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A Series of Medium and High Copy Number Arabinose-Inducible *Escherichia coli* Expression Vectors Compatible with pBR322 and pACYC184

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Abstract

The original pBAD24 plasmid and the derived lower copy number (the pBAD322 series) expression vectors have been widely used in *Escherichia coli*, *Salmonella enterica*, and related bacteria. However, a flexible pBAD expression system has been available only in pMB1 (ColE1) vectors. We report a series of pBAD vectors that replicate using the origin of plasmid RSF1030 that are compatible with pMB1 (ColE1) and p15A (pACYC) vectors. Both high (pBAD24) and medium (~pBAD322) copy number plasmids encoding resistance to ampicillin, chloramphenicol, kanamycin, tetracycline, spectinomycin/streptomycin, gentamycin, or trimethoprim are available.

Keywords

RSF1030; Copy number; Compatibility; pBR322; pBAD24; Expression levels; Toxicity

Introduction

Most molecular cloning and expression studies in *Escherichia coli* and its close relatives (e.g., *Salmonella enterica*) is performed using plasmids containing the replication origins of plasmids pMB1 (e.g., pBR322 and pUC19) or p15A (e.g., pACYC184) (Green and Sambrook, 2012). Plasmids derived from pMB1 can have either a medium (pBR322) or high (pUC19) copy number depending on the presence of the gene encoding Rop, a small (63 residue) protein that modulates copy number. The p15A-derived have a medium copy number (Atlung et al., 1999). This family of plasmids replicate by use of an RNA transcript called RNAII that provides a primer for DNA polymerase I (Davison, 1984). A second RNA called RNAI (or anti-primer) transcribed on the other DNA strand is completely complementary to RNAII and can block priming of DNA replication by hybridizing to

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RNAII (Davison, 1984). Rop acts by binding and stabilizing the RNAI-RNAII hybrid thereby resulting in decreased plasmid copy number. Rop appears specific to the pMB1 origin and is reported to have no effect on replication of p15A origin plasmids (Atlung et al., 1999; Magliery and Regan, 2004). Interestingly, binding of RNA by Rop recognizes structure rather than sequence: this four helix bundle protein recognizes the shape of an RNA hairpin (Magliery and Regan, 2004). RNAI controls not only copy number but also the incompatibility that occurs when two pMB1 derived plasmids are present in the same cell (Davison, 1984; Novick, 1987). The pMB1 and p15A derived plasmids encode different primers and anti-primers and thus the plasmids are compatible (Selzer et al., 1983; Tomizawa and Itoh, 1981). Although it was studied in the early investigations of plasmid replication, plasmid RSF1030 (RSF), another member of this family, has been neglected as a cloning vehicle (Conrad et al., 1979; Selzer et al., 1983; Som and Tomizawa, 1982). To our knowledge there is only a single commercially available RSF1030 origin plasmid, the RSF-1b protein expression plasmid. RSF plasmids are compatible with both pMB1 and p15A derived plasmids (Selzer et al., 1983; Som and Tomizawa, 1982) and thus it should be possible to have cells harboring three plasmids given different selection markers.

In our work on the regulation and mechanisms of synthesis of fatty acids and related coenzymes we have sometimes constructed *E. coli* strains carrying three compatible plasmids each expressing a protein of interest. In a recent example (Smith and Cronan, 2014) two of these plasmids were pMB1 and p15A origin plasmids but the third had to be a low copy plasmid having the origin of pSC101. Although the low copy number of pSC101 origin plasmids was of advantage in this particular investigation, there was no alternative compatible plasmid with a copy number similar to that of the pMB1 and p15A plasmids available. In 2000 Phillips and coworkers (Phillips et al., 2000) reported a series of RSF origin cloning vectors. Although, these plasmids were useful, they lacked the plasmid stabilizing transcription termination sequences plus the strong promoter and appropriately spaced ribosome binding sequences generally found in expression vectors. However, these workers provided a valuable tool by selecting and characterizing a single point mutation within the replication origin that greatly increased plasmid copy number (Phillips et al., 2000).

Our laboratory previously reported the construction of medium copy versions of the widely used arabinose inducible expression plasmid, pBAD24 (Guzman et al., 1995) that encoded Rop and a diverse set of antibiotic resistance cassettes (Cronan, 2006). These plasmids (called the pBAD322 series because the pBR322 origin was reconstructed) have been supplied to over one hundred laboratories and because their dissemination was encouraged, these plasmids are probably found in many other laboratories. Given the success of these vectors it seemed likely that a parallel set of vectors having the RSF origin would be generally useful because their copy numbers would be similar to those of the pBAD322 vectors. Moreover, it seemed that the high copy number mutant with the RSF origin might match the copy number of the original pBAD24 plasmid. Note that in biochemical experiments to be submitted elsewhere we have used a three plasmid system (pMB1, pA15 and RSF1030 origins). At the phenotypic level the elements encoded by each of these plasmids behaved as expected from strains carrying a single plasmid.

Construction of the pBAD-RSF plasmids

The overall scheme used to construct the pBAD-RSF plasmids is outlined in Fig. 1. The first step in construction of the pBAD-RSF plasmids was to ligate a 3365 bp PciI-NgoMIV fragment containing the RSF origin plus a kanamycin resistance determinant purified from either the pDLK29 (medium copy) or pDHK29 (high copy) vectors (Phillips et al., 2000) to a 1880 bp BspHI-NgoMIV fragment of the p15A-derived plasmid, pBAD33 (Guzman et al., 1995). This ligation resulted in the kanamycin-resistant chloramphenicol-sensitive plasmids, pCY1012 and pCY1013, respectively (PciI and BspHI make compatible ends). The pBAD33-derived sequences replaced the truncated *lacZ* gene of the pDHK plasmids and included the *araC* gene, the pBAD promoter and a multiple cloning site having a downstream transcription termination sequence. However, the multiple cloning site (MCS2) lacked the sequences needed for initiation of translation of genes missing such sequences. To provide these elements the BssHII-HindIII fragments of plasmids pCY1012 and pCY1013 were exchanged with that of pBAD322C (Cronan, 2006) resulting in plasmids pCY1014 and pCY1015, respectively (Fig. 1). Having reconstructed the pBAD322/pBAD24 expression module the next task was to exchange the kanamycin resistance determinant with cassettes encoding the panel of antibiotic resistance determinants (Dennis and Zylstra, 1998a; Dennis and Zylstra, 1998b) used in construction of the pBAD322 plasmids (Cronan, 2006). However, the restriction map we obtained for the kanamycin resistance region failed to match that deduced from the sequence provided with the plasmids. Hence, we excised that resistance determinant and replaced it with the spectinomycin resistance cassette of plasmid p34s-Sm2 (Dennis and Zylstra, 1998b) obtained by digestion with KpnI followed by treatment with T4 DNA polymerase plus the 4 dNTPs to remove the 3' overhangs. The resulting blunt-ended cassette was purified and ligated to the 2508 bp HpaI-NaeI fragment of plasmids pCY1014 and pCY1015 to give plasmids pCY1016 and pCY1017, respectively (Fig. 1). In these plasmids the two SacI sites bracket the spectinomycin resistance cassette, a feature common to the antibiotic resistance determinants of the p34s plasmids (Dennis and Zylstra, 1998a; Dennis and Zylstra, 1998b). Straightforward substitution of the SacI-spectinomycin-SacI cassette with the other SacI cassettes resulted in a set of RSF origin arabinose expression plasmids carrying resistance to ampicillin, kanamycin, chloramphenicol, gentamicin, trimethoprim or tetracycline (Table 1). The set contains both the medium and high copy plasmids that carry each of the resistance cassettes except that for tetracycline where the high copy plasmid could not be assembled. This presumably was due to the decreased *E. coli* inner membrane function that results from the high level of expression of tetracycline exporter proteins (Eckert and Beck, 1989; Lee and Edlin, 1985; Moyed et al., 1983).

Plasmid stability and contents in different host strains

The relative contents of the constructed plasmids were determined by quantitation of plasmid preparations by spectrophotometry (Fig. 2) and in a few cases by quantitative PCR (qPCR) analysis of *araC* copy number. Spectrophotometric quantitation provided a direct measure of plasmid concentration because gel electrophoresis indicated that the plasmid preparations were free of chromosomal DNA and RNA. To avoid the loss of plasmids due to high levels of β -lactamase production which can overcome the plasmid maintenance

selection, plasmids encoding chloramphenicol resistance were used. The pBAD1030C and pBAD1031C plasmids were compared with pBAD322C and an ampicillin-sensitive chloramphenicol-resistant derivative of plasmid pBAD24 called pBAD24C (described below). A p15A origin plasmid, pSU21 (Bartolome et al., 1991), was also included. The relative content of plasmid pBAD1031C carrying the mutant RSF origin was found to somewhat exceed that of pBAD24C, the high copy pMB1-derived plasmid lacking *rop*. This was expected from the prior work of Phillips and coworkers (Phillips et al., 2000). In contrast the plasmid carrying the wild type RSF origin, pBAD1030C, had a content quite similar to that of the pMB1 *rop*-containing plasmid, pBAD322C. The relative contents of the two RSF derived plasmids differed by about 8-fold whereas the two pMB1-derived plasmids showed a 5-fold difference (Table 2). We found similar content ratios by qPCR using a derivative of strain MC1061 carrying a wild type *araC* gene plus plasmid pSU21 (which lacks *araC* and allowed growth in the presence of chloramphenicol) as the chromosomal standard (data not shown). The copy numbers obtained were several fold lower than those estimated above. This seems likely to be explained by the fact that rapidly growing *E. coli* cells contain multiple replication forks such that multiple copies of the chromosomal *araC* gene would be present in the control strain (*araC* is located close to the origin of chromosomal replication). The uncertain chromosomal *araC* copy numbers led us to abandon this approach to determine plasmid copy numbers and we turned to plasmid contents. Moreover, since plasmid copy numbers are often altered upon their intended use as cloning vectors, they cannot be taken as fixed. Indeed, even for empty vectors having the same replication origin, the copy numbers reported in the literature vary as much as ten-fold.

Two host genes are known to decrease the copy numbers of pMB1 and p15A plasmids {Davison, 1984 #31; Saramago, 2014 #18; Sarkar, 1996 #15}. These are *polA* and *pcnB* which encode DNA polymerase I and poly (A) polymerase, respectively. DNA polymerase I (*polA*) has already been shown to be required for RSF1030 replication (Grindley and Kelley, 1976) but *pcnB* had not been tested. PcnB regulates plasmid replication by adding a string of adenylates to the 3'-end of RNAI molecules. Addition of this polyadenylation sequence targets RNAI (the anti-primer) for degradation. In the absence of PcnB high levels of RNAI accumulate which compete with priming of DNA replication by RNAII (Sarkar, 1996) and thereby decrease plasmid copy numbers. We found that the lack of PcnB resulted in decreased copy numbers of all the tested plasmids, although the decrease tended to be less for the RSF-derived plasmids and for the plasmids of high copy number (Table 2).

We expected that the host range of the new plasmids would extend to *Salmonella enterica* serovar Typhimurium because the plasmid was first isolated from *S. enterica* serovar Panama (Heffron et al., 1975). However, we tested this directly because the original *S. enterica* strain harbored a second plasmid that could have provided a helper function and plasmid manipulations are known to alter plasmid host range (Hoang et al., 1999). Transformation of *S. enterica* serovar Typhimurium with plasmid pBAD1030C carrying the wild type RSF origin proceeded readily. In contrast, several attempts to transform this bacterium with the high copy mutant RSF origin plasmid, pBAD1031C, gave numerous small colonies indicating successful entry of the plasmid. However, in all cases these colonies failed to grow further and to restreak. We conclude that, unlike *E. coli*, replication

of the high copy number RSF origin in *S. enterica* is limited by the supply of a host protein(s) required for plasmid replication.

The pBAD RSF plasmids are available from the second author and may be freely disseminated. We ask only that an air courier account number be provided to facilitate shipping. The compiled sequences of the plasmids have submitted to GenBank

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Highlights

- RSF1030 origin plasmids are compatible with standard cloning plasmids.
- The RSF1030 origin plasmids are available in both medium and high copy versions.
- A series of arabinose inducible expression vectors with the differing RSF1030 origins have been constructed with a wide variety of antibiotic selections.
- The plasmids are freely disseminable.

Fig. 1.

Assembly of the parental RSF plasmids pCY2016 (pBAD1030S) and pCY2017 (pBAD1031S). Plasmid pBAD33 was chosen as the donor of the arabinose expression cassette due to its favorable restriction sites. The MCS2 multiple cloning site of the resulting plasmids was replaced with the MCS3 of pBAD24 (bottom of the figure). The MCS sequences are named as in Guzman et al., (Guzman et al., 1995). Note that the MCS BamHI site is not unique because a second site lies within one of the AraC binding sites. The restriction sites are: Ng, NgoMIV; Ne, NaeI (which cut the same sequence); B, BspHI; K, KpnI; S, SacI; P, PciI; Bss, BssHII and H, HpaI. All cloning steps were performed in *E. coli* K-12 NEB Turbo except for those involving tetracycline resistance which used strain MC1061.

Fig. 2.

Plasmid contents of various host strains

Panel A. *E. coli* strain MC1061 carrying plasmids pBAD1031C, pBAD1030C, pSU21, pBAD322C or pBAD24C. Plasmid SU21 was also transformed into an *ara*⁺ derivative of MC1061 as an internal control (lanes marked *ara*⁺). The cultures were grown overnight (ON) (OD₆₀₀ of ~3) or to early stationary phase (ES) (OD₆₀₀ of 1.0). The plasmid preparations were made in parallel from 2 ml cultures grown in LB medium supplemented with 25 µg/mL chloramphenicol. Five µl (one-tenth) of each preparation was subjected to gel electrophoresis on 1% agarose gels. Lane M is the TrackIt 1 Kb DNA Ladder (Life Technologies) markers. Panel B. *E. coli* strain MC1061 carrying plasmids pBAD1031C, pBAD1030C, pSU21, pBAD322C or pBAD24C were grown overnight in LB medium supplemented with 25 µg/ml of chloramphenicol. Plasmid preparations were made in parallel from 2 mL cultures normalized to OD₆₀₀ of 3.0. Five µl of each 50 µl preparation

was digested with KpnI-HF (New England Biolabs) and the products were analyzed by gel electrophoresis on a 1% gel. Lane M is the TrackIt 1-Kb DNA ladder (Life Technologies) markers. Panel C. Relative plasmid contents obtained from either *E. coli* MC1061 or *S. enterica* serovar Typhimurium strain LT2. To facilitate comparisons the content of *E. coli* MC1061 carrying pBAD1030C was assigned a value of 100 (the actual values are given in Table 2). A representative experiment is shown. Plasmid preparations were made in parallel from 2 ml cultures normalized to OD₆₀₀ of 3.0. Panel D. Effects of a *pcnB* mutation on plasmid contents. The *E. coli* wild type strain MC1061 or strain MG1655 *pcnB::kan* carrying plasmids pBAD1031C or pBAD1030C were compared. *S. enterica* carrying pBAD1030C was also analyzed. Overnight cultures were grown in LB medium supplemented with 25 µg/ml chloramphenicol and 25 µg/ml kanamycin were processed as in Panel C and digested with KpnI-HF as in panel B.. For these experiments a chloramphenicol-resistant ampicillin-sensitive derivative of plasmid pBAD24 called pBAD24C was constructed by blunt end ligation of the 2987 bp AclI fragment of pBAD24 to a p34s-derived chloramphenicol cassette obtained by KpnI digestion followed by blunting of the single stranded ends by treatment with T4 DNA polymerase and the four dNTPs.

Table 1**Plasmids Constructed**

Wild Type Plasmid Origin	Ab^R Orientation	Mutant Plasmid Origin	Ab^R Orientation	Antibiotic Resistance
pBAD1030A	Syn	pBAD1031A	Anti	Ampicillin
pBAD1030C	Syn	pBAD1031C	Syn	Chloramphenicol
pBAD1030G	Anti	pBAD1031G	Syn	Gentamicin
pBAD1030K	Anti	pBAD1031K	Anti	Kanamycin
pBAD1030S	Syn	pBAD1031S	Syn	Spectinomycin
pBAD1030T	Syn	Not recovered	-	Tetracycline
pBAD1030Tp	Syn	pBAD1031Tp	Anti	Trimethoprim

^aThe orientations of the antibiotic cassettes are given relative to the orientation of the *araBAD* promoter. Syn denotes the same orientation whereas Anti is the opposite orientation. In most cases both orientations were recovered. The antibiotic cassettes are those from the p34s plasmids (Dennis and Zylstra, 1998a; Dennis and Zylstra, 1998b) with two exceptions. The chloramphenicol resistance cassette was that used in construction of pBAD322C (Cronan, 2006) and the ampicillin resistance cassette was obtained by PCR amplification of the β -lactamase gene of plasmid pKD3 (Datsenko and Wanner, 2000) using primers CCACGAGCTCGGAAATGTGCGCGGAACC and CCACGAGCTCGATCTTCACCTAGATCC (the primer SacI sites are underlined). In naming these plasmids we adopted the nomenclature used for the pBAD322 plasmids in which the number designation appended to the pBAD designation denotes the replication origin of the parent plasmid and a single letter denotes the antibiotic resistance (except for trimethoprim where Tp is used). Hence, the ampicillin resistant plasmid having the wild type origin is called pBAD1030A. However, naming the plasmids carrying the mutant (high copy) origin was problematical because, strictly speaking, the origin is no longer that of RSF1030. For this reason we denote this origin as 1031 and thus the ampicillin resistant plasmid having the mutant high copy number origin is called pBAD1031A. The Genbank accession for the pBAD1030 series are pBAD1030A, KP899255; pBAD1030C, KP899256; pBAD1030G, KP899257; pBAD1030K, KP899258; pBAD1030S, KP899254; pBAD1030T, KP899260 and pBAD1030Tp, KP899259. The sequences of the pBAD1031 vectors can be constructed from these sequences by changing nucleotide 2366 from G to T. If *in silico* inversion of antibiotic resistance cassette orientation is required, the central GC of the two SacI sites can be used. Note that neoschizomers of SacI that cut the central SacI GC sequence (e.g., EcoICRI) are known.

Table 2

DNA concentrations of plasmid preparations from overnight cultures

Plasmid	Host Strain	[DNA] ^a (ng/μL)
pBAD1030C	MC1061	28, 45
pBAD1031C	MC1061	225,141
pBAD322C	MC1061	35
pBAD24C	MC1061	142
pSU21	MC1061	35
pBAD1030C	LT2	41, 47
pBAD322C	LT2	33
pBAD24C	LT2	72
pSU21	LT2	67
pBAD1030C	<i>pcnB::kan</i>	9,10
pBAD1031C	<i>pcnB::kan</i>	74, 88
pBAD322C	<i>pcnB::kan</i>	8
pBAD24C	<i>pcnB::kan</i>	18
pSU21	<i>pcnB::kan</i>	7

^aThe plasmid preparations are grouped by host and were quantitated by absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo-Pierce). In some cases the values from two independent biological experiments done several weeks apart are given.

^bThe bacterial cultures were grown at 37 °C and growth was measured by absorbance at 600 nm of suitable dilutions using a Beckman DU600 spectrophotometer. The DNA concentrations are normalized to 0.2 ml of culture having an absorbance of 3.0.