Gene 637 (2017) 14–24

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methodological paper

Module-based systematic construction of plasmids for episomal gene expression in fission yeast

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ARTICLE INFO

Keywords:
Fission yeast
Plasmid
Expression vector
Golden Gate DNA shuffling

ABSTRACT

The fission yeast *Schizosaccharomyces pombe* is a powerful model organism for cell biology and molecular biology, as genetic manipulation is easily achieved. Introduction of exogenous genes cloned in episomal plasmids into yeast cells can be done through well-established transformation methods. For expression of genes in *S. pombe* cells, the multi-copy plasmid pREP1 and its derivatives, including pREP41 and pREP81, have been widely used as vectors. Although recent advancement of technology brought a number of useful genetic elements such as new promoters, selection marker genes and fluorescent protein tags, introduction of those elements into conventional pREP1 requires a large commitment of both time and effort because cloning procedures need to be repeated until the final products are constructed. Here, we introduce materials and methods to construct many pREP1-type plasmids easily and systematically using the Golden Gate shuffling method, which enables one-step ligation of many DNA fragments into a plasmid. These materials and methods support creation of expression plasmids employing a variety of novel genetic elements, which will further facilitate genetic studies using *S. pombe*.

1. Introduction

Expression of exogenous genes in cells using extrachromosomal plasmids is frequently conducted to investigate the effects of the gene products on cells. For the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), a wide variety of expression vectors have been invented and frequently used (Fennessy et al., 2014; Forsburg, 1993; Giga-Hama and Kumagai, 1999; M. B. Moreno et al., 2000; Siam et al., 2004). These include pREP1 and its derivatives (pREP2, pREP41, among others) (Basi et al., 1993; Forsburg, 1993; Maundrell, 1993) and pART1 (McLeod et al., 1987).

pREP1 contains a promoter derived from the gene *nmt1* (no message in thiamine; encoding 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase), which is one of the most highly expressed genes in *S. pombe* (Maundrell, 1990; Verma et al., 2014). Transcription from the *nmt1* promoter (Pnmt1) can be repressed by addition of thiamine to the medium, whereas expression can be induced in the absence of thiamine (Maundrell, 1990). Thus, Pnmt1 is commonly accepted as an inducible/repressible "strong" promoter. In pREP41 and pREP81, mutations were introduced to the TATA box of the promoter to weaken its activity (Basi et al., 1993). Namely, pREP41 contains a moderate-activity promoter (Pnmt41), whereas pREP81 contains a weaker promoter (Pnmt81), and both plasmids retain sensitivity to the thiamine concentration in the medium.

pREP plasmids also contain an autonomously replicating sequence (*ars1*) (Heyer et al., 1986), which is necessary and sufficient for plasmid replication in *S. pombe*; the plasmids also have a selection marker gene such as *LEU2* (derived from the budding yeast *Saccharomyces cerevisiae*) (Beggs, 1978) and *ura4*$^+$ (from *S. pombe*) (Grimm et al., 1988). pREP vectors that contain *ura4*$^+$ along with Pnmt1, Pnmt41 and Pnmt81 are named pREP2, pREP42 and pREP82, respectively (Basi et al., 1993).

The dominant selection marker genes *kan*, *nat*, *hph* and *bsd*, which confer resistance against the specific antibiotics G418, clonNAT, hygromycin B and blasticidin S, respectively, are used routinely during for

Abbreviations: PCR, polymerase chain reaction; GOI, gene of interest; FPtag, fluorescent protein tag; FPcontrol, fluorescent protein for control experiments; GFP, green fluorescent protein; CFP, cyan fluorescent protein; pRGG, pREP-type expression plasmid by the Golden Gate method

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http://dx.doi.org/10.1016/j.gene.2017.09.030
Received 27 March 2017; Received in revised form 13 September 2017; Accepted 15 September 2017
Available online 19 September 2017
0378-1119/

chromosomal integration (Bähler et al., 1998; Hentges et al., 2005; Kimura et al., 1994; Sato et al., 2005). These markers have been used only rarely, however, when constructing episomal expression plasmids, most likely because none of these antibiotics except clonNAT work effectively in standard minimal medium when ammonium chloride is used as the sole nitrogen source (Ahn et al., 2013; Hentges et al., 2005; Matsuyama and Yoshida, 2012).

pART1 contains the promoter of *S. pombe adh1* (Padh1) (McLeod et al., 1987). *adh1* is highly expressed in *S. pombe*, but still at a lower level than *nmt1* (Forsburg, 1993). Similar to Pnmt1, Padh1 has two mutant variants with weaker promoter strength, namely Padh41 and Padh81, and each of these promoters express the downstream gene constitutively (Kawashima et al., 2007; Yokobayashi and Watanabe, 2005). However, Padh41 and Padh81 have not been used extensively in plasmids for *S. pombe* studies.

When a gene of interest (GOI) is expressed, a fluorescent protein tag (FPtag) can be fused to either the N- or C-terminus. FPtags include GFP (green fluorescent protein), mCherry (red fluorescent protein) or CFP (cyan fluorescent protein). This can be achieved by preparing the fusion gene, i.e., GOI plus FPtag, and by cloning the fusion gene into an expression plasmid. Indeed, pREP-based plasmids have been utilized for epitope tagging although the variety of epitope tags is limited (Craven et al., 1998; Forsburg and Sherman, 1997). Thus, the analysis of the cellular effects of an artificially expressed GOI may require many types of plasmids that encode variously colored fluorescent proteins, differential promoter strengths, and various selection markers.

The preparation of a series of varying expression vectors containing such genetic elements would require repeated rounds of DNA cloning, entailing ligation, minipreps, etc. As such, the recent development of new genetic elements has challenged researchers with regard to preparing large numbers of expression plasmids that contain these new elements. We previously introduced materials for constructing plasmids destined to be integrated into *S. pombe* chromosomes (Kakui et al., 2015). The system utilizes the Golden Gate plasmid shuffling method (Agmon et al., 2015; Engler et al., 2009, 2008; Engler and Marillonnet, 2014), which enables on-demand construction of various plasmids containing various gene elements in a one-step/single-pot reaction (see below for details). Although integration plasmids can be created with such materials and methods, it would be useful to similarly prepare episomal plasmids for gene expression. Episomal plasmids facilitate easy transformation of any host strain without genetic crossing, and a 'drop-off' strategy can be chosen when transformed cells are cultivated in non-selective media (Forsburg, 2001). For instance, plasmid drop-off is useful when a non-conditional null phenotype is desired. It is also useful when counter selection is required, e.g., cells that lose plasmids carrying the *ura4*$^+$ marker gene survive in the presence of 5-fluoroorotic acid.

The expansion of the Golden Gate methodology towards construction of episomal plasmids like pREP has removed the barrier that impedes smooth construction of such plasmids for expression of exogenous genes in *S. pombe*. The Golden Gate method has the advantage of using the type II restriction enzyme *Bsa*I. This enzyme recognizes a specific sequence but generates cohesive ends for various sequences. We have prepared 35 plasmids that harbor various kinds of genetic elements (called 'modules'), and these plasmids can be used directly for systematic construction of *S. pombe* integration plasmids (Kakui et al., 2015). Here we describe methods to construct expression plasmids similar to pREPs via the Golden Gate plasmid shuffling method using a new vector module plasmid in combination with a number of existing module plasmids that contain various gene elements.

2. Materials and methods

2.1. Construction and origin of module plasmids

Modules for promoters, FPtags, and a terminator with marker genes were created previously (Kakui et al., 2015), and these elements are used to construct pREP-type plasmids. Briefly, a genetic element(s) in each module was PCR-amplified with a pair of primers that contain particular *Bsa*I sites and was cloned in pCR-Blunt II-TOPO using the cloning kit (Invitrogen). The names ('a'–'g') and their corresponding sequences of *Bsa*I cohesive ends, names of module groups (except for "group IX") in this study are the same as originally defined in (Kakui et al., 2015). For promoter modules (group I), each promoter sequence was amplified to add *Bsa*I sites named a and b, at upstream and downstream ends, respectively. The amplified product (a-Promoter-b) was cloned into vector pCR-Blunt II-TOPO. Two types of FPtags have been prepared: the FPtag-C module for tagging at the C-terminal end and FPtag-N at the N-terminal end of a GOI. FPtag-C modules (group III) were similarly constructed; the sequence encoding a linker sequence was placed in front of an FPtag and flanked by *Bsa*I sites (c-FPtag-d). FPtag-N modules (group VII) contain an FPtag gene followed by the linker sequence (b-FPtag-c).

Terminator + marker modules (group IV) contain the terminator of *adh1* (Tadh) followed by a selection marker gene that confers resistance against an antibiotic, such as *kan* (G418-resistance), *hph* (hygromycin B), *nat* (clonNAT) or *bsd* (blasticidin S), flanked by *Bsa*I sites (d-[Tadh + Marker]-e in the vector). In a previous study, we developed a number of module plasmids containing the *adh1* terminator (Kakui et al., 2015), which has been used in vector pFA6a (Bähler et al., 1998). We used the previous modules containing the *adh1* terminator in this study to construct expression plasmids, although canonical pREP1 vectors use the *nmt1* terminator. Alternatively, a combination of a terminator module (group IVa: d-Term-g) and a marker module (group IVb: g-Marker-e) can be used instead of a group IV module. Kakui et al. utilized the Tadh module for group IVa (Kakui et al., 2015) and auxotrophic marker modules *ura*4$^+$ and *LEU2* as well as antibiotic resistance marker modules *kan* (conferring resistance to geneticin/G418), *hph* (hygromycin B), *nat* (clonNAT) and *bsd* (blasticidin S) for group IVb. All modules were cloned into pCR-Blunt II-TOPO.

GOI can be designed to construct fusion genes with fluorescent protein (FP) genes (group II and group VIII, for C-terminal and N-terminal FP tagging, respectively). In examples shown in Figs. 3 and 4, the *atb2*$^+$ coding sequence was chosen as a GOI. The fragment of the *atb2*$^+$ coding sequence was prepared via PCR with a pair of oligonucleotides 5′-aaaa**GGTCTC**aCTGAaaATGAGAGAGATCATTTCC-3′ and 5′-aaaa**GGTCTC**aAGACTTAGTACTCTTCTTCCATG-3′ (bold bases indicate *Bsa*I recognition sites). Underlined bases indicate *Bsa*I cohesive ends ('c': CTGA and reverse complement of 'd': AGAC).

The pREP-type expression vector module (termed pBMod-exv in the new 'group IX') was constructed in this study as follows (see Fig. 1B). A part of pREP1 that includes the *colE1* origin, the ampicillin resistance gene (*Amp*) and *ars1* was PCR-amplified, during which *Bsa*I sites were added at both ends. The *Bsa*I site termed 'a' (overhang sequence, GTGC) was placed on the *ars1* side, whereas the *Bsa*I site 'e' (ATTC) was placed on the *colE1ori-Amp* side. The primer set was: forward, 5′-TTTTT**GGTCTC**aGCACttttttGCGGCCGCGAATTCGAGTCTAACTCCTTAACC-3′, and reverse, 5′-TTTTT**GGTCTC**aATTCttttttGCGGCCGCAAGCTTGGCGTAATCATGG-3′, where bold and underlined bases indicate *Bsa*I recognition sites and cohesive ends ('a' in reverse complement and 'e'), respectively. Italic sequences indicate *Not*I sites, and lowercase letters represent bases added as spacer. The amplified fragment "e-[*colE1ori-Amp-ars1*]-a" (4377 bp) was cloned into pCR-Blunt II-TOPO (Invitrogen). An internal *Bsa*I site that resides in the *Amp* gene was then eliminated through site-directed mutagenesis as described (Kakui et al., 2015) so that *Amp* in the vector could not be cleaved during the Golden Gate reaction. The resulting plasmid was used as an 'expression vector module' and was termed pBMod-exv (total size: 7896 bp, see Fig. 1B). The cloning vector pCR-Blunt II-TOPO includes an internal *Bsa*I site (with the cohesive end GTTA; see Fig. 2C), although this does not affect yields of final products.