New Multi-Marker Strains and Complementing Genes for *Aspergillus nidulans* Molecular Biology

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Abstract

Technical advances in *Aspergillus nidulans* enable relatively easy deletion of genomic sequences, insertion of sequences into the genome and alteration of genomic sequences. To extend the power of this system we wished to create strains with several selectable markers in a common genetic background to facilitate multiple, sequential transformations. We have developed an approach, using the recycling of the *pyrG* selectable marker, that has allowed us to create new deletions of the *biA*, *pabaA, choA*, and *lysB* genes. We have deleted these genes in a strain that carries the commonly used *pyrG*89, *riboB*2, and *pyroA*4 mutations as well as a deletion of the sterigmatocystin gene cluster and a deletion of the *nkuA* gene, which greatly reduces heterologous integration of transforming sequences. The new deletions are fully, easily and cheaply supplementable. We have created a strain that carries seven selectable markers as well as strains that carry subsets of these markers. We have identified the homologous genes from *Aspergillus terreus*, cloned them and used them as selectable markers to transform our new strains. The newly created strains transform well and the new deletion alleles appear to be complemented fully by the *A. terreus* genes. In addition, we have used deep sequencing data to determine the sequence alterations of the venerable and frequently used *pyrG*89, *riboB*2 and *pyroA*4 alleles and we have reannotated the *choA* gene.

Keywords

Aspergillus; gene targeting; transformation; deletions; selectable markers

1. Introduction

Progress in transformation methods and in production of transforming DNA fragments has greatly facilitated molecular genetic manipulations of *Aspergillus nidulans* (Nayak et al., 2006; Szewczyk et al., 2006; Oakley et al., 2012; Nødvig et al., 2015). These advances allow rapid, sequential molecular genetic manipulations that would have been impossible a few

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years ago. To extend the power of this system and facilitate complex molecular genetic manipulations [e.g. multiple promoter replacements in a single strain (Yeh et al., 2016)], it would be advantageous to have strains carrying several selectable markers as well as cloned genes that complement the markers.

Many selectable mutations have been isolated in *A. nidulans* by conventional mutagenesis and multiply marked strains have been created by crosses. However, many of these mutations were created by heavy mutagenesis (often more than 90% of the mutagenized conidia were killed). This almost certainly creates a heavy background of single nucleotide polymorphisms and may create mutations that are silent under most conditions but manifest themselves under particular conditions or interact synthetically with other mutations [e.g. (Oakley and Morris, 1981)]. In addition, UV mutagenesis, which was used extensively in the isolation of *A. nidulans* mutations, can result in chromosomal abnormalities. Strain G191, the ancestor of many strains that carry *pyrG*89, a mutation that is used extensively as a selectable marker for transformation in *A. nidulans*, carries duplications of portions of chromosomes I and VIII and a likely translocation of a portion of chromosome VIII to chromosome VI (our unpublished data). It is also advantageous to have complementing markers from species other than *A nidulans* because they allow selection but have sufficiently low nucleotide homology to the corresponding *A nidulans* selectable marker that *A nidulans* sequences can be used to target the selectable marker to the desired location in the genome (Nayak et al., 2006). Finally, it is useful for selectable markers to be deletions to minimize or eliminate reversion.

We have used a molecular genetic approach to create new selectable markers by deleting target genes. Using this approach, we have created strains with as many as seven selectable markers in a common genetic background. We have also amplified the corresponding homologs from *Aspergillus terreus*, shown that they complement the deletions and cloned them into a plasmid vector.

2. Materials and Methods

2.1 Media and supplements

Our minimal medium consisted of 6 g/L NaNO$_3$, 0.52 g/L KCl, 0.52 g/L MgSO$_4$ 7H$_2$O, 1.52 g/L KH$_2$PO$_4$, 10 g/L d-glucose, and 400 µl/L of a trace element solution (Vishniac and Santer, 1957), 15 g/L agar and appropriate nutrients to supplement nutritional markers carried by the strains. YG (5 g/L yeast extract, 20 g/L d-glucose, supplemented with 400 µl/L of the same trace element solution) was used as a liquid complete medium and YAG (YG plus 15 g/L agar) was used as a complete solid medium. To support growth of our newly created mutant strains we supplemented minimal media with the following, as required, (final concentrations are listed) p-aminobenzoic acid (PABA), 1.0 µg/mL; biotin 20 ng/mL; L-lysine, 200 µg/mL and choline, 20 µg/mL. Supplements were not required for YAG or YG media.

2.2 Molecular genetic manipulations and transformation

Construction of transforming linear DNA molecules by fusion PCR was as previously described (Szewczyk et al., 2006; Oakley et al., 2012). Phusion [New England Biolabs (M0536)], Q5 Hot Start High-Fidelity [New England Biolabs (M0494)] were used for gene amplification and fusion PCR. OneTaq [New England Biolabs (M0488)] was used for diagnostic PCR. DNA for diagnostic PCR was prepared from conidia using a miniprep procedure (Edgerton-Morgan and Oakley, 2012). Transformation was as previously described (Szewczyk et al., 2006; Oakley et al., 2012) except that 1.0 M sucrose was often used to osmotically balance selection plates instead of 0.6 M KCl. The protoplasting enzyme was VinoTaste Pro (Novo Nordisk) used at 100 mg/mL. Selection of transformants in which the *AtpyrG* gene had been evicted was on osmotically balanced YAG plates containing 1.0 mg/mL 5-fluoroorotic acid (5FOA) (Gold Biotechnology) as well as 1 g/L uracil and 10 mM uridine. Genomic DNA from *A. terreus* strain NIH2624 was supplied by Dr. Kenneth Bruno (Pacific Northwest National Laboratory). *A. terreus pyrG, biA*, *lysB*, *pabaA*, *choA* and *riboB* genes were amplified by PCR from genomic DNA and cloned into the pCR-BluntII-TOPO plasmid vector (Invitrogen) following the manufacturer's instructions. The resulting plasmids (Table 1) have been deposited at the Fungal Genetics Stock Center (www.fgsc.net) and Addgene (www.addgene.org). Multi-marker *A. nidulans* strains (listed in section 3.4) have been deposited at the Fungal Genetics Stock Center.

3. Results and Discussion

3.1 Identification of pyrG89, riboB2 and pyroA4 mutations

Our starting strain was LO4389 (Ahuja et al., 2012) which carries *pyrG*89, *riboB*2, *pyroA*4, a replacement of *nkuA* by *argB*, and a deletion of the sterigmatocystin gene cluster. We used a strain carrying at deletion of the sterigmatocystin gene cluster because sterigmatocystin is a potent toxin and carcinogen. Since we and others have used *pyrG*89, *riboB*2 and *pyroA*4 extensively as selective markers for transformation, we were interested in determining the nature of the mutations in these alleles. For other projects, we have deep sequenced strains carrying these mutations (Oakley et al., 2017) so we compared the sequences of the mutant alleles with the sequences of the same genes in the reference strain. With respect to *pyrG*89, we confirmed the finding of Dr. Miguel Peñalva (personal communication) that the mutation is a G to A transition at nucleotide 511 (with the A of the start codon being nucleotide 1) resulting in a substitution of glycine for serine. With respect to *riboB*2, we found that nucleotide 856 is deleted resulting in a frame shift and premature termination. With respect to *pyroA*4, we found that nucleotides 319-321 (GGA) are deleted and nucleotide 322 is changed from C to T. These changes result in the loss of a glycine and the replacement of a histidine with a tyrosine.

3.2 Creation of strains carrying deletions of selectable genes

In choosing genes to target for deletion, we looked for genes in which strongly selectable mutations had been isolated through classical genetics. In *A. nidulans* most such mutations confer nutritional requirements, and we specifically looked for genes in which classically isolated alleles largely or completely prevent growth on unsupplemented minimal medium and which are supplemented with relatively inexpensive nutrients. To find suitable

candidates we relied on the list of *A. nidulans* gene list compiled by Dr. A. J. Clutterbuck (www.fgsc.net/Aspergillus/gene_list/loci.html, accessed Dec. 12, 2017) and the *Aspergillus* genome database (www.aspgd.org, accessed Dec. 12, 2017). We chose *biA* (AN6644, biotin requirement) (Roper, 1950; Clutterbuck, 1994; Magliano et al., 2011), *lysB* (AN5206, lysine requirement) (Käfer, 1958; Clutterbuck, 1994; Mogensen et al., 2006) (David et al., 2008), and *pabaA* (AN6550, p-aminobenzoic acid requirement) (Pontecorvo et al., 1953; Clutterbuck, 1994; Tüncher et al., 2005). In addition, Dr. A. J. Clutterbuck had tentatively identified AN2154 as *choA* (choline requirement) (Käfer, 1958; Arst, 1968) although it is annotated in the Aspergillus genome database as a possible pseudogene. We compared the AspGD AN2154 sequence to DNA sequences and RNAseq data we have obtained (Oakley et al., 2017) and found that there is a sequence error in the AspGD sequence, an extra G at nucleotide 2277 (with the A of the start codon being nucleotide 1). This error resulted in an erroneous early termination and a consequent mis-annotation of the gene in the current AspGD annotation. Curiously, the stop codon was correctly annotated in an earlier AspGD annotation. We have reannotated AN2154 and the revised sequence is given in Fig. S1. Our results (below) reveal, moreover, that AN2154 is a real gene and its deletion causes a choline requirement. Our results confirm, therefore, the identification of AN2154 as *choA*.

Our strategy for deleting target genes is shown in Fig. 1A. In this strategy, the target gene is initially deleted by replacement with the *pyrG* gene from *A. terreus* strain NIH 2624 (ATEG_09675 using the AspGD designation, herein designated *AtpyrG* for brevity). The transforming fragments were created by fusion PCR and consisted of a 1491 bp fragment from *A. terreus* containing the *AtpyrG* gene flanked by approximately 1 kb of *A. nidulans* genomic sequences that flank the target gene on each side. Transformants carrying a correct replacement of the target gene were verified by diagnostic PCR.

To remove *AtpyrG* and, thus, allow *pyrG* to be used as a selectable marker for subsequent transformations, a fragment was created by fusion PCR that contained only the flanking sequences fused together. This fragment was used to transform a strain in which the target gene had been replaced by *AtpyrG* (Fig. 1B). 5-fluoroorotic acid was used to select transformants in which the *AtpyrG* gene had been replaced (Dunne and Oakley, 1988). The net result was a deletion of the entire coding sequences of the target genes along with a small amount of flanking DNA on each side of the coding sequences. (The deleted regions are given in Table S1). This approach removed all of the *AtpyrG* sequences leaving clean deletions and allowed *AtpyrG* to be used again as a selectable marker.

Using this strategy, we created strain LO9478 by consecutive deletion of the *biA* and *pabaA* genes. In parallel, we created strain LO9487 by consecutive deletion of the *lysB* and *choA* genes. We next deleted the *wA* gene in LO9478 by replacing it with the *A. fumigatus riboB* gene (Nayak et al., 2006), thereby creating strain LO9548, with white conidia caused by the *wA* replacement being a useful color marker for crosses. We then crossed LO9548 with strain LO9487 and tested segregants for nutritional requirements. Among the segregants, we were able to obtain strains carrying all combinations of the newly created selectable markers as shown in Table 2. Notably, strain LO9771 carries all seven selectable markers as well as the *nkuA* deletion and a deletion of the sterigmatocystin gene cluster.

We next tested the "tightness" of the newly created selectable markers and the health of the strains on supplemented media. Results for LO9771, the seven-marker strain, are shown in Fig. 2. LO9771 grew as well as the parental strain LO4389 on fully supplemented minimal medium. It also grew as well as the parental and wild-type strains on complete medium (Fig. S1). Growth was strongly inhibited on medium lacking the appropriate supplement (Fig. 2). Interestingly, although the *biA* deletion completely inhibited growth, we note that the *biA* marker (our deletion or the classically isolated mutation, *biA*1) was particularly susceptible to cross feeding. If a *biA*$^+$ colony was nearby, enough biotin was released by the *biA*$^+$ strain to partially restore growth to the biotin requiring strain. This result is consistent with the fact that *biA*1 and the *biA* deletion are each supplemented fully with very small amounts of biotin (see Materials and Methods).

3.3 Cloning and testing of A. terreus homologs of deleted A. nidulans genes

We identified and cloned genes from *A. terreus* strain NIH 2624 that complement the newly created deletions. We performed BLAST searches of the *A. terreus* genome using the sequences of the deleted *A. nidulans* genes as queries. *A. terreus* homologs of the deleted genes were easily identified (Table 1). We tested them for complementation of the *A. nidulans* deletions by amplifying them from genomic DNA, fusing them to flanking sequences from the *wA* gene and transforming appropriate strains (Fig. 3). Each of the *A. terreus* genes complemented the corresponding *A. nidulans* deletion fully (Fig. 4). Although we have not yet attempted to transform all of the strains we have created, we have encountered no difficulties with the strains we have attempted to transform (LO4389, LO9368, LO9451, LO9478, LO9487, LO9743, LO9770 and LO9771). We discovered quirks, however, in complementing the *choA* deletion by transformation. In attempting to select transformants on plates containing 1.0 M sucrose as an osmotic balancer, we found that the *choA* deletion strain grew too much to allow selection of transformants. We were able to select *choA*$^+$ transformants on plates using 0.6 M KCl as an osmotic balancer, but the transformant colonies grew very slowly. These slow-growing colonies did form conidia, however, and when these conidia were streaked onto plates containing no osmotic balancer normal colonies were formed. The *choA*, marker, was thus quirky but perfectly usable.

Finally, to create stable and easily accessible clones of the complementing genes, we ligated PCR fragments carrying each of the genes into the pCR-BluntII-TOPO vector (Table 1, sequences in Fig. S4-S7). To verify that each of the resultant plasmids carries a fully functional gene, we amplified the genes from each plasmid, created transforming molecules by fusion PCR, transformed appropriate deletion strains with them and verified that the deletions were fully complemented. We also cloned *AtpyrG* and the *A. terreus riboB* gene (*AtriboB*) into pCR-BluntII-TOPO (Table 1, sequences in Fig. S3 and S8).

3.4 Construction of a multi-marker pyrG⁺ strain

The *pyrG* selectable marker is extremely useful in that it affords both positive and negative selections. It has, however, two large liabilities. One is that it requires large amounts of uridine and uracil to complement it, resulting in considerable expense. A second is that it is not fully complemented by uridine and uracil at low temperatures, resulting in a weakly cold sensitive phenotype. We consequently created a multi-marker *pyrG*$^+$ strain by simply

amplifying a 2902 bp sequence containing the *pyrG* allele from *A. nidulans* wild-type DNA and transforming LO9771 with it, selecting for pyrimidine prototrophs. We designated this strain LO10831. Finally, to make these strains generally accessible to the *A. nidulans* research community, we have deposited the following strains at the Fungal Genetics Stock Center: LO4389, LO9368, LO9451, LO9452, LO9749, LO9478, LO9487, LO9734, LO9743, LO9755, LO9768, LO9770, LO9771, LO9772 and LO10831.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We have created four new selectable markers through targeted deletions.

We have determined the sequence changes in three frequently used mutants.

We have created strains of *Aspergillus nidulans* with up to seven selectable markers.

We have cloned *Aspergillus terreus* genes that complement each new selectable marker.

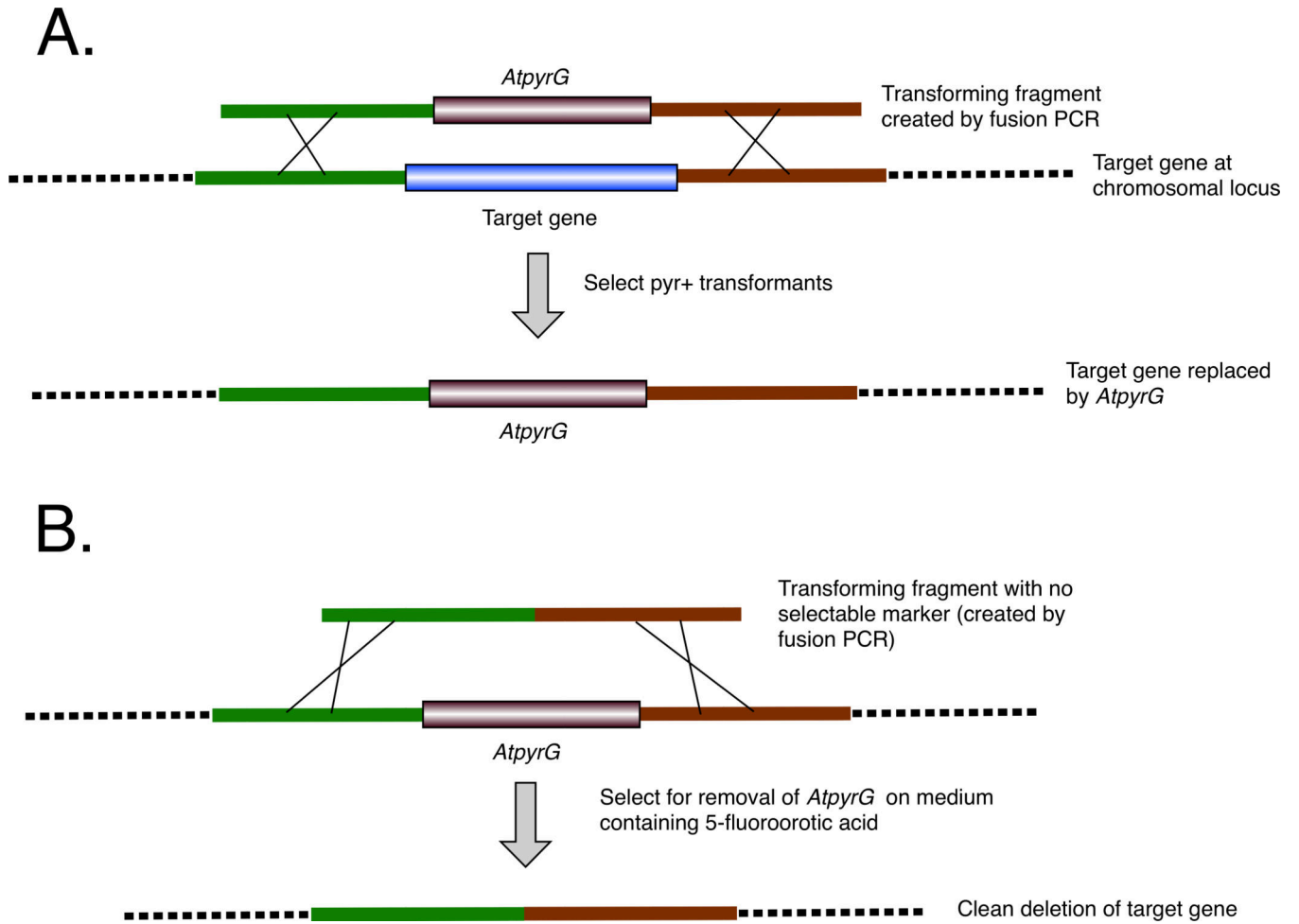These advances will facilitate multiple, sequential transformations.

A.

B.

Fig. 1.
Gene deletion strategy. A. Transformation with a linear molecule containing the *Aspergillus terreus pyrG* gene (*AtpyrG*) flanked by left and right flanking sequences from the target gene to be deleted results in replacement of the target gene by *AtpyrG*. B. Transformation with a linear molecule containing only the left and right flanking sequences from the target gene results in the removal of the *AtpyrG* gene. Transformants in which *AtpyrG* has been removed can be selected on 5-fluoroorotic acid which selects against strains carrying a functional *pyrG* gene. The result is a deletion of the target gene with no selectable marker sequences left behind. The *AtpyrG* gene can be reused in subsequent transformations.
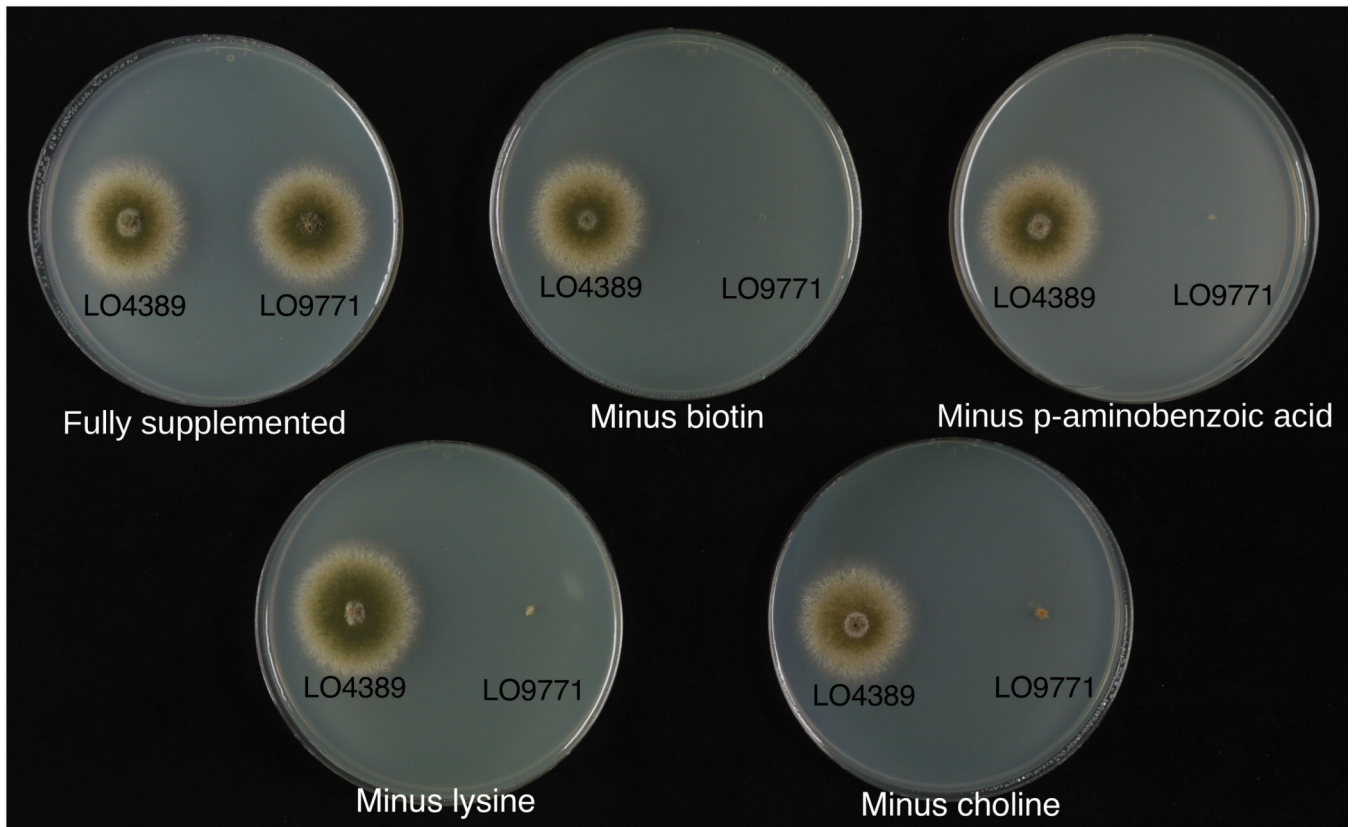
Fig. 2.
Newly created deletions are fully supplementable and strongly selectable. LO9771 carries seven selectable markers. When minimal medium is supplemented for all seven markers, growth is essentially the same as the parental strain LO4389. Selections against the four newly created deletions are shown. Omission of biotin selects against the *biA* deletion, omission of p-aminobenzoic acid selects against the *pabaA* deletion, omission of lysine selects against the *lysB* mutation and omission of choline selects against the *choA* deletion. In the absence of each of these supplements, the growth of the strain is strongly inhibited. For simplicity only the seven-marker strain is shown but similar results were obtained with strains carrying fewer markers.
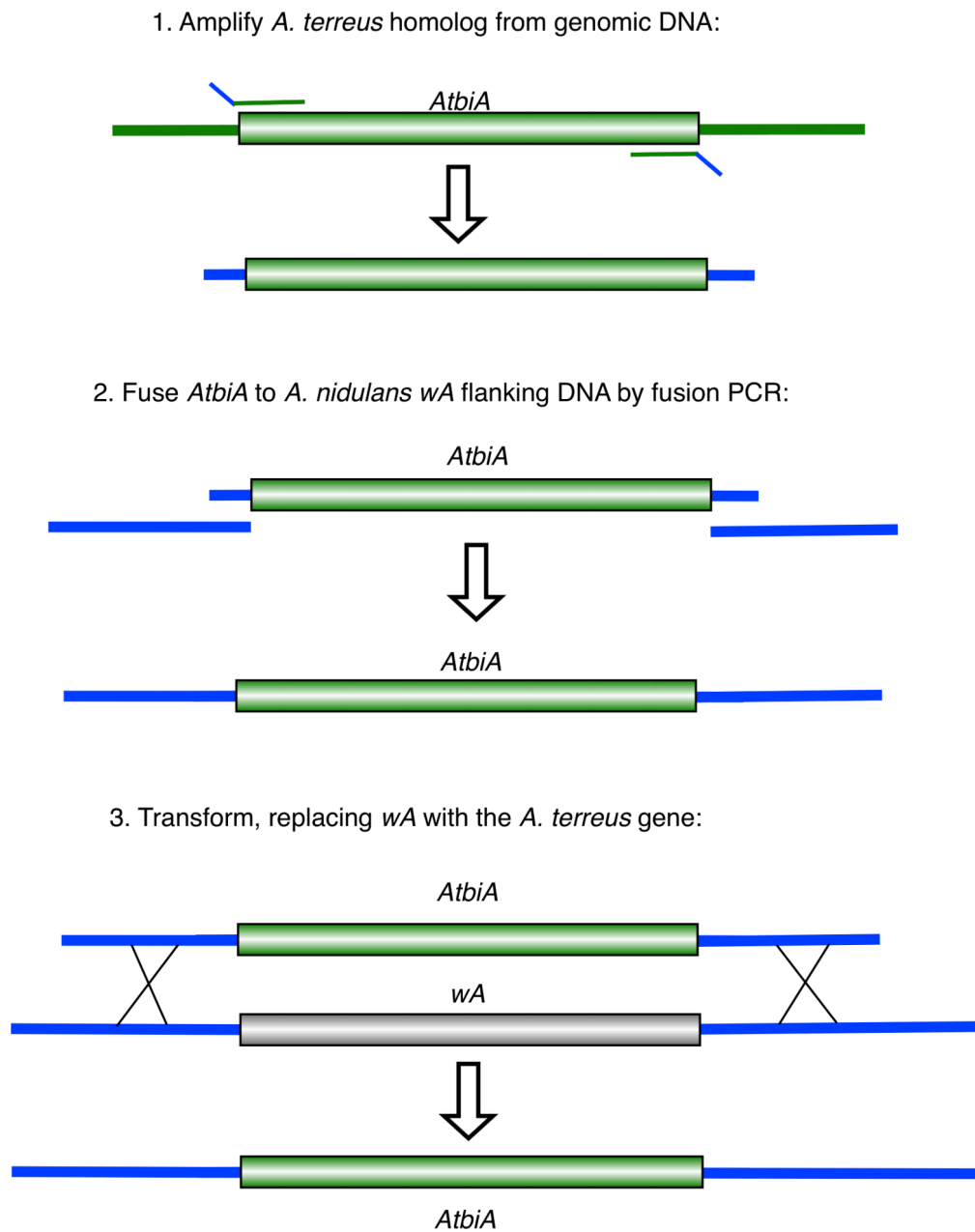
1. Amplify *A. terreus* homolog from genomic DNA:

2. Fuse *AtbiA* to *A. nidulans wA* flanking DNA by fusion PCR:

3. Transform, replacing *wA* with the *A. terreus* gene:

Fig. 3.
Strategy for introducing *A. terreus* homologs of the deleted *A. nidulans* genes at the *wA* locus. The *A. terreus biA* gene (*AtbiA*) is shown as example. This strategy results in replacement of the *wA* gene with the *A. terreus* gene of interest. Correct transformant colonies will be white as shown in Figure 4.

| Minus choline | Minus lysine | Minus p-amino-benzoic acid | Minus biotin |
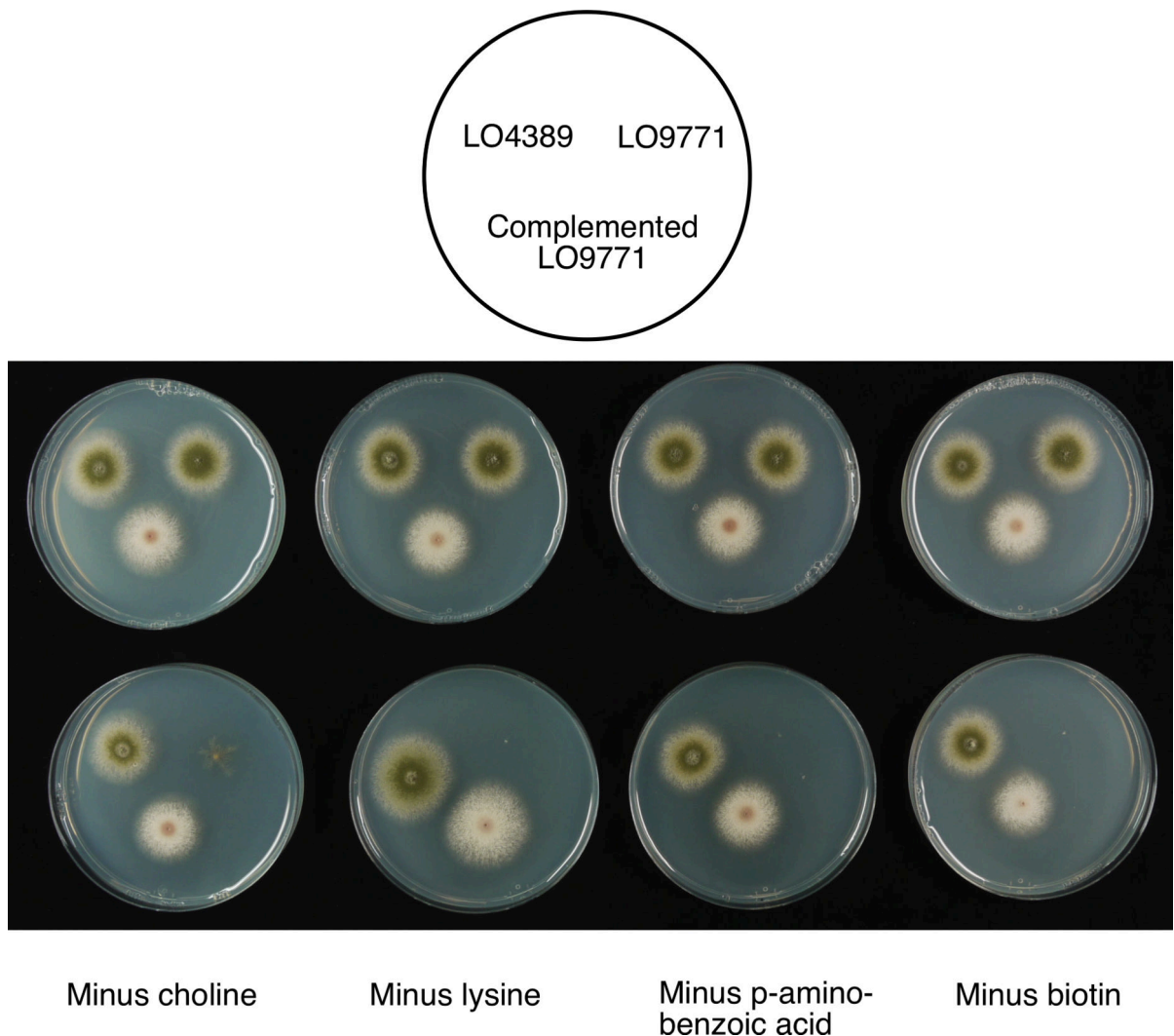
Fig. 4.

Complementation of newly created deletions with homologous *A. terreus* genes. The plates in the top row contain minimal medium fully supplemented for all seven nutritional requirements of LO9771. LO9771 grows as well as the parental strain LO4389. The bottom strain on each plate is LO9771 transformed with a fragment carrying an *A. terreus* gene homologous to one of the genes deleted in LO9771. The bottom row of plates contain minimal medium lacking a supplement required due to one of the newly created deletions. Thus, in the minus choline plate, LO9771 does not grow because of the choline requirement created by the *choA* deletion. However, the white strain at the bottom of the plate is LO9771 that has been transformed with the *AtchoA* gene (inserted at the *wA* locus), and it grows as well as the parental strains. Similarly, the *AtlysB*, *AtpabaA*, and *AtbiA* genes fully complement the corresponding deletions.

Table 1

A. nidulans genes corresponding to previously identified nutritional markers, *A. terreus* homologs of these genes and plasmids carrying the cloned *A. terreus* genes.

Aspergillus nidulans gene	*Aspergillus terreus homolog*	E value	Plasmid carrying *A. terreus* gene
pyrG (AN6157)	ATET_09675	2.0E-121	pLO103
biA (AN6644)	ATET_07102	0.0E+00	pLO101
lysB (AN5206)	ATET_03691	0.0E+00	pLO99
pabaA (AN6550)	ATET_06966	0.0E+00	pLO100
choA (AN2154)	ATET_02347	0.0E+00	pLO102
riboB (AN0670)	ATET_00531	3.0E-167	pLO104

Table 2

Strains carrying multiple selectable markers. Check marks indicate that the strain carries the mutation or deletion.

Strains	Mutations carried				Deletions carried					
	pyrG89	riboB2	pyroA4	Sterigmatocystin cluster (AN7804-7825)	biA (AN6644)	pabaA (AN6550)	lysB (AN5206)	choA (AN2154)	nkuA::argB
LO4389	✓	✓	✓	✓					✓
LO9368-LO9370	✓	✓	✓	✓	✓				✓
LO9451	✓	✓	✓	✓			✓		✓
LO9452	✓	✓	✓	✓		✓			✓
LO9478	✓	✓	✓	✓	✓	✓			✓
LO9487-LO9489	✓	✓	✓	✓			✓	✓	✓
LO9734-LO9736	✓	✓	✓	✓	✓	✓		✓	✓
LO9743	✓	✓	✓	✓	✓			✓	✓
LO9749-LO9751	✓	✓	✓	✓				✓	✓
LO9768	✓	✓	✓	✓	✓	✓	✓		✓
LO9770	✓	✓ (AfriboB::wA)	✓	✓	✓	✓	✓	✓	✓
LO9771	✓	✓	✓	✓	✓	✓	✓	✓	✓
LO9772-LO9774	✓	✓	✓	✓		✓		✓	✓
LO10831		✓	✓	✓	✓	✓	✓	✓	✓