New and Redesigned pRS Plasmid Shuttle Vectors for Genetic Manipulation of Saccharomyces cerevisiae

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ABSTRACT We have constructed a set of 42 plasmid shuttle vectors based on the widely used pRS series for use in the budding yeast Saccharomyces cerevisiae and the bacterium Escherichia coli. This set of pRSII plasmids includes new shuttle vectors that can be used with histidine and adenine auxotrophic laboratory yeast strains carrying mutations in the genes HIS2 and ADE1, respectively. Our pRSII plasmids also include updated versions of commonly used pRS plasmids from which common restriction sites that occur within their yeast-selectable biosynthetic marker genes have been removed to increase the availability of unique restriction sites within their polylinker regions. Hence, our pRSII plasmids are a complete set of integrating, centromere and 2µ episomal plasmids with the biosynthetic marker genes ADE2, HIS3, TRP1, LEU2, URA3, HIS2, and ADE1 and a standardized selection of at least 16 unique restriction sites in their polylinkers. Additionally, we have expanded the range of drug selection options that can be used for PCR-mediated homologous replacement using pRS plasmid templates by replacing the G418-resistance kanMX4 cassette of pRS400 with MX4 cassettes encoding resistance to phleomycin, hygromycin B, nourseothricin, and bialaphos. Finally, in the process of generating the new plasmids, we have determined several errors in existing publicly available sequences for several commonly used yeast plasmids. Using our updated sequences, we constructed pRS plasmid backbones with a unique restriction site for inserting new markers to facilitate future expansion of the pRS series.

KEYWORDS Saccharomyces cerevisiae plasmid shuttle vector auxotrophic marker drug resistance marker polylinker/multiple cloning site

The budding yeast Saccharomyces cerevisiae is an important and widely used model system for studying eukaryotic cell biology that has also become important in the new fields of functional genomics and systems biology (Botstein and Fink 2011). Among the most important tools available for the genetic manipulation of S. cerevisiae are plasmid shuttle vectors, which can be used in both S. cerevisiae and the bacterium Escherichia coli (Da Silva and Srikrishnan 2011; Iserentant 1990). In addition to an antibiotic resistance marker and a bacterial replication origin for propagation in E. coli, these shuttle vectors contain a second yeast-selectable marker. The latter marker is typically either a gene that confers resistance to antibiotics or antifungal toxins (Van Den Berg and Steensma 1997) or a biosynthetic gene that rescues an auxotrophic mutation (Pronk 2002). Today, most commonly encountered S. cerevisiae shuttle vectors belong to one of three classes (Da Silva and Srikrishnan 2011; Iserentant 1990; Romanos et al. 1992): (1) integrating plasmids (YIp), which lack yeast replication origins, must be inserted into the yeast genome in order to be replicated; (2) centromere plasmids (YCp), which contain both a yeast centromere (CEN) and an autonomously replicating sequence (ARS), are replicated in yeast at single or very low copy number; or (3) yeast episomal plasmids (YEp), which contain a 2µ circle replication origin and the cis-acting STB (stability) locus (Mehta et al. 2002), are replicated autonomously in yeast at high copy number.

Among the shuttle vectors most frequently used today by researchers working with S. cerevisiae are the YXplac series (Gietz and Sugino 1988) and the pRS series (Brachmann et al. 1998; Christianson et al. 1992; Sikorski and Hieter 1989). The systematic design and utility of these plasmids have inspired the construction of similar plasmid sets for use in other fungal model organisms (Adams et al. 2005; Chen 1996; Gould et al. 1992). The YXplac series is based...
on the plasmid pUC19 (Gietz and Sugino 1988), whereas the pRS series uses a hybrid backbone built using either the pBluescript or pBluescriptII polylinker/multi-cloning site (MCS) ligated to the pBluescript replication origin (Christianson et al. 1992; Sikorski and Hieter 1989). Compared with older and larger pBR322-based yeast vectors (Botstein et al. 1979; Kuo and Campbell 1983; Tschumper and Carbon 1980), both the YXplac and pRS series offer important advantages of small plasmid sizes (<7 kb), high copy number in bacteria, a good range of unique sites for cloning, the capacity for blue-white screening, and a range of yeast-selectable markers. These markers include the \textit{S. cerevisiae} biosynthetic genes TRP1, LEU2, and URA3, which can be used with almost all commonly encountered laboratory strains that are auxotrophic for tryptophan, leucine, or uracil, respectively. However, there are limitations to both series with respect to histidine and adenine auxotrophy. First, the YXplac series does not include prototrophic markers that can be used with almost all commonly encountered laboratory strains. Second, although the pRS series does include plasmids marked with either \textit{HIS3} (Christianson et al. 1992; Sikorski and Hieter 1989) or \textit{ADE2} (Brachmann et al. 1998), not all common laboratory strains that are \textit{His}− and/or \textit{Ade}− carry \textit{HIS3} and/or \textit{ADE2} mutations; the strains BF264-15D (abbreviated as 15D) (Reed et al. 1985), J17 (Fitzgerald-Hayes et al. 1982), and B93 (Vezhinet et al. 1991) are examples of \textit{his2 ade1} mutants. Given that auxotrophic markers are important for facilitating the genetic manipulation of \textit{S. cerevisiae} (Pronk 2002), the inability to conveniently exploit all the available auxotrophic markers in a given laboratory strain is an unfortunate limitation.

Our need to exploit the \textit{his2} mutation in 15D inspired the construction of the integrating plasmid pRS036H2 (Chee and Haase 2010). Despite its utility, pRS036H2 suffers from an acute shortage of unique sites in its MCS. This highlights another shortcoming of the pRS series plasmids. The choice of restriction sites for cloning constructs into pRS plasmids is marker-dependent and may complicate in \textit{vivo} cloning. This is due to the presence of several restriction sites within the \textit{S. cerevisiae} \textit{HIS3}, TRP1, LEU2, URA3 and other yeast-selectable marker sequences of the pRS plasmids that are also found in the pBluescript/pBluescript II MCS of the pRS plasmid backbone (Brachmann et al. 1998; Eriksson et al. 2004; Sikorski and Hieter 1989). This is in contrast to the YXplac series (Gietz and Sugino 1988) in which TRP1, LEU2, and URA3 markers were mutagenized to remove restriction sites in common with the pUC19 MCS. Hence, all 10 of the 6-bp restriction sites in the pUC19 MCS are unique in every YXplac plasmid (Gietz and Sugino 1988). If constrained by restriction site availability, an investigator seeking to integrate a construct of interest into the yeast genome using a particular pRS plasmid would have to first clone a given construct into another integrating plasmid with a different marker or into an episomal plasmid by using recombination-mediated/gap-repair methods (Ma et al. 1987; Oldenburg et al. 1997) before moving it into the integrating plasmid with the desired marker using 

\textit{Pvu} or 

\textit{Bss}HI fragment exchange (Brachmann et al. 1998; Sikorski and Hieter 1989). However, given the additional labor and time required, this may not be an ideal solution for everyone.

In addition to introducing genetic constructs into yeast and in \textit{vivo} cloning by homologous recombination, the pRS series of vectors can also be used for PCR-mediated homologous replacement of sequences in the budding yeast genome (Baudin et al. 1993; Brachmann et al. 1998; Replogle et al. 1999). This method allows for sequences in the \textit{S. cerevisiae} genome to be replaced by a selectable marker amplified by PCR with 5′ and 3′ flanking sequences matching the sequences upstream and downstream of the sequence of interest (Baudin et al. 1993; Lorenz et al. 1995; Wach 1996). The simplicity and utility of PCR-mediated gene replacement has led to its usage in other fungal model organisms as well (Kaur et al. 1997; Walther and Wendland 2008; Wendland et al. 2000). Due to the standardized design of the pRS series, a single pair of oligonucleotide primers can be used to amplify any prototrophic marker from any pRS plasmid (Brachmann et al. 1998) for transforming yeast.

Heterologous dominant drug resistance markers, such as the \textit{kanMX} module that confers resistance to G418 (Wach et al. 1994), provide some advantages over prototrophic biosynthetic markers for PCR-mediated gene disruption/deletion. Although using a prototrophic marker requires working with a strain that carries the corresponding auxotrophic mutation, no such requirement exists for drug resistance markers. Moreover, whereas the usage of drug resistance genes is more flexible as they lack homology to the \textit{S. cerevisiae} genome (Goldstein and McCusker 1999), prototrophic markers derived from \textit{S. cerevisiae} work best in strains with “designer deletion alleles” (Brachmann et al. 1998; Replogle et al. 1999), in which gene conversion or rescue of the corresponding auxotrophic mutation is prevented. Finally, whereas prototrophic markers have the potential to complicate phenotypic analysis and must be carefully controlled for (Pronk 2002), drug resistance markers reportedly have neutral effects on growth under non-selective conditions (Goldstein and McCusker 1999; Hadfield et al. 1990).

Although plasmids that carry other MX markers, such as \textit{hphMX}, \textit{natMX}, and \textit{patMX} (Goldstein and McCusker 1999; Hentges et al. 2005; Wach et al. 1994), have been developed using the pFA backbone, pRS400 (Brachmann et al. 1998) is the only pRS plasmid in the literature that carries an MX drug resistance cassette, namely, \textit{kanMX4} (Wach et al. 1994). On the other hand, the pRS series offers an unmatched selection of prototrophic markers for PCR-mediated replacement. Hence, researchers may find themselves employing two or more pairs of oligonucleotides to replace a particular gene sequence with markers from different plasmid series.

In this report, we describe our attempts to overcome the limitations described above. First, we have constructed new \textit{his2}- and \textit{ADE1}-marked shuttle vectors by replacing the yeast-selectable marker of existing pRS plasmids. In each of these new plasmid vectors, we have preserved the uniqueness of all 18 common restriction sites found in their polylinker regions, providing valuable new tools for genetic analysis in \textit{his2} and \textit{ade1} laboratory yeast strains.

Second, to expand the availability of unique sites in the MCS of existing pRS plasmids, we have mutagenized the \textit{S. cerevisiae} genes \textit{ADE2}, \textit{HIS3}, \textit{TRP1}, \textit{LEU2}, and \textit{URA3} using a strategy similar to that used during the construction of the YXplac series (Gietz and Sugino 1988). We also swapped the 2μ origin of the pRS episomal vectors with that from the YEpplasmid series so as to remove the \textit{XbaI} site within. Altogether, we have generated 42 pRSII plasmid shuttle vectors with 16 restriction sites in their polylinkers that are unique throughout the entire series: pRSII30x/31x/32x with the pBluescript KS+ MCS (Sikorski and Hieter 1989) and pRS40x/41x/42x with the pBluescript II SK+ MCS (Brachmann et al. 1998; Christianson et al. 1992). The pRSII plasmids are easier to manipulate in \textit{vivo} than their pRS predecessors and will facilitate molecular cloning and yeast plasmid construction.

Third, we have expanded the repertoire of drug resistance cassettes available in pRS plasmids and, hence, the number of markers that can be amplified using a single pair of oligonucleotides for PCR-mediated gene replacement. We replaced the \textit{kanMX4} cassette in pRS400 with four drug resistance genes, derived from other commonly used plasmids (Goldstein and McCusker 1999; Gueldener et al. 2002), that
encode resistance to the antibiotic compounds pleomycin, hygromycin B, nourseothricin, and bialaphos.

Finally, in the course of constructing our new plasmids, we have uncovered several errors in publicly accessible nucleotide sequences for existing yeast plasmids. These errors probably went unnoticed because the restriction maps for these plasmids were based on the published sequences of the different parts used to build them. Some of these errors caused restriction sites to be missed while suggesting the presence of non-existent sites. One error in the sequence for pRS402, pRS412, and pRS422 (Brachmann et al. 1998) is particularly serious as it fails to document the presence of a 163-bp insertion in these plasmids that causes a drastic reduction in yield when one attempts to amplify the ADE2 marker with standard pRS primers. Another error that required rectification was the opposite orientation of the CEN6/ARS4 cassette in pRS313 and pRS413 compared to all other pRS CEN plasmids. We have documented the sequence discrepancies we observed to improve the accuracy of molecular cloning. Importantly, the true sequence of the ADE2 and LEU2 pRS vectors facilitated the construction of pRS backbone plasmids with a unique restriction site (BglII and AgeI, respectively) located between the two pRS primer binding sites. Novel yeast-selectable markers of the user’s choice may therefore be easily introduced to construct additional pRS vectors in the future.

MATERIALS AND METHODS

Plasmid construction

Standard enzymes were used for DNA manipulation. Restriction enzymes were purchased from New England Biolabs, except for PfoI, which was purchased from Fermentas. Ligations were performed using T4 DNA ligase purchased from Invitrogen. Both PCR-mediated site-directed mutagenesis and gene amplification for cloning purposes were performed using either cloned Pfu Turbo DNA polymerase (Stratagene) or KOD HotStart DNA polymerase (Toyobo, Novagen/EMD Chemicals). Antarctic phosphatase (New England Biolabs) was used to treat symmetrical ends of plasmids cut with a single restriction enzyme to prevent recirculation. Plasmid propagation was carried out in Invitrogen MAX Efficiency DH5α bacteria grown in lysogeny broth (LB) (Bertani 2004) supplemented with either 50–100 μg/ml ampicillin sodium salt or 10 μg/ml kanamycin sulfate purchased from Sigma-Aldrich. Bacterial transformants were selected for on LB 2% agar plates supplemented with either 2% DMSO. The reaction parameters we employed were: 94°C for 45 sec, 72°C for 45 sec, 98°C for 10 min. Anneal-denaturation cycles (94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min/kb of expected PCR product size), 72°C for 10 min. Annealing and denaturation times can be shortened to 30 sec, and the extension temperature can be reduced to 70°C or 68°C. Taq DNA polymerase (Denville) was used for marker amplification at 0.05μl.

PCR protocol for amplifying pRS/pRSII plasmid yeast-selectable markers

Similarly to what has previously been described (Brachmann et al. 1998), we used primers starting with 40–50 nucleotides of gene-specific sequence at the 5’ end and followed by either 5’-CAGATTG TACTGAGGTGCG-3’ (pRS forward primer binding site) or 5’-CCTTACGATCTGTGCGG-3’ (pRS reverse primer binding site) to amplify the yeast-selectable marker sequences in any of the pRS or pRSII plasmids; examples of primer pairs used to target the genes KIP1, CIN8, and ADE2 are provided in Table S3. As noted before (Goldstein and McCusker 1999), PCR amplification of the natMX4 and patMX4 drug resistance cassettes requires the addition of 5% DMSO. The reaction parameters we employed were: 94°C for 1 min followed by 34 amplification cycles (94°C for 45 sec, 55°C for 45 sec, 72°C for 1 min/kb of expected PCR product size), 72°C for 10 min. Annealing and denaturation times can be shortened to 30 sec, and the extension temperature can be reduced to 70°C or 68°C. Taq DNA polymerase (Denville) was used for marker amplification at 0.05μl.

Yeast transformation

Yeast were transformed using high-efficiency methods involving lithium acetate, polyethylene glycol, and denatured, single-stranded salmon sperm DNA (Gietz and Schiestl 2007; Gietz and Woods 2001). To transform the wild-type strains 15Daub and W303a using pRS/pRSII plasmids, we used either 200 ng of integrating plasmid linearized by restriction at a unique site within the yeast-selectable prototrophic marker sequence or 50 ng of CEN/2μ plasmid. Prototrophic transformants were selected for by plating on synthetic complete dropout plates (0.67% yeast nitrogen base, 2% dextrose, 2% agar) lacking the appropriate amino acid or nucleobase. To select for drug-resistant transformants, we suggest referring to previously published protocols for guidelines (Baudin et al. 1993; Gatignol et al. 1987; Goldstein and McCusker 1999; Wenzel et al. 1992). Selection conditions that we have tested ourselves and suggestions for users who experience difficulty with drug selection are described in File S1.
The transformation of yeast with PCR-amplified MX4 drug resistance cassettes is described in detail in File S1.

RESULTS AND DISCUSSION

New HIS2-marked yeast-bacteria shuttle vectors
Whereas *S. cerevisiae* **HIS3** encodes imidazolglycerol-phosphate dehydratase, **HIS2** encodes histidinolphosphatase. Both of these enzymes function in histidine biosynthesis but catalyze different steps (Alifano et al. 1996; Gorman and Hu 1969; Struhl and Davis 1980). Despite the availability of plasmids that can be used with histidine auxotrophic laboratory strains of budding yeast that are *his3* mutants, these plasmids cannot be used with His− strains that are *his2* mutants. The latter includes include strains such as 15D (Reed et al. 1985), which is widely used in cell-cycle research. The comparative scarcity of **HIS2**-marked yeast vectors poses an unnecessary limitation when working with **his2** strains. Our first attempt at making an integrating vector with a **HIS2** marker involved the disruption of the **URA3** marker in pRS306 (Sikorski and Hieter 1989) with a wild-type **HIS2** allele, resulting in pRS306H2 (Chee and Haase 2010). Although this plasmid has been successfully used to both integrate genetic constructs into the *S. cerevisiae* genome (Chee and Haase 2010) and to delete genes of interest by PCR (unpublished data), it suffers from a shortage of unique sites in its MCS, contains extraneous sequences, and could be streamlined (Figure 1A). Moreover, **HIS2**-marked centromere and 2µ episomal versions of pRS306H2 have yet to be constructed.

To improve upon pRS306H2, we have completely rebuilt it using a different strategy (File S1). Using a site-directed mutagenesis strategy similar to that of Gietz and Sugino (1988), we removed the BamHI and XhoI sites present in the wild-type **HIS2** gene (Table S1 and File S1). We subsequently used the mutated **HIS2**, PCR-amplified with NdeI and NsiI ends, to replace almost the entire **URA3** gene in pRS306. The resulting plasmid, which we have dubbed pRSII309, is the updated successor to pRS306H2. pRSII309 is 0.4 kb smaller than its predecessor due mostly to the near-complete excision of the **URA3** marker from pRS306 (Figure 1); in pRS306H2, the **URA3** marker was disrupted between the NcoI and NsiI sites. Moreover, all of the 18 common restriction sites in the pRSIII09 polylinker region (the pBluescript KS+ MCS) are unique. This replacement method is similar to the one we used to generate pRS306H2 in that it can be used to convert other **URA3**-marked yeast plasmids to the **HIS2** marker (Chee and Haase 2010). We subsequently constructed **CEN** and 2µ episomal derivatives of pRSII309, pRSII319, and pRSII329, respectively, as well as pRSII409/419/429, which carry the pBluescript II SK+ MCS (Table 1 and File S1). The significance of the pRSII designation is explained below.

New ADE1-marked shuttle vectors
Adenine auxotrophy presents an analogous problem to that we have encountered for histidine auxotrophy in *S. cerevisiae*. *S. cerevisiae* **ADE1** encodes N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase, whereas **ADE2** encodes phosphoribosylaminoimidazole carboxylase, enzymes required for distinct steps in *de novo* purine biosynthesis (Jones and Fink 1982; Myasnikov et al. 1991; Stotz and Linder 1990). Ade− strains that carry adel and/or ade2 mutations accumulate a red pigment that distinguishes them from Ade+ yeast, which are white (Fisher 1969; Silver and Eaton 1969). Hence, adel, ade2 as well as adel ade2 mutants are valuable for visual red-white screening of transformants and other color-based assays (Uoglini and Bruschi 1996; Weng and Nickoloff 1997). However, as **ADE2**-marked plasmids are not useful when working with **adel** mutant strains, investigators would benefit from having a set of **ADE1**-marked pRS plasmids available to complement existing **ADE2** pRS plasmids (Brachmann et al. 1998).

Without pre-existing **ADE1** shuttle vectors in hand, we chose the **ADE2**-marked pRS402 (Brachmann et al. 1998) to build an **ADE1**-marked integrating plasmid. Based on its GenBank sequence (accession no. U93717.1), the **ADE2** marker in pRS402 is flanked by BgIII sites and is thus easily replaced (Figure 2A); however, we discovered disagreements between the actual and the GenBank sequences of pRS402 when performing restriction analysis and Sanger sequencing. First, restricting pRS402 (Brachmann et al. 1998) with NdeI yields two fragments (1.9 and 3.8 kb) instead of the single 5.5 kb molecule predicted by its GenBank sequence. Moreover, we could not sequence pRS402 using a standard pRS reverse primer (5′-CCCTTACGCACTGGTGC-3′) as Sanger capillary sequencing reactions consistently returned overlapping electropherograms, strongly suggesting that the primer was annealing to two different sites on the plasmid.

By sequencing with other primers (Table S4), we determined the presence of an undocumented insertion in pRS402 (Figure 2A) that contains an unwanted second pRS reverse primer binding site, which we had to remove along with the **ADE2** marker, and then generate a pRS backbone plasmid with a unique BgIII site (Figure 2A). We also mutated the **ADE1** gene to remove five restriction sites that are found in the pbluescript/pbluescript II MCS similarly to what we did to **HIS2** (Table S1 and File S1). Next, we subcloned the mutated **ADE1** marker into the BgIII site to generate pRSII408 (Figure 2B). As the same unwanted insertion was found in both pSR412 and pSR422 (Brachmann et al. 1998), we used a similar strategy to construct pRSII418 (**CEN** and pRSII428 (2µ) and subsequently generated pRSII308/318/328. As with their **HIS2**-marked counterparts, all 18 common restriction sites in the polylinker region of the new **ADE1** pRSII plasmids are unique (Table 1).

A second generation of pRS plasmids (pRSII) with expanded unique restriction site selection within the polylinker region

Due to the existence of restriction sites common to both their yeast-selectable marker sequences as well as their polylinker regions, unique site selection within the MCS of current pRS plasmids is marker-dependent (Christianson et al. 1992; Sikorski and Hieter 1989). As shown in Table S1, only 9 of the 18 common restriction sites in the MCS of existing pRS vectors marked with either **ADE2**, **HIS3**, **TRP1**, **LEU2**, or **URA3** are unique across the board; this number drops to 7 if the **MET15**, **LYS2**, and **ADE8** markers found in other pRS series plasmids (Brachmann et al. 1998; Eriksson et al. 2004; Tomlin et al. 2001) are also considered (data not shown). Additionally, the 2µ pRS plasmids (Christianson et al. 1992) carry an XbaI site within the 2µ replication origin originally derived from YEp24 (Hartley and Donelson 1980). As a consequence, the XbaI site in the pRS42x MCS is not unique.

In contrast to the pRS series, the *S. cerevisiae* **TRP1**, **LEU2**, and **URA3** alleles used to construct the YXplac series of shuttle vectors were mutagenized to remove all 6-bp restriction sites that are also found in the pUC19 MCS (Gietz and Sugino 1988) of that series. Additionally, the XbaI site within the 2µ origin from YEp24 (Hartley and Donelson 1980) was removed before it was incorporated into the 2µ YXplac (YEplac) series (Gietz and Sugino 1988). Hence, all 10 of the 6-bp sites in the pUC19 MCS (5′-EcoRI-SacI-KpnI-Smal-BamHI-XbaI-SalI-PstI-SphiI-HindIII-3′) are unique throughout the YXplac series. Removal of the XbaI site in the 2µ origin of YEplac195
(Gietz and Sugino 1988) as well as the non-YXplac series plasmids YEp351 and YEp352 (Hill et al. 1986) does not appear to significantly alter their copy number, estimated by Southern blotting, when compared to the pRS42x plasmids (Christianson et al. 1992; Li and Johnston 2001; Vashee and Kodadek 1995; Velmurugan et al. 2000).

Removing common restriction sites outside the MCS: When building our new HIS2 and ADE1 shuttle vectors, we emulated the efforts of Gietz and Sugino (1988) and kept all the common restriction sites in the polylinker region unique by mutating the two marker genes. We subsequently explored the feasibility of altering the prototrophic marker sequences (Figure 3) of other commonly used pRS plasmids to both increase the availability of unique sites in their polylinkers as well as to standardize unique site selection across the series. To do so in an efficient manner, we wanted to subclone the TRP1, LEU2, and URA3 alleles developed for the YXplac series into the pRS series.
Table 1  pRSII series plasmids

| Plasmid Names | Yeast-selectable Marker | Yeast Replication Origin | MCS | Non-unique Restriction Sites Remaining in MCS | Addgene ID |
|---------------|-------------------------|--------------------------|-----|--------------------------------------------|------------|
| pRSI302       | ADE2                    | None                     | pBluescript KS+ | EcoRV, BstXI                               | 35433      |
| pRSI402       | ADE2                    | None                     | pBluescript II SK+ | EcoRV, BstXI                              | 35434      |
| pRSI303       | HIS3                    | None                     | pBluescript KS+ | BstXI                                      | 35435      |
| pRSI403       | HIS3                    | None                     | pBluescript II SK+ | BstXI                                      | 35436      |
| pRSI304       | TRP1                    | None                     | pBluescript KS+ | EcoRV, BstXI                               | 35437      |
| pRSI404       | TRP1                    | None                     | pBluescript II SK+ | EcoRV, BstXI                              | 35438      |
| pRSI305       | LEU2                    | None                     | pBluescript KS+ | EcoRV, BstXI                               | 35439      |
| pRSI405       | LEU2                    | None                     | pBluescript II SK+ | EcoRV, BstXI                              | 35440      |
| pRSI306       | URA3                    | None                     | pBluescript KS+ | EcoRV                                      | 35441      |
| pRSI406       | URA3                    | None                     | pBluescript II SK+ | EcoRV                                      | 35442      |
| pRSI308       | ADE1                    | None                     | pBluescript KS+ | None                                       | 35443      |
| pRSI408       | ADE1                    | None                     | pBluescript II SK+ | None                                       | 35444      |
| pRSI309       | HIS2                    | None                     | pBluescript KS+ | None                                       | 35445      |
| pRSI409       | HIS2                    | None                     | pBluescript II SK+ | None                                       | 35446      |
| pRSI312       | ADE2                    | CEN6/ARSH4               | pBluescript KS+ | EcoRV, BstXI                               | 35447      |
| pRSI412       | ADE2                    | CEN6/ARSH4               | pBluescript II SK+ | EcoRV, BstXI                              | 35448      |
| pRSI313       | HIS3                    | CEN6/ARSH4               | pBluescript KS+ | BstXI                                      | 35449      |
| pRSI413       | HIS3                    | CEN6/ARSH4               | pBluescript II SK+ | BstXI                                      | 35450      |
| pRSI314       | TRP1                    | CEN6/ARSH4               | pBluescript KS+ | EcoRV, BstXI                               | 35451      |
| pRSI414       | TRP1                    | CEN6/ARSH4               | pBluescript II SK+ | EcoRV, BstXI                              | 35452      |
| pRSI315       | LEU2                    | CEN6/ARSH4               | pBluescript KS+ | EcoRV, BstXI                               | 35453      |
| pRSI415       | LEU2                    | CEN6/ARSH4               | pBluescript II SK+ | EcoRV, BstXI                              | 35454      |
| pRSI316       | URA3                    | CEN6/ARSH4               | pBluescript KS+ | EcoRV                                      | 35455      |
| pRSI416       | URA3                    | CEN6/ARSH4               | pBluescript II SK+ | EcoRV                                      | 35456      |
| pRSI318       | ADE1                    | CEN6/ARSH4               | pBluescript KS+ | None                                       | 35457      |
| pRSI418       | ADE1                    | CEN6/ARSH4               | pBluescript II SK+ | None                                       | 35458      |
| pRSI319       | HIS2                    | CEN6/ARSH4               | pBluescript KS+ | None                                       | 35459      |
| pRSI419       | HIS2                    | CEN6/ARSH4               | pBluescript II SK+ | None                                       | 35460      |
| pRSI322       | ADE2                    | 2μ ORI-STB               | pBluescript KS+ | EcoRV, BstXI                               | 35461      |
| pRSI422       | ADE2                    | 2μ ORI-STB               | pBluescript II SK+ | EcoRV, BstXI                              | 35462      |
| pRSI323       | HIS3                    | 2μ ORI-STB               | pBluescript KS+ | BstXI                                      | 35463      |
| pRSI423       | HIS3                    | 2μ ORI-STB               | pBluescript II SK+ | BstXI                                      | 35464      |
| pRSI324       | TRP1                    | 2μ ORI-STB               | pBluescript KS+ | EcoRV, BstXI                               | 35465      |
| pRSI424       | TRP1                    | 2μ ORI-STB               | pBluescript II SK+ | EcoRV, BstXI                              | 35466      |
| pRSI325       | LEU2                    | 2μ ORI-STB               | pBluescript KS+ | EcoRV, BstXI                               | 35467      |
| pRSI425       | LEU2                    | 2μ ORI-STB               | pBluescript II SK+ | EcoRV, BstXI                              | 35468      |
| pRSI326       | URA3                    | 2μ ORI-STB               | pBluescript KS+ | EcoRV                                      | 35469      |
| pRSI426       | URA3                    | 2μ ORI-STB               | pBluescript II SK+ | EcoRV                                      | 35470      |
| pRSI328       | ADE1                    | 2μ ORI-STB               | pBluescript KS+ | None                                       | 35471      |
| pRSI428       | ADE1                    | 2μ ORI-STB               | pBluescript II SK+ | None                                       | 35472      |
| pRSI329       | HIS2                    | 2μ ORI-STB               | pBluescript KS+ | None                                       | 35473      |
| pRSI429       | HIS2                    | 2μ ORI-STB               | pBluescript II SK+ | None                                       | 35474      |

plasmids where convenient and separately mutagenize HIS3 and ADE2. Additionally, to make the XbaI site within the MCS of the pRS 2μ plasmids unique, we wanted to replace their 2μ origin with that from YEplac195 (Gietz and Sugino 1988).

After the reconstruction detailed in File S1, we have reduced restriction site overlap between the five markers and the pBluescript/pBluescript II MCS to the point where 16 of the 18 common restriction sites in the polylinker region of our pRSII plasmid series are universal; EcoRV and BstXI, one or both of which occur in all five mutagenized markers (Figure 3), were the only sites we left intact (Table 1). We did not initially plan to mutagenize the Apal site in the URA3 marker because it overlaps with a dcm methylation site (Larimer 1987) and most laboratory bacterial strains are dcm⁺; however, we found that Apal is able to cleave pRS306 isolated from dcm⁺ DH5α E. coli at this site (data not shown), underscoring the difficulty of predicting inhibitory effects by site-specific DNA methylation (McClelland et al. 1994). As a result, we removed the site altogether.

The ability of the modified prototrophic markers to rescue their corresponding auxotrophic mutations in yeast was verified by transforming the auxotrophic wild-type strains 15Daub (Kaiser et al. 1999) and W303a (Ellion et al. 1993) with the new pRSII plasmids. Side-by-side transformations were done with existing pRS plasmids for comparison, except for the modified HIS2 and ADE1 alleles described above. For HIS2 and ADE1, comparisons were made by transforming 15Daub with TA cloning plasmids containing either the unmodified or the mutagenized alleles (pGEM-T-HIS2 and pDrive-ADE1, File S1), which act as yeast integrating plasmids when linearized (see Materials and Methods). We observed no significant differences in transformation efficiency (data not shown).

Correcting aberrant features in existing pRS plasmids: In the course of building our pRSII plasmids, we discovered aberrant features in five pRS plasmids that contradict the intended uniform design of that plasmid series. We have either removed or corrected these in our
pRSII series plasmids to eliminate confusion and to standardize their design. Significantly, we rebuilt the three ADE2 pRS plasmids to remove the undocumented insertion mentioned earlier that is found in pRS402/412/422 (Figure 2A). This 163-bp insertion is a near-identical repeat of 163 nucleotides 3’ of the ADE2 marker and hence carries an extra pRS reverse primer binding site (highlighted). This repeat was removed to generate the pRS backbone plasmid pRS40BgII (B) that was subsequently used to construct pRSII402 and pRSII408. (C) Restriction maps of pRSII408, pRSII418, and pRSII428. Unique restriction sites are shown in black, and non-unique BgII and NdeI sites are shown in red; isoschizomers are also indicated.

**Figure 2** Features of existing S. cerevisiae ADE2 and new ADE1-marked plasmid shuttle vectors. (A) Restriction maps of pRS402 built using existing GenBank (left) and experimentally determined (right) sequence data. A previously undocumented 163-base pair insertion indicated in dark purple; this insertion is a nearly identical repeat of 163 nucleotides 3’ of the ADE2 marker and hence carries an extra pRS reverse primer binding site (highlighted). This repeat was removed to generate the pRS backbone plasmid pRS40BgII (B) that was subsequently used to construct pRSII402 and pRSII408. (C) Restriction maps of pRSII408, pRSII418, and pRSII428. Unique restriction sites are shown in black, and non-unique BgII and NdeI sites are shown in red; isoschizomers are also indicated.

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**pRSII plasmid features:** Our initial set of 42 pRSII integrating, centromere and 2μ episomal plasmids are listed in Table 1; as these plasmids will be made available through Addgene, the corresponding Addgene plasmid IDs are indicated. The naming conventions established for the pRS plasmid series (Brachmann et al. 1998; Christianson et al. 1992; Sikorski and Hieter 1989) also apply to the pRSII plasmids (Table 1 and Table S1). The elimination of common 6-bp restriction sites like KpnI, HindIII, EcoRI, and XbaI from the seven prototrophic marker gene sequences (Figure 3) makes it more convenient to clone inserts into the pRSII polylinker and also simplifies the movement of inserts between pRSII plasmids. As we have also maintained the characteristic uniform structure of the pRS series in our pRSII plasmids, they are compatible with the many sets of pRS-based plasmids that have been
designed for uses as varied as epitope tagging, heterologous gene expression in yeast, and recombination cloning. By adapting the added features of such existing plasmids to the pRSII backbone, derivatives with a standardized MCS, differing only in the yeast-selectable marker that they carry, can easily be generated. Users should take note of the addendum in the original paper that described the initial set of pRS plasmids (Sikorski and Hieter 1989). The MCS of the pRS300 (and hence the pRSII300) series plasmids contains a single base pair deletion found in all of Stratagene’s pBluescript KS plasmids. This deletion removed a G immediately upstream of the Kpn I site and downstream of the lacZ reporter’s ATG start codon. Blue-white screens still work (by an unknown mechanism) with the pRS300 (and pRSII300) series plasmids, but users who plan to generate LacZ fusion proteins should be aware of this frameshift. The pRS400 (and pRSII400) plasmids are not affected by this deletion as their MCS is derived from pBluescript II KS+ (Sikorski and Hieter 1989).

New pRS plasmids with drug resistance markers for PCR-mediated gene disruption/deletion

The introduction of the plasmid pRS400 made drug selection possible for users of the pRS series seeking to either disrupt or delete sequences of interest in the budding yeast genome by PCR-mediated homologous replacement (Brachmann et al. 1998). pRS400 (Figure 4A) contains a heterologous kanMX4 module (Wach et al. 1994), in which the E. coli transposon Tn903 kan gene (Grindley and Joyce 1980) is under the control of the constitutive Ashbya gossypii TEF1 promoter. Tn903 kan encodes aminoglycoside phosphotransferase, which confers resistance to kanamycin/G418 by phosphorylating the antibiotic (Oka et al. 1981). It should be noted, however, that the kanMX4 cassette in pRS400 is oriented in the opposite direction to what its GenBank sequence (accession no. U93713.1) indicates (Figure 4A).

To expand the repertoire of drug resistance markers in the pRS plasmid series, we replaced the kanMX4 cassette of pRS400 with MX4 cassettes containing drug resistance genes from other commonly used plasmids (Goldstein and McCusker 1999; Gueldener et al. 2002) and generated four new pRS plasmids that will also be made available through Addgene (Table 2 and Figure 4B): (1) pRS40B contains the gene ble, originally cloned from transposon Tn5 isolated from Klebsiella pneumoniae, which encodes a protein that binds with high affinity to phleomycin/bleomycin family antibiotics (Gatignol et al. 1987; Genilloud et al. 1984), such as Zeocin (Invitrogen); (2) pRS40H contains hph from K. pneumoniae, which encodes hygromycin B phosphotransferase for hygromycin B resistance (Gritz and Davies 1983); (3) pRS40N contains nat1, from Streptomyces noursei, which encodes nourseothricin N-acetyltransferase for resistance toward

Figure 3 Schematic diagrams of the prototrophic biosynthetic marker genes found in pRSII series plasmids. Restriction sites in each marker that were targeted for removal before incorporation into pRSII series plasmids are indicated, as are the restriction sites that immediately flank the ADE2, LEU2, ADE1, and HIS2 markers within the pRS or pRSII plasmids. The ORF in each marker is indicated by a block arrow. A complete list of pRSII plasmids is found in Table 1, and the oligonucleotides used for site-directed mutagenesis of restriction sites are found in Table S2. The BamHI site found in the ADE1 genomic sequence was previously removed from the ADE1 allele (Nagley et al. 1988) used to generate pRSII408. The BglI site found in the ADE2 genomic sequence was also previously removed (Stotz and Linder 1990) from the ADE2 allele used to generate pRSII402. Although the Apal site in URA3 overlaps with a dcm methylation site, plasmid DNA isolated from DH5α dcm− bacteria is still cleaved at this site by Apal. The gene diagrams shown are drawn to scale.
nourseothricin, a mixture of streptothricins (Kriegl et al. 1993); and (4) pRS40P contains *pat*, from *Streptomyces viridochromogenes*, which encodes phosphinothricin N-acetyltransferase for resistance to bialaphos (Strauch et al. 1988; Wohlleben et al. 1988), to make pRS40P.

When using the above drugs to select for yeast transformants, we recommend referring to previously suggested drug concentrations and media recipes for guidelines (Gatignol et al. 1987; Goldstein and McCusker 1999; Wenzel et al. 1992). We successfully tested our

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**Figure 4** Features of pRS400 and plasmids derived from it carrying new dominant drug resistance MX4 cassettes that can be amplified by PCR for gene disruption/deletion in yeast. (A) Restriction maps of pRS400 with *kanMX4* cassette for G418 resistance, based on existing GenBank (left) and experimentally derived (right) nucleotide sequences. The orientation of the *kanMX4* cassette is inverted in the Genbank sequence. (B) New MX4 plasmids derived from pRS400. Top, pRS40B with *bleMX4* cassette for phleomycin resistance (left) and pRS40H with *hphMX4* cassette for hygromycin B resistance (right). Bottom, pRS40N with *natMX4* cassette for nourseothricin resistance (left) and pRS40P with *patMX4* cassette for bialaphos resistance (right). Unique restriction sites are shown in black, whereas the *Nco*I sites we found to be non-unique in the *patMX4* cassette (File S1) are shown in red; isoschizomers are also indicated.
new drug resistance plasmids by targeted replacement of the ADE2 gene (Figure S2 and Table S5) in the wild-type yeast strain S288C (Mortimer and Johnston 1986) and have included selection conditions that we used (File S1).

There are now a total of 5 drug resistance MX4 markers and 10 prototrophic markers (MET15, ADE2, HIS3, TRP1, LEU2, URA3, LYS2, ADE1, HIS2, and ADE8) that can be amplified using a single pair of oligonucleotides (examples given in Table S3) from known pRS/pRSII plasmids (Brachmann et al. 1998; Sikorski and Hieter 1989; Tomlin et al. 2001) for targeted homologous replacement in budding yeast. A suggested PCR protocol compatible with all pRS/pRSII plasmids is provided in Materials and Methods. As many of the drugs used for selection are compatible with minimal media (File S1), it is possible to design double selection schemes involving both nutritional and drug selection. Marker exchange within a deletion/disruption strain is also possible using the same pair of oligonucleotides; given the identical TEF1 oligonucleotides; given the identical absence of cross-resistance between the antibiotic resistance markers allows for strains carrying more than one marker to be selected on media containing two or more drugs (Goldstein and McCusker 1999).

Errors in publicly available sequences for existing yeast plasmids

In addition to the errors in existing sequences for plasmids that we described earlier in this report, we observed inconsistencies in existing sequence data available for several other yeast plasmids we worked with in the course of this study. Errors were sometimes first detected by unexpected differences observed in the number and sizes of restriction fragments; however, a large number were first determined by Sanger sequencing, such as during the construction of pRSII304 (File S1). Sequencing the pRS plasmids was necessary to verify suspected errors, as the restriction maps and sequences for the first pRS plasmids were generated based on published sequences of their components available at the time (Sikorski and Hieter 1989). Plasmids were sequenced using the oligonucleotide primers listed in Table S4, and errors were verified by sequencing related plasmids for comparison. For example, errors identified in the pRS402 GenBank sequence were verified by sequencing pRS412 and pRS422. Errors in the backbone sequence common across the pRS series were also identified in this way. Our findings reinforce sequence errors that have been reported elsewhere for pRS416 and pRS426 (Tomlin et al. 2001) as well as the HIS3-marked pRS vectors (http://genome-www.stanford.edu/vecordb/vector.html). We have included details of the most significant errors we determined in sequences deposited in public databases such as GenBank in File S1 and have listed sequences in need of updating in Table S6. Accurate sequence data will greatly benefit cloning using the affected plasmids.

Table 2 pRS400-based drug resistance MX4 marker plasmids

| Plasmid Name | Drug Resistance Gene in MX4 Cassette | Species of Origin for Resistance Gene | Source Plasmid for MX4 Cassette | Addgene ID |
|--------------|-------------------------------------|--------------------------------------|--------------------------------|-----------|
| pRS40B       | ble                                 | Klebsiella pneumoniae, transposon Tn5| pUG66 (Gueldener et al., 2002) | 35478     |
| pRS40H       | hph                                 | Klebsiella pneumoniae                | pAG32 (Goldstein and McCusker 1999) | 35479     |
| pRS40N       | nat†                                | Streptomyces noursei                 | pAG25 (Goldstein and McCusker 1999) | 35480     |
| pRS40P       | pat                                 | Streptomyces viridochromogenes       | pAG29 (Goldstein and McCusker 1999) | 35481     |

pRS backbone plasmids for generating future pRS plasmids with new yeast-selectable markers

As mentioned above, using the updated sequences for the ADE2-marked pRS plasmids, we were able to excise the additional undesired pRS reverse primer binding site and generate pRS backbone vectors with a unique BglII site for the insertion of new marker sequences (File S1). Similarly, the updated sequence data that we have collected has allowed us to generate a second set of pRS backbone vectors with a unique AgeI site by excising the LEU2 marker from the LEU2-marked pRS plasmids using Thh111 and AgeI and subsequently recircularizing the backbone (File S1); the presence of an AgeI site flanking the LEU2 marker in pRS305/315/405/415/425 was previously undetermined. Previous efforts to build new pRS plasmids with novel yeast-selectable markers involved lengthy cloning processes with multiple steps (Eriksen et al. 2004; Tomlin et al. 2001). With two non-overlapping sets of restriction enzymes that generate cohesive ends compatible with either BglII or AgeI, our new backbone vectors provide greater flexibility and should simplify the future construction of pRS/pRSII plasmids with additional yeast-selectable markers. Our new backbone vectors are also intended to complement the existing markerless pRS plasmids pJK142 (integrating), pGC25 (CEN), and pGC26 (2μ) (Brachmann et al. 1998), which have a unique NdeI site for inserting yeast-selectable markers.

We hope that the new yeast plasmids introduced in this report as well as the updated sequences for existing plasmids will provide a sufficiently complete and cost-effective set of tools for starting research projects that employ budding yeast as a model. We also hope that they will facilitate the development of new molecular genetic tools for yeast research.

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