bp including the promoter) and hence most cloned genes would be larger. If so, the frequency of inactivation of the toxic gene could exceed that of *tetR*.

Tet at 5–10 µg/ml gave the best growth in most strain backgrounds (tested on derivatives of *E. coli* K-12 strains MC1061, MG1655 and W3110). However, most strains grew well in the presence of 0.1 or 0.5 µg/ml Tet even in the absence of an efflux pump and this was sufficient to support growth of strains carrying pCY1107 (although Tet-independent strains are more likely to be selected at these concentrations). Vector pAM34, the IPTG-dependent parental plasmid was also included in these analyses. The plasmid contents of these strains were compared to that of pBAD322S, a 5535 bp spectinomycin-dependent vector that carries the wild type pBR322 origin and is of a size quite similar to that of pCY1107 (Cronan, 2006). Note that all the plasmids carry the same *aadA* gene which allowed

spectinomycin to be used to select for maintenance of all plasmids. Vector pBAD322S was assigned a copy number of 20 by averaging various literature values (Balbas et al., 1988; Balbas et al., 1986; Cesareni et al., 1982; Lee et al., 2006; Twigg and Sherratt, 1980). The maximal copy numbers of pCY1107 and pAM34 were found to exceed that of the wild type pBR322 origin plasmid, pBAD322S. Gil and Bouché (Gil and Bouche, 1991) reported the maximal copy number of pAM34 to be about 8 copies per cell a value about 3 to 4-fold lower than those estimated in Fig. 2. However, these workers did not directly determine plasmid content but rather obtained their value by extrapolation of the rate of plasmid loss and reported it as a minimal estimate (Gil and Bouche, 1991). The increased copy number relative to the wild type pBR322 origin standard is readily explained by the greater strengths of the *tetA* and *lacZYA* promoters relative to the RNAl promoter that normally produces the primer for pBR322 DNA replication (Bertrand et al., 1984; Cesareni et al., 1982). An increase in RNAl transcripts should result in increased copy numbers by titrating the inhibitory anti-primer transcripts from the more active RNAI promoter, although the modulating activities of the Rop protein and RNA degradation will limit the effects of increased transcription (Balbas and Bolivar, 2004; Balbas et al., 1986).

1.3 Efflux pumps facilitate rapid loss of plasmids dependent on the *tetA* promoter

In preliminary experiments cultures of strains carrying one of the Tet-dependent plasmids were grown in the presence of Tet or AhTet (plus antibiotics selecting for plasmid maintenance) were washed and plated on the selective medium lacking tetracyclines. Small colonies were formed indicating remaining residual expression of the *tetA* promoter. A straightforward approach to cope with the rebinding possibility would be active expulsion of the antibiotic by an efflux pump such as the TetA(B) protein encoded by Tn10 or the related TetA(C) determinant encoded by pBR322 and pACYC184. Indeed, loss of pAM34 in the absence of IPTG is probably facilitated by the YdeA pump (Carole et al., 1999). However, in the case of AhTet this approach is precluded. AhTet is (at best) only a very weak substrate for these and some other efflux pumps (Chopra et al., 1982; Guay and Rothstein, 1993; Oliva and Chopra, 1992). Expression of the TetA(B) or TetA(C) pumps had essentially no effect on the lethal dose of anhydrotetracycline to *E. coli* (Guay and Rothstein, 1993; Oliva and Chopra, 1992). In contrast, the Mg⁺⁺-Tet complex is efficiently pumped from *E. coli* cells and thus we tested if expression of an efflux pump would eliminate the residual colony formation of Tet grown cultures.

The efficiency of loss of plasmid pCY1107 was assayed by plating dilutions of Tet or AhTet induced cultures on medium that contained only spectinomycin and ampicillin (Fig. 3). Plasmid loss was tested in strains that either expressed or lacked an efflux pump. The host strains that encoded efflux pumps contained either a Tn10 element inserted in the chromosome or plasmid pACYC184. The two pumps have similar efficiencies of Tet efflux in *E. coli* (Oliva and Chopra, 1992; Oliva et al., 1992). In the presence of either efflux pump >99% of the Tet grown cells failed to form colonies on the selective medium (Fig. 3, plate columns 1 & 3) whereas in the absence of an efflux pump small colonies were formed in numbers equivalent to those formed in the presence of Tet (Fig. 3, plate column 2). Similar results were observed for cultures previously grown on plates containing Tet at 10, 5, 1 or 0.5 mg/ml indicating that the rate of efflux was sufficient to rid the cells of the higher Tet

concentrations. The presence or absence of an efflux pump had no detectable effect on the small colonies formed by AhTet grown cells (Fig. 3, plate columns 4 & 5) in agreement with reports that AhTet is not a substrate of these efflux pumps (Chopra et al., 1982; Guay and Rothstein, 1993; Oliva and Chopra, 1992).

Plasmid pACYC184, one of the original p15A cloning vectors (Chang and Cohen, 1978), encodes the TetA(C) efflux pump and chloramphenicol resistance and is compatible with the pBR322 origin of the *tetA* promoter-dependent plasmids. In physiological experiments where the *tetA* promoter-dependent plasmid was to be eliminated, the use of pACYC184 as the efflux pump source was favored because the p15A vector can be readily co-transformed into any host strain together with the *tetA* promoter plasmid (Hanahan, 1983). Moreover, pACYC184 is intrinsically unstable in the absence of selection (Chang and Cohen, 1978; Lenski and Bouma, 1987; Ray and Skurray, 1984; Vernet et al., 1985) and thus after colonies grown with Tet were suspended in nonselective liquid medium and grown overnight to eliminate the Tet-dependent plasmid, 20–25% of the colonies had also lost pACYC184. Note that a higher efficiency of pACYC184 curing can be obtained by growth in LB medium containing 2–6 mM Ni⁺⁺ salts (Podolsky et al., 1996; Stavropoulos and Strathdee, 2000; Tas et al., 2015).

A disadvantage of the use of pACYC184 is that the presence of a second plasmid precludes straightforward purification of Tet-dependent plasmids. Hence, a host strain that carried a Tn*10* element inserted into the chromosome was used to prepare Tet-dependent plasmids (an F' factor carrying a Tn*10* insertion should also suffice). Following *tetA* plasmid transformation of a Tn*10*-carrying host, the medium used in the usual outgrowth for expression of plasmid antibiotic resistance proteins should contain Tet (1–5 µg/ml) to induce plasmid replication. In the absence of induction reduced colony formation is seen because the Tet-dependent plasmid must initiate replication in a TetR repressor-filled cytosol. An excellent host for Tet-dependent plasmid preparation is strain ER2984 due to its chromosomal Tn*10* insertion, unusually rapid growth and the *endA1* mutation that gives improved plasmid quality (it is the parent of the Tet-sensitive NEB Turbo). Note that high TetA levels inhibit growth and result in decreased tetracycline resistance due to membrane damage (Eckert and Beck, 1989; Lee and Edlin, 1985; Lenski and Bouma, 1987; Moyed et al., 1983; Nguyen et al., 1989). Hence, there is no benefit to increasing TetA(B) or TetA(C) levels above the levels encoded by a single Tn*10* insertion or by a medium copy number plasmid (e.g., pACYC184). Moreover, plasmid copy numbers are often altered upon their intended use as cloning vectors and hence they cannot be taken as constants.

1.4. Curing and replication of plasmid pCY1108 in *S. enterica*

The Tet-dependent plasmids were also tested in *S. enterica* serovar Typhimurium strain LT2 because we had observed some differences between *E. coli* and *S. enterica* in replication of other plasmids (Chakravarty and Cronan, 2015). Given the extensive use of pBR322-derived plasmids and Tn*10* in this bacterium (Maloy, 1990), the Tet-dependent plasmids were expected to behave as they did in *E. coli* strains carrying Tn*10*. This was the case. Plasmid pCY1108 was readily introduced into a strain carrying a chromosomal Tn*10* insertion and also into the wild type LT2 strain when co-transformed with pACYC184. In

excellent agreement with the *E. coli* data (Fig. 3) cultures of the pCY1108-containing Tn10 host strain grown with 10 µg/ml Tet overnight gave no colonies when plated on selective medium lacking Tet (Fig. 4A, lane 1). However, in contrast to *E. coli*, when a parallel experiment was performed on the wild type LT2 host strain carrying pCY1108 and pACYC184, small colonies were formed on the plates lacking tetracycline suggesting that the pACYC184 TetA(C) efflux pump was less effective than the Tn10 TetA(B) pump in *S. enterica* (Fig. 4A, lane 2). Plasmid preparations from the pACYC184 plus CY1108 cultures grown with 10 µg/ml Tet showed that high levels of pACYC184 were present (Fig. 4B, lane 3) and thus the observed difference between *E. coli* and *S. enterica* must be at another level (e.g., TetA(C) expression, membrane insertion or function). Consistent with this interpretation, when the Tet concentration of overnight cultures was decreased to 1 µg/ml, no small colonies were formed on selective medium lacking Tet (Fig. 4A, lane 3). Finally, the pCY1108 contents of overnight cultures of the *S. enterica* Tn10 host strain grown with 0.1, 1.0 or 10 µg/ml Tet showed a modest dependence on inducer concentration similar to that seen in *E. coli* (Fig. 4C).

The pCY1108 and pCY1109 plasmids are available from the author and may be freely disseminated. An air courier account number should be provided to facilitate shipping. The compiled sequences of plasmids pCY1108 and pCY1109 have submitted to GenBank and their respective accession numbers are KU667315 and KU667316. *E. coli* host strain ER2984 is available upon request from New England Biolabs.

1.5 Conclusions

Plasmids pCY1108 and pCY1109 provide new vectors that are stably maintained but readily eliminated and that are compatible with IPTG inducible promoters. Rapid elimination requires the presence of a tetracycline efflux pump but these are readily supplied. A possible simplification of the system would be to place a gene encoding a tetracycline efflux pump on the Tet-dependent plasmid. However, this would tie the efflux pump protein level to plasmid copy number and these parameters could work at cross purposes since efflux competes with Tet-dependent replication. Indeed, attempts to replace the β -lactamase gene of pCY1108 with the pBR322 Tet efflux pump gene resulted in transformed colonies that failed to grow upon subsequent restreaking or in liquid medium. The same result was seen when transformations were plated with differing Tet concentrations. The failure of the approach could be due to efflux outstripping Tet entry such that replication was not stably maintained or toxic accumulation of excess efflux pumps in the cytoplasmic membrane (Eckert and Beck, 1989; Lee and Edlin, 1985; Lenski and Bouma, 1987; Moyed et al., 1983; Nguyen et al., 1989). A combination of these factors and perhaps others also seems possible.

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Highlights

- Conditional Replication
- Efflux
- TetR
- Tetracycline

Fig. 1.

Assembly of plasmids pCY1107, pCY1108 and pCY1109. The construction proceeded in several steps. The KpnI site present in one of the pAM34 multiple cloning sites was removed by BamHI digestion. *In vivo* resolution of the resulting palindrome deleted the BamHI site a about 120 additional bp to give pAM34 BamHI. The *lacZYA* promoter and *lacI* gene of pAM34 BamHI were then replaced with the Tet regulatory region by ligating the NheI(B)-KpnI fragment of pWQ572 to the AatII(B)-KpnI fragment of pAM34 BamHI to give the Tet-dependent ampicillin-resistant plasmid pCY1103. The PciI-FspI fragment of pCY1103 was then replaced with the same fragment of pAM34 to restore the *aadA* gene, the two multiple cloning sites and the two omega (Ω) elements to give pCY1107. The redundant EcoRI and KpnI sites plus the intervening NcoI site were removed from pCY1107 by replacing its 33 bp BaeI fragment with a synthetic cassette of the same length that restored the *O*₁ operator of the *tetA-tetR* control region and introduced a BsiWI site resulting in pCY1108. Plasmid pCY1109, a kanamycin-resistant and ampicillin-sensitive derivative of pCY1108, was constructed by replacing most of the β-lactamase coding sequence with a

kanamycin cassette that is transcribed both from its native promoter and the β -lactamase promoter. The multiple cloning sites that bracket the *aadA* spectinomycin resistance gene are given at the base of the figure. Antibiotics were added as follows (in $\mu\text{g/ml}$) sodium ampicillin, 100; spectinomycin sulfate, 50; chloramphenicol, 10; anhydrotetracycline HCl, 0.01 or 0.1 and tetracycline HCl, 10. The antibiotics were from the Sigma Chemical Company except anhydrotetracycline HCl (Cayman Chemical Company). Cloning procedures were as previously described (Chakravarty and Cronan, 2015; Cronan, 2006).

Fig. 2.

Panel A. The gel was loaded with 10% of pCY1107 plasmid preparations made from 2 ml of cultures normalized to an O.D. of 3.0 (Chakravartty and Cronan, 2015). Panel B. The gel was loaded with 10% of *Sma*I digested plasmid preparations made from 2 ml of cells grown to an O.D. of 1.0 (Chakravartty and Cronan, 2015). All cultures were LB medium containing spectinomycin. Lanes 1–8 are strain ER2984 cultures containing pCY1107 grown plus tetracycline (Tet) or anhydrotetracycline (AhTet) as shown. Lane 9 is pBAD322S in strain ER2984. Lanes 10 and 11 are pAM34 in strains ER2984 or MC1061 (MC1061 was used by Gil and Bouché (Gil and Bouche, 1991)), respectively. The copy numbers given are relative to that of the wild type pBR322 origin plasmid pBAD322S (lane 9) that was assigned the literature value of 20. The ligand concentrations are given in the units generally used for that ligand. The flanking lanes of panel B are size standards. The left lane contained the New England Biolabs 2-log DNA ladder whereas the right lane contained an *Eco*RI-*Hind*III digest of phage λ DNA. The sizes of the relevant bands are given in Kbp. The medium used was L broth (in g/l: tryptone, 10; yeast extract, 5 and NaCl, 5). Growth was at 37°C. Copy numbers were determined as previously described (Chakravartty and Cronan, 2015) except that DNA concentrations were determined by scanning ethidium bromide stained agarose gels. Gels loaded with differing known DNA concentrations were used to establish that the scans were within the linear range. The *E. coli* K-12 host strains used were MC1061 (*araD139* *(araA-leu)*7697 *(lac)*X74 *galK16 galE15 rpsL150(strR)*) and ER2984 (*F'* *proA⁺B⁺* *lacF^I* *lacZM15/fhuA2* *(lac-proAB)* *glnV galK16 galE15 R(zgb-210::Tn10)*Tet^R *endA1 thi-1* *(hsdS-mcrB)*5). Strain ER2984, is available from New England Biolabs. It is the parent of the New England Biolabs Tet sensitive strain NEB Turbo.

Fig. 3.

Curing of the Tet-dependent plasmid pCY1107. Fresh colonies were taken from plates containing various concentrations of either Tet or AhTet (top line of figure), dispersed and diluted in LB medium. Suitable dilutions (generally at least a 10^{-4} dilution of the dispersios) were spread on plates (top row of plates) that contained either 5 µg/ml Tet (lanes 1–3) or 100 ng/ml AhTet (columns 4 and 5). The same dilutions were spread on ampicillin-spectinomycin plates lacking any tetracycline (middle row of plates) whereas the bottom row of plates was spread with a 10-fold greater cell concentration (i.e., a 10-fold lesser dilution). The plates were incubated at 37°C for 24 h and scanned. The TetA(B) host strain was ER2984 whereas the Tet(C) strain was MC1061 carrying pACYC184 (maintained by chloramphenicol). MC1061 was the strain lacking an efflux pump.

Fig. 4. Curing and replication of pCY1108 in *S. enterica*

Panel A. Curing of Tet-dependent plasmid pCY1108 in *S. enterica*. Curing was determined exactly as in Fig. 3 except the spectinomycin concentration was doubled. In Column 1 Strain TT1374, a *panC::Tn10* derivative of *S. enterica* serovar Typhimurium strain LT2 transformed with pCY1108 was grown overnight with 10 μ g/ml Tet and then diluted and plated as in Fig. 3. In Columns 2 and 3 the wild type LT2 strain was transformed with a mixture of pCY1108 and pACYC184 and transformants carrying both plasmids were selected on medium containing tetracycline, spectinomycin, ampicillin and chloramphenicol. Cultures of these transformants were grown overnight on either 10 μ g/ml Tet (Column 2) or 1 μ g/ml Tet (Column 3) and then diluted and plated as in Fig. 3.

Panel B. Lanes 1–3. A plasmid preparation from an overnight culture of *S. enterica* LT2 carrying pACYC184 and pCY1108 grown in LB containing 10 μ g/ml Tet, ampicillin and spectinomycin was digested with either SacI or SacII or with both enzymes as shown. SacI

cuts pCY1108 once but does not cut pACYC184 whereas SacII cuts pACYC184 once but does not cut pCY1108. Lane 4 is a pCY1108 preparation from the Tn10 host TT1374 cut with SacI. The broken arrows identify the two plasmids whereas the small white arrows at the right edge denote (top to bottom the 6, 5 and 4 kbp bands of the Trackit standard (Tr). λ denotes the λ standard used in Fig. 2. Panel C. Lanes 1–3 contain plasmid preparations from overnight cultures of *S. enterica* strain TT1374 carrying pCY1108 grown with 0.1, 1 or 10 $\mu\text{g/ml}$ of Tet whereas lane 4 contains a plasmid preparation from *E. coli* ER2984 carrying pCY1108 grown with 10 $\mu\text{g/ml}$ of Tet.