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Repair of plasmid DNA used for transformation of *Rhizopus oryzae* by gene conversion

Received: 10 December 2003 / Revised: 6 February 2004 / Accepted: 13 February 2004 / Published online: 9 March 2004

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**Abstract** Techniques for genetic manipulation of the filamentous fungus *Rhizopus* have been hampered due to a lack of understanding regarding the recombination and replication mechanisms that affect the fate of introduced DNA. The ability to target chromosomal integration of a plasmid has been difficult because DNA transformed into *Rhizopus* rarely integrates and is autonomously replicated in a high molecular weight concatenated arrangement (i.e., series or chain). Linearization of the plasmid prior to transformation at a site having homology with the genomic DNA yields the highest frequency of integration, but repair of the double-strand break by end-joining is still the predominant event. We recently attempted to circumvent replication of the plasmid by introducing frameshift mutations in *pyrG*, the *R. oryzae* orotidine-5'-monophosphate decarboxylase gene used for selection of the vector. It was hypothesized that autonomous replication of the mutated plasmids would be incapable of restoring prototrophic growth, since the genomic *pyrG* also contained a mutation. However, homologous integration of the plasmid results in duplication of the *pyrG* gene, which can create a functional copy of *pyrG* if both the genomic and plasmid mutations are paired on the same duplicate copy. While this event was detected in one of the isolates, it represented less than 8% of the total transformants. The majority of transformants contained plasmid replicating autonomously in a concatenated arrangement. Sequence analysis showed that prototrophic growth was restored by repairing the non-functional *pyrG* sequence in the plasmid, while the genomic *pyrG* gene was unaltered. Frequent transfer of the genomic *pyrG* mutation to the plasmid suggests that gene conversion is likely occurring by recombination pathways involving break-induced replication or synthesis-dependent strand annealing.

**Keywords** *Rhizopus* · Recombination · Synthesis-dependent strand annealing · Break-induced replication · Transformation

**Introduction**

*Rhizopus* is a valuable filamentous fungus that is used for the production of fermented foods, industrial enzymes (e.g., glucoamylase, lipase), organic acids (e.g., lactate, fumarate), and corticosteroids, while also being a food spoilage organism, a plant pathogen, and an opportunistic human pathogen. Yet even with such importance, the techniques for genetic manipulation of this organism are still in an early stage of development, compared with those used for many other fungi. In general, Mucorales fungi (e.g., *Rhizopus*, *Mucor*, *Absidia*, *Phycomyces*, *Rhizomucor*), an order of the class Zygomycetes, share this same dilemma, mainly due to a lack of understanding regarding the recombination and replication mechanisms that affect the fate of introduced DNA. Unlike most filamentous fungi, DNA transformed into Mucorales fungi rarely integrates into the chromosome, but is replicated extrachromosomally in high molecular weight concatenated structures (i.e., linked together in a series or chain). Since replication of these plasmids can be unstable, integration into the genome is the most ideal method of ensuring that the introduced DNA is maintained. Furthermore, methods to disrupt genes require the ability to target integration into specific loci.

Autonomous replication of plasmids in Mucorales fungi does not appear to require a defined origin of...
replication (Benito et al. 1995; van Heeswijck 1986; Skory 2002; Wostemeyer et al. 1987; Revuelta and Jayaram 1986; Yanai et al. 1990), as is typical with many other higher eukaryotes (Gilbert 2001). In *Rhizopus*, a circular plasmid will only integrate at homologous loci at frequencies less than 5%, with the remainder of transformants having autonomous replication of concatenated plasmids. This frequency of integration can increase up to 20% of the total transformants, if plasmids used for transformation are linearized with a double-strand break (DSB) within a region having homology with chromosomal DNA. Integration almost always occurs at the homologous locus in a manner frequently described as type I, additive, or crossover integration (Skory 2002; Hinnen et al. 1978). The mechanisms of this type of integration become obvious with an understanding of current models of double-strand break repair (DSBR), which are nicely reviewed in several recent publications (Prado et al. 2003; Lewis and Resnick 2000; Heyer et al. 2003; Paques and Haber 1999).

One of the reasons that the total number of transformants having integration is not higher is due to the efficient end-joining of the DSB (Skory 2002). When plasmids are linearized with a single restriction endonuclease, the predominant repair event is re-ligation of the plasmid. Ligation of compatible overhangs, such as those created by restriction endonucleases, is almost always precise and restores the original sequence. This type of repair is most common in higher eukaryotes and is often referred to as non-homologous end-joining (NHEJ; Ray and Langer 2002; Frank-Vaillant and Marcand 2002; Lieber 1999). While end-joining is usually precise, NHEJ also has the ability to fill-in and delete nucleotides to allow repair of DSB having non-homologous ends (Sandoval and Labhart 2002). However, we have not detected end-joining of non-homologous overhangs in *Rhizopus*. If repeated sequences are oriented in the same direction on both sides of the DSB, rejoining of the plasmid ends may also occur by single-strand annealing (SSA), which allows recombination between the duplicated regions (Lin et al. 1984; Lin et al. 1985; Skory 2002).

All of these repair mechanisms result in efficient recircularization of the plasmid, thus permitting autonomous replication of plasmids instead of homologous integration. It was originally hypothesized that introduction of a mutation into the vector pyrG gene used for selection could prevent prototrophic growth with autonomous replication, even if the plasmid re-circularized. The recombined plasmid would still contain a mutation and be incapable of producing functional orotidine 5'-monophosphate (OMP) decarboxylase. However, chromosomal insertion of this plasmid by type I homologous integration would result in at least two copies of the gene, with one having the potential to be functional (Fig. 1a). This only occurs if the genomic and plasmid mutations are paired on the same copy of the duplicated gene. Otherwise, both genes are non-functional (Fig. 1b). Testing this hypothesis involved making two sets of plasmids containing frameshift mutations at either the *MfeI* or *StyI* restriction endonuclease site of *pyrG*. Linearization of the plasmid prior to transformation was performed at the non-mutated restriction site. We knew the location of the mutation within the genomic *pyrG* and postulated that homologous integration should only duplicate a fully functional copy of a genomic *pyrG* gene when integration occurs with the mutated *StyI* plasmid, *Pyr31XVI-A*. This idea proved to be correct, but was in fact a rare event. The majority of transformants actually restored growth by repairing the mutations within the plasmids. In this paper, we present evidence which suggests that difficulties with achieving integration in *Rhizopus* are due to ineffective formation or cleavage of Holliday junctions (HJs) during strand invasion. Instead, this organism seems to rely primarily on the non-crossover mechanism such as break-induced replication (BIR) or synthesis-dependent strand annealing (SDSA).
Materials and methods

Strains and plasmids

*R. oryzae* (syn. *R. arrhizus*) NRRL 395 was the source strain for the OMP dehydrogenase auxotroph *R. oryzae* PYR-17 (pyr181) used in this study. This strain was described in detail by Skory (2002) and contained a G-to-A transversion 181 bp downstream of the start codon, at the 5' end of a splice junction (Skory 2002). Plasmid pPyr225 contained the *R. oryzae pyrG* gene and was capable of restoring prototrophic growth when transformed into *R. oryzae* PYR-17. Frameshift mutations were introduced into the coding region of the *pyrG* gene at either the *MfeI* or *Styl* restriction sites, using T4 polymerase fill-in and self-ligation. Sequence analysis confirmed that the *MfeI* and *Styl* sites were modified by the addition of four extra nucleotides for plasmids pPyr31XVI-A and pPyr31XVI-B, respectively.

Fungal transformation

Transformations of *R. oryzae* PYR-17 used microprojectile particle bombardment (BioRad, Hercules, Calif.) as described by Skory (2002). Unerminated spores were transformed directly on RZ minimal medium plates, since no recovery time for auxotrophic selection was required. Approximately 5-7 days following bombardment, spores were collected and diluted in sterile water to obtain single-spore isolates. Only one isolate per plate was used for further analysis, to avoid multiple progeny originating from the same transformation event. Transformations were performed with undigested plasmids pPyr31XVI-A and pPyr31XVI-B, or linearized with *MfeI* and *Styl*, respectively.

Molecular analyses

Southern analyses were performed using the Genius system (Boehringer Mannheim, Indianapolis, Ind.), according to manufacturer's recommendations. Digoxigenin-labeled lambda DNA, cleaved with *HindIII*, was included in Southern hybridizations as a DNA molecular weight marker. Preparation of probes for the analysis of transformants used the primers 5'-ata gcg agc gtg cca aac aac-3' and 5'-ttc aag ata tgc gtc cca aca-3' for PCR amplification and labeling of an internal 848-bp region of the *pyrG* gene.

Undigested total DNA from *R. oryzae* transformants was transformed into *Escherichia coli* TOP10 cells (Invitrogen, La Jolla, Calif.), using ampicillin selection, to recover replicating plasmids. Approximately five different *E. coli* isolates from each *R. oryzae* transformant were analyzed in this study. Plasmids were examined for multimer formation by gel analysis of undigested DNA, with a Supercoiled DNA ladder (Invitrogen) as a molecular weight standard. The presence of functional *MfeI* and *Styl* sites was tested by RE digestion. Furthermore, the entire coding region of the *pyrG* gene was sequenced for all recovered plasmids. The chromosomal *pyrG* coding region for each transformant was analyzed by PCR amplification, using the primer sets P1 (5'-cgc cct tac cta tctg tat ctc a-3') and P2 (5'-aat cca tcc tctg ttc cca aca aac taa taa gga gtt-3') and P4 (5'-cgc ggt gaa gat gat gat gat gat gat gat-3'). Primers P1 and P4 annealed to targets upstream and downstream, respectively, of the 2.25-kb region used on the selectable marker. Both sets amplified a 1.8-kb chromosomal DNA fragment (Fig. 1a), but had specificity towards either the left or right flanking *pyrG* copy when integration occurred.

Results

Biostatic transformation with the mutated plasmid pPyr31XVI-A readily generated numerous prototrophic colonies that appeared within 3-4 days. Efficiency of transformation was highest, with a frequency of approximately 50 transformants/µg DNA, when plasmid pPyr31XVI-A was linearized with *MfeI*. Undigested pPyr31XVI-A did yield transformant colonies, but at a frequency estimated to be at least ten-fold less than linearized. Plasmid pPyr31XVI-B was originally thought to be incapable of restoring growth, since no transformants appeared within 4 days. However, we eventually realized that the transformation frequency was less than 1 transformant/µg DNA and that prototrophic growth did not appear until 8-10 days later.
Southern analysis was performed on several of the above transformants. We chose one isolate each from those transformed with undigested plasmid. Eight isolates from MfeI-linearized pPyr31XVI-A and three isolates from StyI-linearized pPyr31XVI-B were selected. The restriction enzyme BstB1 was used to digest genomic DNA, since it did not linearize the plasmid and allowed us to easily determine whether integration occurred in the chromosomal pyrG gene locus. Surprisingly, only two of the transformants, isolate 2 from undigested pPyr31XVI-B and isolate 4 from MfeI-linearized pPyr31XVI-A, showed evidence of integration into the pyrG, as indicated by a molecular weight shift of the 4.7-kb hybridizing BstB1 fragment (Fig. 2a). The remaining isolates had the unmistakable indication of a multi-copy high-molecular-weight concatenated plasmid that is very obviously greater than 23 kb and is even trapped within the wells of the gel.

As further confirmation of integration for isolates 2 and 4, these transformants were passed through ten generations of sporulation on medium containing non-selective uracil. Spores isolated from both transformants continued to maintain 100% prototrophy, although growth from isolate 2 was slow and required approximately twice the time to achieve an equivalent amount of mycelium (data not shown). Southern analysis of DNA from isolates 2 and 4, after non-selective growth, confirmed that integration of the plasmids had occurred (Fig. 2b). Restriction enzymes BstB1 and XhoI cut outside of the pyrG gene and resulted in fragments of 4.7 kb and 3.9 kb, respectively, for the untransformed control. For isolate 4, we estimated that approximately 4–5 copies of plasmid pPyr31XVI-A recombined into the chromosomal pyrG locus by additive integration, as shown in Fig. 1a. The 6.8-kb and 2.3-kb XhoI fragments were created on the flanking ends of the integration site, while all subsequent copies of the plasmid were internal and are represented by the band hybridizing at 5.2 kb. The faint 10.4-kb band seen with XhoI digestion for isolate 4 is believed to be the result of incomplete digestion of multimeric plasmids integrated in tandem. Isolate 2 appears to have 2–3 copies of plasmid pPyr31XVI-B integrated in the pyrG locus. A 2.3-kb XhoI fragment resulted from this integration, as with the previous plasmid, followed by an additional 1–2 copies of the 5.2-kb plasmid in tandem. A 6.8-kb XhoI fragment should have resulted from this integration (Fig. 1b). Instead, isolate 2 contained a 3.6-kb fragment, indicating a rearrangement or deletion in the upstream region of the chromosomal pyrG locus.

PCR amplification was performed on all isolates, using primers P1/P2 and P3/P4 (Fig. 1a) that amplified only the genomic pyrG gene fragment. These two combinations of primers allowed selective amplification of the right or left flanking copy of the pyrG gene for isolates with integrated plasmids. The sequence from every isolate, except 2 and 4, which are known to have integrated plasmids, were identical to the R. oryzae Pyr17 recipient strain containing the pyr181 mutation with functional MfeI and StyI restriction sites. Isolate 4, transformed with MfeI-linearized pPyr31XVI-A, revealed no mutations in the right flanking copy of the pyrG gene, as shown in Fig. 1a. However, the left flanking copy of the duplicated gene, which was amplified with primers P1/P2, only contained the pyr181 mutation and had a functional StyI restriction site. Thus, the StyI mutation in plasmid pPyr31XVI-A was somehow repaired during or prior to integration. The right flanking copy of the pyrG gene from isolate 2 only possessed a mutation in the MfeI restriction site, as shown in Fig. 1b. Oddly, a combination of the wild-type and the mutant pyr181 and MfeI genotypes was amplified from the left flanking pyrG gene from this transformant, while all StyI restriction sites were functional. Amplification with primers P1/P2 resulted in a full-length PCR fragment, suggesting that the deletion in the 6.8-kb XhoI fragment, as seen in the Southern analysis (Fig. 2b, isolate 2), must be located within the adjacent vector sequence.

We originally attempted to analyze the pyrG gene on the plasmid by sequencing fragments amplified from transformant DNA, using primers that annealed to the vector sequence. However, it became obvious that multiple nucleotide polymorphism existed within the regions for the pyr181 mutation site and the MfeI and StyI restriction sites. Therefore, individual plasmids were recovered from each of the Rhizopus isolates by transformation of uncut DNA into E. coli. Approximately five bacterial isolates were chosen from each transformation, to get a representation of the different types of plasmid configurations. Plasmids were easily recovered from all of the Rhizopus transformants, including isolates 2 and 4 prior to cultivating multiple generations on a non-selective medium. Restriction enzyme and sequence analysis revealed that numerous modifications had occurred in the pyrG gene contained on the plasmid.

Plasmids recovered from each Rhizopus isolate typically were a mix of different configurations. The different types of configurations identified are shown in Table 1 and include monomers and dimers of plasmids often modified from the original plasmid used for transformation.

The distribution of plasmid configurations for each transformation set varied significantly (Table 2). The majority of the plasmid configurations recovered from the Rhizopus transformants had non-functional pyrG genes. However, it was usually possible to detect plasmids from each Rhizopus transformant that had been restored to the pyrG wild-type genotype, either as a monomer configuration (M2) or as dimers (D1, D2, D4). No functional copies of pyrG were detected for isolates 5, 7, and 11.

Plasmids recovered from Rhizopus transformed with circular pPyr31XVI-A were primarily in their original M1 configuration, with M2 and D3 configurations also found. The majority of plasmids recovered from Rhizopus transformed with circular pPyr31XVI-B were actually in the dimer configuration D4, with M5 and D5
Table 1 Plasmid configurations recovered from *Rhizopus* transformants

<table>
<thead>
<tr>
<th>Configuration</th>
<th>pyr181&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MfeI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>StyI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Monomers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>wt</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>wt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M3</td>
<td>mut</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>mut</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>wt</td>
<td>-</td>
<td></td>
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<tr>
<td>Dimers</td>
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<td>D1</td>
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<tr>
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<td>wt</td>
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<tr>
<td>D4</td>
<td>wt</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D5</td>
<td>wt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D6</td>
<td>mut</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Wild-type genotype for *pyr181* mutation locus, *mut* *pyr181* mutation
<sup>b</sup>+ Functional restriction endonuclease site, - non-functional site
<sup>c</sup>Same configuration as plasmid pPyr31XVI-A
<sup>d</sup>Same configuration as plasmid pPyr31XVI-B

representing a small proportion. We detected the largest number of different plasmid configurations from *Rhizopus* transformed with MfeI-linearized pPyr31XVI-A. This was probably because this was the set that had the greatest transformation efficiency and the most transformants analyzed. Approximately two-thirds of the recovered plasmids from this set were evenly distributed between M2 and M3 configurations. The remaining monomers were M1 and M4, which represented 11% and 6%, respectively, of the total transformants for this set. The recovered dimers were D1, D2, D3, and D6, found at frequencies of 6, 9, 3, and 3% for this set. The final set of plasmids recovered from *Rhizopus* transformed with StyI-linearized pPyr31XVI-B were M5 (60%), M2 (20%), and M4, D1, and D4 (7% each).

Table 2 Analysis of *pyrG* sequences from *Rhizopus* transformants

<table>
<thead>
<tr>
<th>Plasmid used for transformation</th>
<th>Plasmid configuration&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Topology</td>
</tr>
<tr>
<td>pPyr31XVI-A</td>
<td>Circular</td>
</tr>
<tr>
<td>pPyr31XVI-B</td>
<td>Circular</td>
</tr>
<tr>
<td>pPyr31XVI-A</td>
<td>MfeI</td>
</tr>
<tr>
<td>pPyr31XVI-B</td>
<td>StyI</td>
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<tr>
<td>pPyr31XVI-A</td>
<td>MfeI</td>
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<tr>
<td>pPyr31XVI-B</td>
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<td>pPyr31XVI-B</td>
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<td>pPyr31XVI-B</td>
<td>StyI</td>
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<tr>
<td>pPyr31XVI-B</td>
<td>StyI</td>
</tr>
<tr>
<td>pPyr31XVI-B</td>
<td>StyI</td>
</tr>
<tr>
<td>pPyr31XVI-B</td>
<td>StyI</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of *E. coli* transformants identified with plasmid genotype described in Table 1.

Discussion

Initially, it was hypothesized that introducing a site-specific mutation in the *pyrG* marker contained on the plasmid could be used to select for only those transformants where integration had occurred in a manner as shown in Fig. 1a. The primary objective of this approach was to prevent prototrophic growth from autonomous replication of plasmid, while still providing a route to complement the auxotrophic mutation in the recipient strain. The two sets of plasmids designed in this study, pPyr31XVI-A and B, contained frameshift mutations within the coding region of the *pyrG* gene and were believed to be incapable of providing functional OMP decarboxylase, unless integrated into the *pyrG* locus by a type I crossover. However, this integration must occur within the region of DNA between the chromosomal and plasmid mutation in order for the two mutations to be paired together on the same copy of the duplicated *pyrG* gene (Fig. 1a). The remaining duplicated gene would therefore be functional, assuming that no mutations were introduced during the homologous crossover recombination process. Any additional copies of the plasmid that were integrated would remain non-functional. In theory, both sets of plasmids are capable of restoring a functional copy of the *pyrG* gene in this manner, if integration occurs in the region of DNA between the chromosomal and plasmid mutation. Both sets of plasmids were transformed as circular DNA to determine whether integration must follow this postulate in order for duplication of a functional *pyrG* to occur. In addition, plasmids were linearized with either MfeI or StyI to more efficiently direct crossover and integration at these loci. It was recognized that the majority of the linearized plasmids would re-circularize by NHEJ, but these plasmids were thought to be incapable of expressing functional *pyrG* unless integrated and hence would not be maintained.

The results of this work clearly show that integration was not the sole mechanism capable of restoring growth
Fig. 3 Possible route for repair of plasmid mutations through DSBR recombination. a Initiation of DSBR by 5’-3’ end-processing to yield 3’ single-strand overhangs. b 3’ ends invade homologous chromosomal DNA molecule to form D-loop and prime new DNA synthesis. The newly replicated DNA is then captured by the resected 5’ ends to form a double HJ, which can migrate along the DNA chain. c-d Cleavage of each HJ must proceed past the branched target to allow for recombination and repair at this mutated site. It is assumed that the other HJ can also migrate beyond the pyr181 site to allow recombination of the chromosomal mutation (Fig. 3c-d). Likewise, branch migration of the D-loop or HJ must proceed past the Mfel site of plasmid pPyr31XV1-B to allow for recombination and repair at this mutated site. Continuation of this branch migration beyond the pyr181 is required for recombination at this site.

Cleavage of each HJ at points “C” and “D” (Fig. 3, points c/d) would release the modified plasmids, while cleavage of the HJ at points a/d or b/c would result in integration of the plasmid. Unfortunately, there are several inconsistencies when using this model of recombination to explain the observed mutation repair of the plasmids. It was originally proposed that resolution of the HJ occurs with almost equal frequency through either plane of the rotated linkage, resulting in approximately 50% crossover (Orr-Weaver and Szostak 1983). More recent experimental evidence suggests that resolution of the HJ is not entirely stochastic, which may help to explain the variability of crossover events (Ira et al. 2003; Prado and Aguilera 2003; Merker et al. 2003). We still expected that integration from cleavage of the HJs in opposite planes would be detected with our mutated constructs more frequently than observed in this study. Alternative cleavage at points c/d to release the modified plasmids, followed by mismatch repair or post-mitotic segregation, could yield several different recombinant plasmids that were observed in this study, including those with repaired mutations. However, this type of recombination for plasmid pPyr31XV1-A should result in some isolates having a Styl mutation at the genomic pyrG locus. There would not be any selective pressure against this mutation if a repaired pyrG existed on one of the plasmids. Yet, this event was not detected in any of our isolates. Resolution of the HJ in either plane appeared to be a rare event, suggesting that other models of recombination must be responsible for the gene conversions.
Fig. 4 Proposed model for the repair of plasmid mutations through SDSA mechanisms. a 5'-3' end-processing to generate single-strand overhangs beyond plasmid mutations, followed by 3' strand invasion to form a D-loop. b-1 DNA replication primed from the 3' ends uses the chromosomal homologue as template to restore one strand of plasmid DNA to a functional genotype. Strands can then re-anneal without any recombination, but must be repaired to resolved mismatched bases. b-2 Alternative route of SDSA when DNA synthesis extends beyond the chromosomal pyr181 mutation. Diagram on left represents MfeI-linearized pPyr31XVI-A, diagram on right represents StyI-linearized pPyr31XVI-B. Top strands Plasmid DNA shown without vector sequence being contiguous with pyrG flanking ends, bottom strands chromosomal DNA.

We propose that other mechanisms, such as SDSA (Hastings 1988; McGill et al. 1989; Nassif et al. 1994) or BIR, are more likely responsible for repair or gene conversion of the plasmids (Fig. 4). With SDSA, the chromosomal DNA serves as a template to correct the mutations without crossovers and non-reciprocal exchange of DNA between homologues. SDSA still requires 5'-3' resection of the DSB, strand invasion of the 3' end, and new DNA synthesis, as shown in Fig. 4a. Unlike DSBR, this type of recombination does not involve capturing the 5' ends of the DSB and results in the formation of a three-way junction (Prado and Aguilera 2003) instead of the HJ. Since cleavage cannot occur at a three-way HJ, DNA synthesized using the opposite homologue as template, re-anneals to the original strand and any resulting gaps are filled-in (Fig. 4b-1). Cross-over of the DNA does not occur with SDSA and therefore integration of the plasmid cannot result.

It is important to realize that new DNA synthesis must extend past the plasmid mutations in order to allow gene conversion to occur by SDSA (Fig. 4a). The DNA containing the mutation site could be removed by 5'-3' end-processing of the DSB or the extra flap could be cleaved after re-annealing has occurred (Prado et al. 2003; Paques and Haber 1999; Heyer et al. 2003). Strand invasion could occur with either end of the DSB (Hunter and Kleckner 2001; Allers and Lichten 2001), which would dictate whether the coding or non-coding strand of the chromosomal DNA serves as template for new synthesis on the invading strand. Gene conversion that results in the same end-products is possible if we assume that branch migration occurs equally with invasion of either strand. However, it is possible that the efficiency of branch migration of the three-way junction may be altered in the region of the plasmid mutations introduced at MfeI and StyI. These sites have four additional nucleotides, compared with the genomic homologue, which would result in a bulge when paired together. In contrast, the pyr181 chromosomal mutation is only a single nucleotide transversion, where branch migration would be expected to be uninhibited. Therefore, we suggest that it is more likely that gene conversions with the two plasmids involve strand invasion of opposite strands in order to bypass migration of the three-way junction through the plasmid mutation. For plasmid pPyr31XVI-A, this would involve invasion with the opposite 3' plasmid strand from that shown in Fig. 4a, while SDSA with plasmid pPyr31XVI-B would be as shown.

Even after SDSA gene conversion, the mismatched bases on the modified plasmids would still have to be repaired in order to restore a functional pyrG. We suggest that DNA strand segregation during subsequent mitosis is probably responsible for this event and the reason why transformation efficiency with StyI-linearized pPyr31XVI-B is so low, compared with MfeI-linearized pPyr31XVI-A. Each model of strand invasion, DNA synthesis, and branch migration should work equally well with gene conversion on both plasmids. The main difference between these two plasmids is how the
mutations segregate with SDSA. Plasmid pPyr31XVI-A should always result in the plasmid DNA containing one functional strand and one mutated strand, prior to mismatch repair. In contrast, plasmid pPyr31XVI-B will only result in the plasmid DNA containing one functional strand if the new DNA synthesis extends just through the MfeI site, prior to re-annealing of the strands. Both strands will be mutated if synthesis continues beyond the chromosomal `pyr181. If the mismatch repair mechanism were primarily responsible for correcting improperly paired nucleotides, we would again expect equal transformation efficiency between the plasmid sets. However, if strand segregation during mitosis occurred prior to mismatch repair, then both strands would be mutated and restoration to a functional `pyrG would be less likely to occur. This could help in explaining why isolates transformed with plasmid pPyr31XVI-B took 8–10 days to appear on the initial selection medium.

While SDSA helps to explain the recombination events observed in this study, the possibility of other repair mechanisms, such as BIR, are not excluded. BIR is very similar to SDSA, except that it involves recombination of only one end of the DSB (Kraus et al. 2001). DNA would still have to be removed from the plasmid by 5'–3' end-processing of the DSB or flap removal after SSA. It is also tempting to hypothesize that BIR could provide an alternative pathway that avoids branch migration through the mismatched nucleotides of the mutation site. BIR is typically associated with long gene conversions (up to several hundred kilobases), while SDSA is becoming more accepted as the primary recombination event for gene conversions during mitosis (Prado et al. 2003). There are also examples where both events are detected for the recombination of flanking markers (Merker et al. 2003).

It is not entirely clear how repair occurred when uncut circular plasmids were used for transformation. The most likely explanation is that a small percentage of plasmid used for transformation contained DSBs from physical and/or endonuclease cleavage within the homologous `pyrG gene. SDSA or BIR could then proceed as described above for repair of plasmid pPyr31XVI-A. Plasmid pPyr31XVI-B probably was repaired in the same manner. However, since integration of plasmid pPyr31XVI-B should not have been capable of restoring prototrophic growth (Fig. 1b), crossover was probably preceded by recombination with SDSA or BIR.

It was also interesting to note the high percentage of mutated plasmids detected in Rhizopus transformants, even though there was no selective pressure for their maintenance. There were even a few transformants where we could only find a mutated plasmid, although we assume that a functional plasmid must have been present. This demonstrates that plasmid replication and maintenance in Rhizopus is not necessarily contingent on selective pressure. We were surprised to find so many dimers with several different combinations of modified plasmids. It is difficult to speculate on the mechanisms involved in these formations, since the dimers represent such a small fraction of the total concatenated plasmid in Rhizopus isolates. It is possible that these combinations could arise from recombination between the various modified plasmids in the Rhizopus transformant. Also, we should not reject the possibility that some recombination of the plasmid dimers might have occurred in the E. coli used for plasmid recovery. We feel that any recombination that might have occurred in E. coli probably represents a very small percentage of the total isolates. Instability or recombination of Rhizopus DNA has never been a problem in our laboratory when we have used recA strains, as in this study. Furthermore, similar results to those described in this paper have been obtained with alternative E. coli recombination mutants having defective genes in recB/recJ/sbcC, uvrC (UV repair), and umuC (SOS repair; data not shown).

References


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Supplied by the U.S. Department of Agriculture, National Center for Agricultural Utilization Research, Peoria, Illinois