A new vector for efficient gene targeting to the pyrG locus in Aspergillus niger

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Abstract

Background: The possibility for efficient gene targeting for the controlled integration of DNA constructs is an important tool in fungal genetics.

Findings: In this study, we report a new targeting vector based on the pyrG marker in Aspergillus niger. The DNA sequence to be targeted is surrounded by two fragments of the pyrG gene to allow homologous recombination of the recombinant DNA at the pyrG locus. The 5’ end of the targeting cassette contains a non-functional truncated pyrG open reading frame (first 112 bases deleted) and the 3’ untranslated region (3’ UTR). At the 3’ end, the targeting cassette consists of the 3’ flanking region of the pyrG gene. A unique NotI site between the flanks allows the insertion of a gene of interest. The linearized targeting cassette is transformed to the A. niger pyrG mutant strain AB4.1 or a derivative thereof. By using a constitutively expressed luciferase reporter gene (mluc) as an example, it is shown that the targeting system is efficient as 4 out of 6 (67%) AB4.1 transformants and 51 out of 66 (77%) MA169.4 (ku70−) transformants contained the reporter gene at the pyrG locus. A luciferase (lux) activity assay, performed with independently obtained transformants in which the mluc reporter was integrated at the pyrG locus, showed comparable and reproducible lux activities.

Conclusion: The new pyrG targeting vector is an important improvement to the existing method for gene targeting in A. niger. Although the vector is specific for A. niger, the presented design and approach is easily applicable for constructing integration vectors for other fungi.

Keywords: Reporter gene, Luciferase activity, Promoter analysis

Finding
The pyrG gene of Aspergillus niger (An12g035570) is homologous to the Saccharomyces cerevisiae URA3 gene. In A. niger, as well as in other fungi, the pyrG marker is popular because it is a stringent auxotrophic marker which can be fully supplemented with uridine or uracil [1-3]. Another advantage of the pyrG marker over other auxotrophic markers is that it is counter selectable and pyrG mutants can be obtained by isolating 5-fluoro-orotic resistant mutants [4,5]. The pyrG mutant strain AB4.1 is often used in A. niger genetics [1], for instance in numerous studies to create gene deletion mutants using the pyrG gene of A. oryzae as a heterologous marker, initially developed by de Ruijter-Jacobs et al. [6]. The A. niger Δku70, pyrG− mutant MA70.15 [7] and ku70−, pyrG− mutant MA169.4 [8], are derived from AB4.1. Sequence analysis of the pyrG gene in the AB4.1 mutant showed a deletion of a cytosine at position 378 bp in the pyrG gene, causing an out of frame mutation after 103 amino acids. The AB4.1 mutant has been used to set up a system for targeted integration of e.g. reporter constructs to the pyrG locus [9]. This system, referred to as the pyrG* (pyrG star) system, makes use of the AB4.1 mutant, in combination with a plasmid based mutated A. niger pyrG gene. The mutation in the plasmid based pyrG gene was introduced by filling in the BamHI restriction site (position 828 bp in the pyrG ORF) that is present in the protein-coding region of the gene, causing a frame shift. The vector containing the pyrGmut* will not be functional when ectopically integrated and will therefore not give rise to uridine prototrophic transformants. However, targeted integration of the pyrGmut*BamHI at the pyrG locus of strain AB4.1 via a single cross over will result in the integration of the vector at the pyrG locus (Figure 1). The pyrG* system has been successfully used in several studies for the targeted integration of reporters in
gene expression studies [10,11], or for the controlled integration of expression cassettes [12] and GFP-fusion proteins [13].

However, there are certain drawbacks of using the pyrG* method. Transformation frequencies are on average 10 to 20 times lower compared to regular pyrG transformations, often resulting in only one or two transformants per transformation. In addition, the percentage of successful integration of the reporter construct, varying between 10 to 50%, is rather low, probably due to a recombination or repair event that restores the mutation in the pyrG gene in AB4.1. Another disadvantage of the pyrG* system is that after targeted integration, two pyrG repeats are present around the construct, making the loss of the reporter construct via a loop out event possible (Figure 1). Finally, despite the well established positive effect on gene targeting efficiencies in ku70 mutants [7,8], we noticed that deletion of the ku70 gene did not improve transformation or targeted integration frequencies of the pyrG* plasmid. The reason for this is not known and systematic studies to analyse homologous integration using circular (pyrG*) or linear DNA fragments in wild type or ku70 strains to clarify this have not been reported. These drawbacks make the construction of A. niger strains with targeted integration, such as of reporter constructs, a time consuming and laborious exercise.

In this study, a new pyrG targeting vector is presented to facilitate the construction of strains in which a reporter construct or another gene of interest is targeted to the pyrG locus, which is named pyrG** (pyrG double star). The targeting vector (pMA334) is schematically depicted in Figure 2A and consists of a 5’ truncated non-functional pyrG gene, the pyrG 3’ UTR, a unique NotI site, and a 3’ pyrG flanking region. The position of the NotI site has been chosen directly behind the end of the 3’ UTR of the pyrG mRNA and is based on RNA seq data (personal communication with P. Punt). The NotI site can be used to clone a particular DNA fragment (e.g. promoter reporter construct) in the targeting vector. Transformation of the linear DNA fragment will only result in a functional pyrG gene when the targeting vector integrates at the pyrG locus via a double cross over event (Figure 2B). The first cross over must occur between the 5’ end of the truncated pyrG gene on the plasmid and the pyrG mutation in AB4.1. The second cross over must occur at the 3’ flank for a complete integration of the cassette. In theory, a double crossover event could also occur between the 5’ truncated non-functional pyrG gene and the pyrG 3’ UTR, giving rise to pyrG prototrophic transformants that do not contain the reporter gene.

To test the efficiency of the pyrG** targeting system, the luciferase reporter gene mluc [12] was cloned behind the constitutive gpdA promoter and ligated into targeting vector pMA334, giving plasmid pMA349 (Figure 2A). Linear DNA was isolated by digestion of pMA349 using Ascl and after purification, 20 μg of DNA was transformed [5] into A. niger strain AB4.1 (pyrG*) (for strains and primers used in this study, see Table 1 and Table 2 respectively), resulting in 6 primary transformants. To check if integration of the reporter construct on the pyrG locus has occurred, all transformants were purified and analysed by Southern blot (Figure 3A). The result of the Southern blot analysis shows that 4 out of 6 transformants contain the reporter construct at the pyrG locus, while the other 2 transformants have a wild-type pyrG locus. The luciferase activity of these transformants was determined in a lux activity assay (Figure 3B), and only the strains that contain the reporter construct show lux activity.

In the pyrG** system, the reporter construct has to integrate at the pyrG locus via homologous integration. In order to test the performance of the pyrG** system in a ku70 background, strain MA169.4 (pyrG**, ku70”) was

![Figure 1 Schematic representation of integration of a reporter construct after a single crossover event using the pyrG* targeting system.](Image)
**Table 1 Strains used in this study**

| Strain  | Genotype            | Description                               | Reference |
|---------|---------------------|-------------------------------------------|-----------|
| N402    | cspA1               | derivative of N400                        | [14]      |
| AB4.1   | cspA1, pyrG378      | UV mutant of N402                         | [1]       |
| MA169.4 | cspA1, pyrG378, kusA::DR-amdS-DR | ku70 disruption in AB4.1 | [8]       |
| MA317.1-6 | cspA1, PgpaA-mluc-TtrpC on pyrG locus | This study |
| MA565.1-66 | cspA1, kusA::DR-amdS-DR, PgpaA-mluc-TtrpC on pyrG locus | This study |

**Table 2 Primers used in this study**

| Primer name | Sequence 5'-3' | Used for |
|-------------|----------------|----------|
| ABpyrGP12for-EcoRI-AscI | CGGAAATTCCGG CGGCCCCGCG | pyrG-3’ UTR PCR (Figure 2A) |
| ABpyrGP10rev-NotI | AAGGAAAAAA GCGGCCGCA | pyrG-3’ UTR PCR (Figure 2A) |
| ABpyrGP11for-NotI | AAGGAAAAAA GCGGCCGCA | pyrG PCR (Figure 2A) |
| ABpyrGP13rev-SacI-AscI | CGGACCTCGGC CGCCCTCCTG | pyrG PCR (Figure 2A) |
| SL1 | ATTTGCGGCC GCAGAACGCC | PgpaA-mluc PCR (Figure 2A) |
| TtrpCP2rev-NotI | AAGGAAAAAA GCCGCGCCGTC | PgpaA-mluc PCR (Figure 2A) |

*Relevant restriction sites are shown in italic.

**Figure 2 Schematic representation of integration of a reporter construct after a double crossover event using the pyrG**<sup>**</sup>** targeting system.**

A) The truncated pyrG gene (-112) + 3’ UTR fragment (1255 bp) was amplified by PCR using primers ABpyrGP12f and ABpyrGP10r. The 3’ pyrG fragment (684 bp) was amplified by PCR using primers ABpyrGP11f and ABpyrGP13r. Both PCR products were digested (EcoRI-NotI for pyrG-3’ UTR and NotI-SstII for 3’ pyrG) and ligated in EcoRI-StII opened pBluescriptSK, yielding plasmid pMA334. The mluc reporter cassette was obtained by PCR using SL1 and TtrpCP2rev-NotI as primers and pMA313 (containing PgpaA-mluc-TtrpC-AOpyrG, unpublished vector) as template. pMA334 was opened with NotI and the NotI digested PgpaA-mluc-TtrpC fragment was inserted, giving plasmid pMA349. Both plasmids have been deposited at Fungal Genetic Stock Centre. pMA349 was digested with AscI to release the complete pyrG**<sup>**</sup>** targeting transformation DNA. B) Integration of the pyrG**<sup>**</sup>** targeting construct via a double cross over at the pyrG locus.
transformed with 10 μg of linear DNA, isolated by digestion of plasmid pMA349 with AscI. In total 66 primary transformants were obtained and purified. These transformants were analysed for their luciferase activity in a lux assay (data not shown), resulting in 51 out of 66 (77%) strains that show lux activity. Southern blot analysis (data not shown) of 15 selected strains showed that 13 strains, which showed comparable lux activities in the lux assay, contain the lux reporter construct at the pyrG locus. In the other two strains, that were negative in the lux assay, the reporter construct was not present. No ectopic integrations were detected in these 15 transformants.

**Figure 3** Analysis of *A. niger* MA317 transformants containing the *Pgpd-mluc* reporter construct using the pyrG** targeting method.

**A**) Southern blot analysis. Genomic DNA was restricted with *Eco*RI or *Kpn*I and size fractioned by electrophoresis on a 1.0% agarose gel. For hybridisation, 32P-labelled *pyrG* probe (1255 bp, Figure 2A) or 3' *pyrG* probe (684 bp, Figure 2A) were used. When digested with *Eco*RI and using the *pyrG* probe (upper panel), a signal of 9.0 kb corresponds with the wild-type *pyrG* locus, while a signal of 4.9 kb corresponds with integration of the *Pgpd-mluc* cassette at the *pyrG* locus. When digested with *Kpn*I and using the 3' *pyrG* probe (lower panel), a signal of 3.3 kb corresponds with the wild-type *pyrG* locus, while a signal of 4.8 kb corresponds with integration of the *Pgpd-mluc* cassette at the *pyrG* locus. Strains MA317.1 and MA317.3 have the wild-type *pyrG* locus, while strains MA317.2 and MA317.4-6 contain the *Pgpd-mluc* cassette at the *pyrG* locus.

**B**) Lux activity assay. The lux activity assay described by Meyer et al. [12] has been slightly modified. Briefly, 100 μL of 2 x Minimal Medium [5] with 0.006% yeast extract (w/v), 76 μL deionized water, 4 μL 25 mM luciferin (Promega, E1605) and 20 μL spore suspension (7.5*10^5 spores/mL) was pipetted together (in triplicate) in a well of a white, clear bottom, 96 wells plate (Greiner Bio-one, ref 655095) and incubated for 24 hours at 30°C in the EnSpire Multiplate Reader (Perkin) with continuous measuring of lux and OD. Lux activities after 16 h of incubation are shown here. Strains MA317.1 and MA317.3 have no lux activity, while strains MA317.2 and MA317.4-6 show comparable lux activities.
In the experiments described above, the results indicate that the pyrG** targeting system is both useful in wild-type and ku70− strains, even though the transformation frequency in the wild-type strain is much lower. It is likely that this lower frequency of transformation is due to the ectopic integration of the cassette, which does not result in transformants as this integration does not reconstitute the pyrG gene.

The new targeting system has recently been successfully used in two independent studies in our laboratory. In the first study (A-M Burggraaf-van Welzen, unpublished results) 4 different ku70− strains and 2 different reporter genes were used. Out of 28 transformants analysed, 23 transformants contained the reporter construct at the pyrG locus (82%). In the second study (J. Niu, unpublished results), a Δku70 strain was transformed with 6 different reporter constructs. Out of 122 primary transformants analysed, 105 transformants contained the reporter construct (86%). Southern analysis of 24 transformants (four of each construct) confirmed integration at the pyrG locus in all transformants analysed. These studies further confirm the efficiency and ease at which transformants with targeted integrations are obtained.

The described method to obtain transformants with targeted integration is not restricted to pyrG mutants, but can also be used for other auxotrophic markers. Pre-requisite is that the mutation in the auxotrophic marker is determined to allow design of the targeting cassette.

Availability of supporting data
The data set supporting the results of this article is included within the article. Plasmids and plasmids maps and DNA sequences are deposited at Fungal Genetics Stock Centre.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MA carried out the molecular genetic studies. MA and EL designed and carried out the luciferase experiments. MA and AR designed the study and drafted the manuscript. All authors contributed to the writing. All authors read and approved the final manuscript.

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