A set of isogenic auxotrophic strains for constructing multiple gene deletion mutants and parasexual crossings in *Aspergillus niger*

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**Introduction**

*Aspergillus niger* has attracted considerable interest as cell factories for the production of organic compounds (citric acid and secondary metabolites) or (recombinant) proteins (Andersen et al. 2013; Meyer et al. 2015; Pel et al. 2007; Ward 2012). *A. niger* is not only an important cell factory, it also has become an important model system for fungal development (Krijgsheld et al. 2013; Wösten et al. 2013). System biology-based approaches in combination with targeted metabolic engineering techniques are important tools to study and optimize production processes (Caspeta and Nielsen 2013; Jacobs et al. 2009). With relative ease, gene knockouts can be made using the *ku70* mutants (Carvalho et al. 2010; Meyer et al. 2007) in combination with split marker approaches (Nielsen et al. 2006; Goswami 2012; Arentshorst et al. 2015a). Together with tools for controlled overexpression of genes using the tetracycline promoter system (Meyer et al. 2011), metabolic engineering can be efficiently performed. A limiting factor for metabolic engineering in *A. niger* is the limited number of isogenic auxotrophic mutants with multiple auxotrophic markers, in which multiple gene deletion mutants can be made quickly without the need to recycle the selection markers. Selection markers such as the *pyrG* marker or the *amdS* marker are counter-selectable, but when multiple deletions need to be made, these markers need to be recycled, which is time-consuming. To overcome this limitation, we have selected the *nicB* gene (encoding nicotinate mononucleotide pyrophosphorylase; Verdoes et al. 1994), the *argB* gene (encoding ornithine carbamoyltransferase; Lenouvel et al. 2007), and the *adeA* gene (encoding adenosine deaminase; Stroobants et al. 2003) as multiple auxotrophic markers.

**Abstract**

To construct a set of isogenic auxotrophic strains in *Aspergillus niger* suited for creating multiple gene deletion mutants and executing parasexual crossings, we have combined mutations in genes involved in colour pigmentation (*fwnA* and *olvA*) with well-selectable auxotrophic markers (*pyrG*, *nicB*, *argB*, and *adeA*). All markers, except for the *pyrG* marker, were introduced by targeted deletion, omitting UV mutagenesis of the strains. *Aspergillus oryzae* orthologous genes of the *argB*, *nicB*, and *adeA* markers were used as heterologous selection markers, and all markers were shown to complement to respective auxotrophic *A. niger* mutants. A quadruple auxotrophic marker was further constructed suitable for multiple gene deletions. Genome sequencing of two auxotrophic colour mutants JN3.2 (*olvA::pyrG, argB::hygB*) and JN6.2 (*olvA::pyrG, nicB::hygB*) revealed four SNPs between them in non-coding regions, indicating a high level of isogenicity between both strains. The availability of near-isogenic complementary auxotrophic colour mutants facilitates the selection of diploids and the isolation of haploid segregants from the diploid using the parasexual cycle.

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2002), and the adeA gene (encoding phosphoribosylaminooimidazole-succinocarboxamidase synthase) (Jin et al. 2004; Ugolini and Bruschi 1996) of A. niger to construct near-isogenic auxotrophic marker strains containing four auxotrophic markers (pyrG, nicB, adeA, and argB). In combination with dominant selection markers such as hygromycin resistance (Punt and van den Hondel 1992), phleomycin resistance (Punt and van den Hondel 1992), and AmdS selection (Kelly and Hynes 1985), seven different markers are available for strain construction.

The lack of a sexual cycle in A. niger limits easy crossing of two strains to combine interesting properties or to construct double mutants. Despite the lack of a sexual cycle, the parasexual cycle can be used to combine genetic traits in A. niger (Pontecorvo et al. 1953; Swart et al. 2001). The parasexual cycle includes the selection of a heterokaryon and subsequently the selection of a diploid strain. The frequency by which diploids are formed from a heterokaryotic mycelium in A. niger is very low, and selection of diploids can be accomplished by crossing strains that have complementary auxotrophic and complementary spore colour markers. Only when a diploid is formed, the resulting colony will produce solely black conidiospores which can be easily detected by eye. The genes encoding proteins involved in spore melanin production in A. niger have been identified (Jørgensen et al. 2011). Several studies, mainly conducted by Bos et al., have reported on the isolation of A. niger colour and auxotrophic mutants [see for review (Swart et al. 2001)]. However, most of these mutants were isolated by UV treatment. Although carried out with caution and relative high survival rates, unwanted random mutations are inevitable, leading to possible growth defects. By targeted deletion of spore colour genes and auxotrophies, we constructed a set of near-isogenic strains suitable for parasexual crossings (Niu et al. 2016). We performed genome sequencing of two auxotrophic colour mutants and confirmed the near-isogenicity between these auxotrophic mutants.

### Materials and methods

#### Strains and growth conditions

The A. niger strains used in this study are listed in Table 1. Auxotrophic strains are deposited at the Fungal Genetic Stock Centre. A. niger strains were grown on minimal medium (MM) (Bennet and Lasure 1991) or on complete medium (CM) consisting of minimal medium with the addition of 5 g/L yeast extract and 1 g/L casamino acids. When required, 10 mM uridine, 200 μg/mL L-arginine, 2.5 μg/mL nicotinamide, 100 μg/mL hygromycin, or 40 μg/mL phleomycin was added. Adenine was directly added from the solid stock to the medium to a final concentration of 200 mg/L after autoclaving and dissolved by mixing. Fluoroacetamide (FAA) and 5-fluoroorotic acid (5-FOA) counter-selection was performed as described (Carvalho et al. 2010; Arentshorst et al. 2012) to remove the amdS marker and the pyrG marker, respectively.

#### Molecular biological techniques

Transformation of A. niger and chromosomal DNA isolation of A. niger and Aspergillus oryzae were performed

| Name | Genotype/description | Reference/source |
|------|----------------------|-----------------|
| N402 | cspA1, derivative of N400 | Bos et al. (1988) |
| A. oryzae | ATCC16868 | – |
| MA169.4 | kusA::amdS, pyrG^- | Carvalho et al. (2010) |
| MA100.1 | cspA1, fwnA::hylG, kusA::amdS, pyrG^- | Jørgensen et al. (2011) |
| AW8.4 | cspA1, olvA::AopyrG in MA169.4 | Jørgensen et al. (2011) |
| JN3.2 | argB::hylG, olvA::AopyrG (derived from AW8.4) | This study |
| JN6.2 | nicB::hylG, olvA::AopyrG (derived from AW8.4) | This study |
| JN1.17.1 | argB::hylG in MA169.4 | This study |
| OJP3.1 | nicB::phleo in MA169.4 | This study |
| OJP1.1 | adeA::pyrG in MA169.4 | This study |
| MA322.2 | ku70::amdS, nicB::AopyrG in MA169.4 | This study |
| MA323.1 | ku70::amdS, ΔnicB^-, pyrG^- | This study |
| MA328.2 | ku70::amdS, ΔnicB^-, adeA::AopyrG | This study |
| MA329.1 | ku70::amdS, ΔnicB^-, ΔadeA^-, pyrG^- | This study |
| MA334.2 | ku70::amdS, ΔnicB^-, ΔadeA^-, argB::AopyrG | This study |
| MA335.3 | ku70::amdS, ΔnicB^-, ΔadeA^-, ΔargB^-, pyrG^- | This study |
A. niger strain MA169.4 (transformation to the purified linear PCR fragment was used for subsequent DNA as template with primers argBKO1 and argBKO4 and gene deletion cassette was amplified by PCR using pJN4.5 disruption plasmid pJN4.5. The argB result in the Δ obtained from plasmid pIII/3-kb flank of 3 with obtain plasmid pJN3.3. Subsequently, pJN3.3 was digested of +ers listed in Supplementary Table 1. The PCR products, followed by cloning of the three purified PCR products, were amplified from wild-type A. niger A. oryzae genes, including their promoter and terminator regions, were amplified from N402 genomic DNA with primers Fw_adeA_5' and Rev_adeA_3' to obtain the 0.9-kb 5'flanking region and Fw_adeA_3' and Rev_adeA_3' to obtain the 0.7-kb 3'flanking region (Supplementary Table 1). The 1.8-kb A. nidulans pyrG selection marker was introduced by PCR from the plasmid pCRpyrGAN (Ouedraogo et al. 2015) with the primers Fw_pyrG_adeA and Rev_pyrG_adeA which contain complementary sequence of Rev_adeA-5' and Fw_adeA-3', respectively (Supplementary Table 1). The adeA::Anid_pyrG deletion cassette was obtained by a fusion PCR of the three purified PCR products, followed by cloning of the 3.4-kb fusion PCR product into pJet1.2, resulting in plasmid pOPJ1 and used for transformation to A. niger strain MA169.4 (ku70', pyrG') to give OJP1.1 (ku70', ΔadeA::pyrG). Proper deletion of the nicB, adeA, and argB genes was confirmed by Southern blot analysis (Supplementary Figures. 1–3).

For complementation studies, argB, nicB, and adeA genes, including their promoter and terminator regions, were amplified from wild-type A. oryzae and A. niger genomic DNA with appropriate primer pairs described in the Supplementary Table 1. The respective complementing gene fragments were cloned into pJet1.2 (Thermo Scientific) and sequenced (Table 2). The plasmids pOPJ5 (pJet1.2_AoniargB), pOPJ4 (pJet1.2_Aoni nicB), pOPJ3 (pJet1.2_Aoni adeA), pJN29 (pJet1.2_Aory argB), pJN30 (pJet1.2_Aory nicB), and pJN31 (pJet1.2_Aory adeA) were used to complement the respective auxotrophic mutants.

Recyclable split marker strategy for creation of a strain with multiple auxotrophies

To construct an A. niger strain with multiple auxotrophies, it was necessary to use a recyclable split marker approach. Therefore, auxotrophic marker-specific direct repeats (DR) surrounding the AOprrG selection marker were introduced by PCR. By selecting on 5-FOA, the AOprrG marker was removed. The recyclable split marker approach is outlined in Fig. 1; see Supplementary Table 1 for primer sequences. Strain MA169.4 (ku70', pyrG') was used as starting strain to first delete the nicB gene and, subsequently, adeA and the
argB marker. All strains containing single, double, triple, and the quadruple auxotrophic strain are listed in Table 1. Correct integration of split marker fragments and successful loop out of the \textit{AOpyrG} was confirmed by Southern blot analysis for all strains and shown for MA335.3 in Supplementary Figures 1–3).

### Table 2  Plasmids used in this study

| Name          | Description                              | Reference/source |
|---------------|------------------------------------------|------------------|
| pJN3.3        | 5′ flank of \textit{argB} in pBluescript II SK(+) | This study       |
| pΔ2380        | Δ\textit{ugmB}:\textit{hygB} deletion cassette | Damveld et al. (2008) |
| pJN4.5        | pBluescript\_\textit{argB}:\textit{hygB} | This study       |
| pJN8.1        | 5′ flank of \textit{nicB} in pBluescript II SK(+) | This study       |
| pMA299        | pBluescript\_\textit{phleo} | This study |
| pJN10.1       | pBluescript\_\textit{nicB}:\textit{phleo} | This study |
| pCKpyrGAN     | Containing the full gene of \textit{A. nidulans pyrG} | Ouedraogo et al. (2015) |
| pOJP1         | pJet1.2\_\textit{adeA}:\textit{pyrG} | This study |
| pOJP5         | pJet1.2\_\textit{Anig.argB} | This study |
| pOJP4         | pJet1.2\_\textit{Anig.nicB} | This study |
| pOJP3         | pJet1.2\_\textit{Anig.adeA} | This study |
| pJN29         | pJet1.2\_\textit{Aory.argB} | This study |
| pJN30         | pJet1.2\_\textit{Aory.nicB} | This study |
| pJN31         | pJet1.2\_\textit{Aory.adeA} | This study |
| pAO4-13       | Containing full \textit{pyrG} gene of \textit{A. oryzae} | de Ruiter-Jacobs et al. (1989) |

**A. niger parasexual cycle**

Heterokaryon formation and selection for diploids was performed as described (Pontecorvo et al. 1953). Segregation of diploids by benomyl was performed essentially as described (Bos et al. 1988) with slight modifications (Niu et al. 2016).

### Sequencing and analysis

Genome sequencing of JN3.2 (\textit{olvA}:\textit{pyrG}, \textit{argB}:\textit{hygB}) and JN6.2 (\textit{olvA}:\textit{pyrG}, \textit{nicB}:\textit{hygB}) was performed using NGS platform (Illumina GA) as described (Park et al. 2014). Sequencing was performed at ServiceXS, Leiden, The Netherlands. SNPs between JN3.2 and JN6.2 were identified using \textit{A. niger} strain ATCC1015 (http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Aspni5) as reference genome. For each SNP, it was verified whether the SNP was in a predicted protein-encoding region using the \textit{A. niger} 3.0 genome at JGI using the SNP coordinates (Park et al. 2014).

### Results and discussion

**Construction and characterization of \textit{argB}, \textit{nicB}, and \textit{adeA} auxotrophic mutants**

Deletion constructs \textit{nicB}:\textit{hygB}, \textit{argB}:\textit{phleo}, and \textit{adeA}:\textit{pyrG} were transformed to strain MA169.4 (\textit{ku70}−, \textit{pyrG}−), and hygromycin, phleomycin resistant, or uridine prototrophic transformants were obtained and purified. Proper deletion of the respective markers was verified by diagnostic PCRs (data not shown) and by testing the growth...
on MM plates containing the relevant supplements. As shown in Fig. 2, the \textit{nicB}, \textit{argB}, and \textit{adeA} mutants required the addition of the nicotinamide, L-arginine, or adenine to allow growth.

To determine the minimal concentrations of nicotinamide, arginine, or adenine for full supplementation, spores of the auxotrophic mutants were spotted on plates containing a concentration series of the respective supplement and incubated at 30 °C for 3 days for arginine and nicotinamide supplementation test and for 4 days for adenine supplementation test.

![Supplementation test of the auxotrophic \textit{A. niger} mutants. 10 µL of a spore stock (1 × 10^7 conidia/mL) of each auxotrophic strain and the parental strain (MA169.4) was inoculated on an MM plate without and with serial concentrations of the respective supplement and incubated at 30 °C for 3 days for arginine and nicotinamide supplementation test and for 4 days for adenine supplementation test.](image)

| Supplement          | Concentration (mg/L) | JN1.17.1 | OJP3.1 | OJP1.1 |
|---------------------|----------------------|----------|--------|--------|
| L-arginine          | 0                    | 0.1      | 2      | 20     |
| nicotinamide        | 0                    | 0.25     | 1.25   | 2.5    |
| adenine             | 0                    | 10       | 50     | 100    |

The \textit{nicB}, \textit{argB}, and \textit{adeA} genes from \textit{A. oryzae} are suitable markers for \textit{A. niger} transformation

To prove that auxotrophic mutants can be complemented by heterologous and homologous markers, DNA fragments containing the \textit{argB}, the \textit{nicB}, and the \textit{adeA} genes from \textit{A. oryzae} and \textit{A. niger}, including their promoters and 3′ untranslated sequences, were used for the complementation of the respective \textit{A. niger} auxotrophic mutants. Protoplasts of JN1.17.1 (\textit{argB::hygB}), OJP3.1 (\textit{nicB::phleo}), and OJP1.1 (\textit{adeA::pyrG}) were transformed with plasmids containing the corresponding marker genes from...
A. oryzae or A. niger. Transformants were obtained for the A. oryzae heterologous markers, which demonstrated that nicB, argB, and adeA of A. oryzae complemented the auxotrophy and therefore are suitable markers for A. niger transformations. As expected, also all A. niger genes (argB, nicB, and adeA) were able to complement the respective auxotrophic A. niger mutants. The obtained transformants were further analysed to determine whether the A. oryzae marker also complemented the auxotrophies. As shown in Fig. 4, all heterologous genes complement similarly to the homologous A. niger genes. A heterologous marker for gene disruption experiments is preferred as it reduces the homologous integration of the marker gene in the disruption cassette at the homologous site. We have compared the DNA sequence of the different genes markers of A. niger to those of A. oryzae by BLASTN (http://blast.ncbi.nlm.nih.gov/) using standard settings. The identity of the coding regions between the different gene markers was 73.3, 72.0, and 77.8 % for argB, nicB, and adeA genes, respectively. These values are comparable to the value obtained when comparing the pyrG genes markers of both Aspergillus species. The pyrG gene of A. oryzae is identical to the pyrG gene of A. niger at 78.6 % and has been so far successfully used to transform A. niger and vice versa (Carvalho et al. 2010; Mattern et al. 1987). It should be noted that complementation analysis in the Δku70 background is not efficient because of the low frequencies of ectopic integration the complementing fragment. To circumvent this limitation, we constructed a curable ku70 deletion strategy (Carvalho et al. 2010). The presence of ku70 repeats around the AmdS selection marker used to disrupt the ku70 gene allows efficient loop out of the AmdS marker via fluorooacetamide counter-selection as described (Arentshorst et al. 2012). An alternative method for easy complementation, which omits the need for curing the ku70 locus, is the use of a second auxotrophic marker which can be used to target the complementing gene to this locus. For the pyrG marker, an efficient gene targeting method has recently become available (Arentshorst et al. 2015b) which allows targeted
integration when the complementing fragment is cloned in the pyrG targeting vector. For example, one could start with a nicB−, pyrG− strain and use the nicB selection marker for initial deletion of the gene of interest, followed by a complementation experiment in which the complementing fragment is cloned in the pyrG targeting vector which is that transformed to the deletion strain.

Isogenic auxotrophic colour mutants for parasexual crossing in A. niger

Combining mutations by crossing strains is a powerful genetic tool for strain construction. In Aspergillus nidulans, this method is well established and used in many studies to construct double mutants (Todd et al. 2007). The lack of a sexual cycle in A. niger has limited the use of crossings to combine mutations. However, the use of the parasexual cycle in A. niger (Pontecorvo et al. 1953) has been used extensively for linkage studies in A. niger and can be used to combine mutations (Bos et al. 1988). Straightforward crossing in A. niger requires complementing auxotrophies to select for a heterokaryotic mycelium and preferably colour makers to select for a diploid strain. The frequency by which A. niger forms diploids is generally very low (1 in 106–107 spores), and diploids are not easily detected if wild-type strains are used that produce black conidia. By using complementing colour markers, a diploid can be selected as only this diploid will produce black spores, whereas a heterokaryotic mycelium will produce a mix of heterogeneously coloured spores (Pontecorvo et al. 1953). By combining colour mutants (fwnA and olvA) with complementary auxotrophic markers such as pyrG, nicB, or argB, heterokaryons and diploids can be easily selected. We constructed several auxotrophic colour mutant strains including MA100.1 (fwnA::hygB, pyrG−), JN3.2 (olvA::pyrG, argB::hygB), and JN6.2 (olvA::pyrG, nicB::hygB) (Table 1). In a recently conducted study, JN3.2 has been used for parasexual crossings to obtain haploid segregants (Niu et al. 2016). With these segregants, a bulk segregant analysis was performed to identify SNPs that are closely linked or responsible for the mutant phenotypes (Niu et al. 2016).

To test the isogenicity between two auxotrophic colour mutants JN3.2 (olvA::pyrG, argB::hygB) and JN6.2 (olvA::pyrG, nicB::hygB), the genomes of these strains were sequenced and compared to the genome of the reference ATCC strain. In total, 155 SNPs were found for JN3.2 and JN6.2, respectively, when compared to the ATCC reference strain (Supplementary Table 2). Two SNPs were found to be specific for JN3.2, and two SNPs were specific for JN6.2. None of them were found in predicted open reading frames (Table 3), demonstrating that JN3.2 and JN6.2 are likely to have no mutation affected its phenotype and that they are near-isogenic.

In conclusion, new auxotrophic strains carrying targeted deletions in the argB, nicB, and adeA genes of A. niger were constructed. The orthologous genes argB, nicB, and adeA of A. oryzae complemented the arginine, nicotinamide, and adenine auxotrophic mutants similar to the endogenous genes and are therefore suitable selection markers for A. niger transformations. The quadruple auxotrophic strain MA335.3 (argB−, nicB−, adeA−, and pyrG−) allows rapid deletion of multiple genes the need to recycle selection markers. The targeted deletion of auxotrophic markers instead of selection of auxotrophic strains after UV mutagenesis significantly reduces the occurrence of mutations as genome sequencing of two auxotrophic mutants (JN3.2 and JN6.2) revealed only four SNP between them.

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Table 3 SNP comparison JN6.2 and JN3.2

| Position | Allel ATCC | JN6.2 | JN3.2 | Details mutation |
|----------|------------|-------|-------|-----------------|
| chr_1_2  | 726,573    | T     | C     | Intergenic     |
| chr_3_4  | 45,864     | T     | A     | Intergenic     |
| chr_8_2  | 2,725,044  | G     | A     | Intergenic     |
| chr_8_2  | 2,725,045  | T     | A     | Intergenic     |
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