Versatile Enzyme Expression and Characterization System for Aspergillus nidulans, with the Penicillium brevicompactum Polyketide Synthase Gene from the Mycophenolic Acid Gene Cluster as a Test Case

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Assigning functions to newly discovered genes constitutes one of the major challenges en route to fully exploiting the data becoming available from the genome sequencing initiatives. Heterologous expression in an appropriate host is central in functional genomics studies. In this context, filamentous fungi offer many advantages over bacterial and yeast systems. To facilitate the use of filamentous fungi in functional genomics, we present a versatile cloning system that allows a gene of interest to be expressed from a defined genomic location of Aspergillus nidulans. By a single USER cloning step, genes are easily inserted into a combined targeting-expression cassette ready for rapid integration and analysis. The system comprises a vector set that allows genes to be expressed either from the constitutive PgdA promoter or from the inducible PalcA promoter. Moreover, by using the vector set, protein variants can easily be made and expressed from the same locus, which is mandatory for proper comparative analyses. Lastly, all individual elements of the vectors can easily be substituted for other similar elements, ensuring the flexibility of the system. We have demonstrated the potential of the system by transferring the 7,745-bp large mpaC gene from Penicillium brevicompactum to A. nidulans. In parallel, we produced defined mutant derivatives of mpaC, and the combined analysis of A. nidulans strains expressing mpaC or mutated mpaC genes unequivocally demonstrated that mpaC indeed encodes a polyketide synthase that produces the first intermediate in the production of the medically important immunosuppressant mycophenolic acid.

Filamentous fungi have the ability to produce a plethora of bioactive metabolites and enzymes enabling them to thrive in competitive environments. Among the metabolites are not only mycotoxins but also compounds that are used as drugs, e.g., the antibiotic penicillin and the immunosuppressant mycophenolic acid (MPA). Since many of the desirable products are naturally secreted in large amounts, fungi possess considerable potential as expression hosts for the production of small molecules as well as proteins. The wide interest in fungi has led to the sequencing of an increasing number of fungal genomes, and this number is expected to increase dramatically in the coming years (30). This resource constitutes a tremendous industrial exploitation of fungal biology. For example, the number of gene clusters predicted to produce secondary metabolites, such as polyketides and nonribosomal peptides, constantly increases as new fungal genome sequences are released. However, at present, the compound produced by the enzymes encoded by the gene cluster has been identified for only a few of these gene clusters. Since for most organisms no or inefficient gene-targeting technology exists, it is often difficult to assign secondary metabolites to specific genes in the natural host. This problem can be solved by transferring genes of interest to a suitable heterologous host that can provide a wide range of genetic tools for gene characterizations. Considering the large number of genes to be analyzed, it is important to develop high-throughput methods that facilitate this process.

Preferentially, the foreign gene should be expressed from a defined, well-characterized location. Compared to random integration, this provides several advantages. First, integration at a random site may cause mutation or alter expression of neighboring genes, causing unpredictable pleiotropic effects (3). Second, since integrated genes are differentially expressed, depending on the genomic context at the site of integration, it eliminates undesired positioning effects by ensuring that the novel genes are integrated at a location that accommodates a high expression level (29). Third, it allows for comparative studies where the phenotypes of strains expressing wild-type and mutated alleles can be reliably compared, as the gene
for strain constructions when argB nkuA-trS plasmids were propagated in of a point-mutated mpaC strains that express suppressant mycophenolic acid. By analyzing the biosynthetic enzymes for the production of the immuno-al. [25].

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variants are expressed from the same locus in the different strains. *Aspergillus nidulans* serves as a widely used model for filamentous fungi and has been extensively used for basic genetic research. Many genetic tools are therefore available, including efficient gene targeting in strains where the nonhomologous end-joining pathway for DNA integration has been eliminated (14, 16, 17). However, compared to the yeast *Saccharomyces cerevisiae* (where only 20 to 50 bp are needed), gene-targeting substrates need to contain large. >1,500-bp, homologous sequences to ensure integration at the selected locus. Accordingly, a gene-targeting substrate that contains an expression cassette and a selectable marker is constructed from six pieces of DNA and often exceeds a total size of 10 kb, complicating its construction (Fig. 1). Construction of gene-targeting substrates therefore constitutes a potential bottleneck in a high-throughput gene analysis process.

Here we present a vector set based on the USER (uracil-specific excision reagent) cloning technique, which allows rapid and easy generation of constructs for targeted integration and heterologous expression of a gene of interest in *A. nidulans*. As a proof of concept, the vector set was used to express the heterologous *mpaC* gene from *Penicillium brevicompactum* (see the accompanying manuscript in this issue by Regueira et al. [25]). *mpaC* is located in a gene cluster predicted to encode the biosynthetic enzymes for the production of the immuno-suppressant mycophenolic acid. By analyzing *A. nidulans* strains that express *mpaC* under the control of both constitutive and inducible promoters, as well as constitutive expression of a point-mutated *mpaC*, we conclusively show that MpaC catalyzes the production of 5-methylorsellinic acid (5-MOA), the first intermediate in mycophenolic acid production.

### MATERIALS AND METHODS

#### Strains and media.

The *A. nidulans* strains IBT28738 (argB2 veA1 pyrG89 nkaA-Ars:AFpyrG) and IBT29539 (argB2 veA1 pyrG89 Delta4) (17) were used for strain constructions when argB or pyrG was used as selection marker, respectively. The IBT30750 strain (veA1 pyrG89), NID127, was used as reference strain in growth experiments. A full list of *A. nidulans* strains is provided in Table 1. All plasmids were propagated in *Escherichia coli* strain DH5α. Minimal medium (MM) contained 1% glucose, 10 mM NaNO₃, 1× salt solution (4), and 2% agar for solid media. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), and 4 mM L-arginine (Arg) when necessary. Solid plates containing 5-fluoroorotic acid (5-FOA; Sigma-Aldrich) were made as MM plus Uri and Ura medium supplemented with filter-sterilized 5-FOA to a final concentration of 1.3 mg/ml. YES medium (yeast extract-sucrose) was made as previously described (9) and supplemented with 10 mM Uri, 10 mM Ura, and 4 mM Arg when necessary. PcaA induction medium consisted of 100 mM t-threonine, 100 mM glycerol, 10 mM NaNO₃, mineral mix (1×), 2 g agar/liter. Pgdpa LacZ activity was determined on MM supplemented with 0.12 mM 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), 10 mM uracil, and 10 mM uridine.

#### PCR and USER cloning.

Amplification of DNA by PCR to produce DNA fragments suitable for USER cloning was performed in 30 PCR cycles using the proofreading PfuTurbo CX Hotstart polymerase (Stratagene) or PfuX7 (21) in 50 μl according to the manufacturer’s instructions. USER cloning was performed as previously described (22) with minor modifications. The USER vectors were digested for 6 h with AsISI for the AsISINb.BsmI and AsISI.Nb.BstI USER vectors or PacI for the PacI/Nt.BbvCI USER vectors A and B, followed by digestion with the appropriate nicking endonuclease for 1 h. Purified digested vector (0.1 pmol) was mixed with 1 pmol purified PCR products amplified with primers that were extended by the appropriate tails for USER cloning into a designated USER cassette (see Fig. S1, S2, S3, S4, and S5 in the supplemental material). When more than one PCR product was cloned simultaneously, the combined concentration of PCR product was kept at 1 pmol and the same concentration of each PCR product was used. The DNA mix was adjusted to 8 μl by adding Milli-Q purified water followed by addition of 1 μl of 10× TE buffer (100 mM Tris-HCl, 1 mM EDTA; pH 8.0) and 1 μL of USER enzyme mix (New England BioLabs). The reaction mixture was incubated for 20 min at 37°C, followed by 20 min at 25°C. Next, the 10-μl reaction mix was used directly to transform chemically competent *E. coli* cells.

#### Construction of USER vectors.

Four different USER cassettes were designed to allow for the construction of the USER vector set: the two PacI/Nt.BbvCI USER vectors A and B (see Fig. S3 in the supplemental material) (8), one AsISINb.BsmI USER vector (see Fig. S1 in the supplemental material), and an AsISINb.BstI USER vector (see Fig. S2 in the supplemental material). The founder vector used to construct the USER vector set, pU0002, was custom made by DNA2.0 (Menlo Park, CA). pU0002 is based on pJ204 and contains a USER linker containing two successive PacI/Nt.BbvCI USER cassettes, A and B. The USER linker is flanked by SwaI and NolI restriction sites on both sides. The cloning strategies and cloning into the individual USER cassettes and vectors are outlined in Fig. S1, S2, S3, S4, and S5 in the supplemental material. Sequences and descriptions of primers used toward creating this vector set are found in Tables S1 and S2 in the supplemental material.

#### Nomenclature for the USER vector set.

Nomenclature for our USER vector set follows the general system pU QXYZ-IS. Q describes the marker present in the plasmid (use for after *A. nidulans* transformation, with 0 indicating no marker, 1 indicating argB, 2 indicating AfpyrG (flanked by direct repeats to allow marker excision), and 3 indicating bfe (resistance to the genotoxin bleomycin). X denotes which promoter is present in the plasmid, with 0 indicating no promoter, 1 indicating Pgdpa, and 2 indicating PalcA. Y denotes which terminator is present in the plasmid, with 0 indicating no terminator and 1 indicating TtrpC. Z is defined by the USER cassettes that are present in the plasmid, with 0 indicating no USER cassette, 1 indicating the AsISINb.BstI cassette, 2 indicating PacI/Nt.BbvCI USER cassettes A and B, 3 indicating AsISINb.BstI cassette and PacI/Nt.BbvCI USER cassettes A and B, and 4 indicating AsISINb.BsmI cassette and PacELNI.BbvcI cassette and A, B. An IS is present in the name of the plasmid if a PCR fragment for targeting has been inserted into the PacI/Nt.BbvCI USER cassettes A and B, with 1 indicating targeting regions for homologous recombination into insertion site 1 (IS1) are present.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NID66</td>
<td>argB2 pyrG89 veA1 ΔnkaA IS1::PalcA-mpaC-TtrpC-AfpyrG</td>
</tr>
<tr>
<td>NID127</td>
<td>pyrG89 veA1</td>
</tr>
<tr>
<td>NID189</td>
<td>pyrG89 veA1 IS1::Pgdpa-mpaC1622A-TtrpC-argB</td>
</tr>
<tr>
<td>NID190</td>
<td>pyrG89 veA1 IS1::Pgdpa-mpaC1622A-TtrpC-argB</td>
</tr>
<tr>
<td>NID192</td>
<td>pyrG89 veA1 IS1::Pgdpa-lacZ-TtrpC-argB</td>
</tr>
<tr>
<td>NID210</td>
<td>pyrG89 veA1 IS1::Pgdpa-TtrpC-argB</td>
</tr>
<tr>
<td>NID211</td>
<td>pyrG89 veA1 IS1::Pgdpa-mpaC-TtrpC-argB</td>
</tr>
<tr>
<td>NID212</td>
<td>pyrG89 veA1 IS1::Pgdpa-mpaC-TtrpC-argB</td>
</tr>
</tbody>
</table>
USER cloning of lacZ, RFP, and mpac into USER vectors. E. coli lacZ was amplified from pWJ1042 (7) using the primers BGHA503 and BGHA504. The red fluorescent protein gene (RFP) was amplified from pSK800 (28) using the primers BGHA564 and BGHA565. The P. brevicaeruleum mpac coding sequence, including introns, was amplified from BAC1E13 (23) using the primers BGHA296 and BGHA297. Purified PCR products were USER cloned into the AsBBI/Nb.BslI USER cassette in pU2111-1, pU2221-1, pU1111-1, and pU1221-1, PCR-generated sections of plasmids were sequenced (StarSeq, Germany) to confirm that no mutations were introduced into PCR errors.

A. nidulans strain constructions. Prototaxing and gene-targeting procedures were performed as described previously (11, 20). Five micrograms of plasmid DNA was digested with NotI (argB- and bl-constituting construct) or with SwaI (pyrG-containing constructs) to liberate the gene-targeting substrate, which was used for transformation of IBT28738 (using argB as the selection marker) or IBT29039 (using pyrG as the selection marker). Streak-purified transformants were grown on 5-FOA medium to select for recombinants where the nkuA locus was restored to wild type, as described previously (17). All gene-targeting events were verified by analytical PCR using Taq polymerase (Sigma-Aldrich) and genomic DNA obtained from individual transformants. A list of primers for verifying transformants in IS1 can be found in Table S3 of the supplemental material. The region upstream of the integration events was tested using primer BGG180, which is upstream of the open reading frame, and a pair of anneals to the inserted promoter (BGHA502 for FpdA and BGHA267 for Pala) (see Fig. S6 in the supplemental material). Similarly, the downstream region of the integration event was tested using primer BGHA162, which anneals downstream of the TS2 sequence, and one that anneals to the selectable marker (BGHA98 for argB and BGHA182 for AfpG).

Creation of point mutations in mpac. Perturbations in the DSL motif of the acr carboxy protein (ACP) domain in mpac were created by using a variation of the USER fusion method previously described (10). The DSL-to-ASL mutation was obtained by USER fusing two PCR fragments generated by primer pairs (BGHA456 with BGHA297 and BGHA457 with BGHA296, respectively), using mpac as template. Simultaneously, the fusion event was USER cloned into the pU1111-1 vector fragment, which was included in the same reaction mixture (see Results and Discussion for further details). The DSL-to-DAL mutation was made in the same way except that the two PCR fragments were generated by primer pairs BGHA456 with BGHA297 and BGHA457 with BGHA296. The two mutated mpac genes were inserted into IS1 by the method described above.

Batch fermentation. Batch fermentations of A. nidulans were performed in 2-liter Braun fermentors with a working volume of 1.6 liters and equipped with two Rushton four-blade disk turbines. The bioreactor was sparged with air, and the concentrations of carbon dioxide in the exhaust gas were measured in a gas analyzer. The temperature was maintained at 30°C, and the pH was kept constant at 5.5, controlled by automatic addition of either 2 M NaOH or 2 M HCl. Agitation and aeration were controlled throughout the cultivations. For inoculation of the bioreactor and germination of spores, the stirring rate was set to 200 rpm and aeration was set to 0.2 liter/min. The bioreactor was equipped with a diode array detector and coupled to an HPLC-UV/Vis high-resolution mass spectrometry (LC-HRMS) analysis was performed with an Agilent 1100 system (Waldbronn, Germany) equipped with a diode array detector and coupled to an LCT apparatus (Micromass, Manchester, United Kingdom) equipped with an electrospray ionization system (18, 19). Separations of 1 to 5 μl of sample were performed on a 100- by 2-mm (inner diameter), 2.6-μm Kinetex Eclip, column (Phenomenex, Torrance, CA), using a linear water-ACN gradient at a flow rate of 0.400 ml/min from 10 to 65% ACN within 14 min, then to 100% ACN in 3 min, followed by a plateau at 100% ACN for 3 min. Both solvents contained 20 mM formic acid. Samples were analyzed both in ES+ and ES− modes. For compound identification, each peak was matched against an internal reference standard database (∼800 compounds) (18). 3-Methylforsellinic acid (Ambinter, Paris, France) and orsellinic acid (Apiin Chemicals, Oxon, United Kingdom) were coanalyzed. Other peaks were tentatively identified by matching data from previous studies in our lab and searching the accurate mass in the ∼13,000 fungal metabolites reported in Antibase 2009 (12). Here UV/Vis ionization efficiencies in ES+ versus ES−, and retention times were used.

RESULTS AND DISCUSSION

Identification of a genomic insertion site for heterologous gene expression. An ideal all-around genomic integration site for characterization of heterologous genes and their products should accommodate the new genes without interfering with the fitness of the strain and allow for high and stable expression levels in a tissue-unspecific manner. To identify such a site, we exploited the existing transcriptome microarray data of A. nidulans (1), obtained during exponential growth on different carbon sources, to identify genomic regions that supported high expression under all the different growth conditions investigated. One possible site of integration, IS1, which fulfilled these criteria and is situated 202 bp downstream of AN6638 and 245 bp upstream of AN6639, was selected for further characterization. To evaluate the usefulness of IS1 as a site for heterologous expression, we inserted lacZ and RFP into this locus (NID192 and NID257, respectively) by taking advantage of the vector set described below. Expression of lacZ from IS1
panels show hyphal tips, and the bottom panels show conidia spores. RFP, as indicated. The top panels show a full mycelium, the middle bright-field (BF) microscopy and by fluorescence microscopy detecting f1ation, converted setup. Crude protein extracts prepared from NID192 mycelia, well-controlled bioreactors by using a batch fermentation level of protein production, NID192 was grown in MM in three successive restabbings (data not shown). To examine the time as judged by visual inspection of colonies obtained from A. nidulans (13). The reference strain, NID127, was analyzed was first visualized by growing NID192 on X-Gal-containing plates. As expected, NID192 colonies were bright blue due to β-galactosidase production, whereas colonies from a reference strain (NID127), which does not contain lacZ, were pale (Fig. 2A). Importantly, expression of lacZ appeared stable over time, as the blue appearance of colonies did not change over time as judged by visual inspection of colonies obtained from three successive restabbings (data not shown). To examine the level of protein production, NID192 was grown in MM in well-controlled bioreactors by using a batch fermentation setup. Crude protein extracts prepared from NID192 mycelia, which were harvested in the stationary phase of the fermentation, converted o-nitrophenyl-β-D-galactoside at a rate of 5.3 µmol/min/mg of total protein. This level of activity is similar to what was obtained by Lubertozi and Keasling, who inserted PgdA::lacZ into three different loci, argB, trpC, and niaD, of A. nidulans (13). The reference strain, NID127, was analyzed in parallel and produced no detectable β-galactosidase activity (see Fig. S7 in the supplemental material). Importantly, we noted that given these experimental conditions the growth rates of NID192 and NID127 were not significantly different (P > 0.2), indicating that insertion of foreign DNA into IS/ does not impair fitness of the strain in MM. Inspection of NID257, which expresses RFP, by fluorescence microscopy revealed easily detectable RFP distributed uniformly throughout the mycelia (Fig. 2B). Similarly, RFP was also present in the spores (Fig. 2B), indicating that RFP is expressed from IS/ in a tissue-independent manner. We also investigated whether inserting an expression cassette into IS/ influenced expression of the flanking genes, AN6638 and AN6639. The expression levels of the two genes were determined in a reference strain as well as in three different strains containing the PgdA/TtrpC expression cassette and the argB selection marker. In the three strains, the cassette contained either nothing, RFP, or mpAC. Of the two flanking genes, only AN6638, which is located next to the constitutive promoter PgdA, was significantly affected (~2.5-fold increase) in these three strains. To investigate whether other genes close to this locus were affected by the presence of an expression cassette in IS/, the same analysis was performed for AN6636, AN10837, and AN6639. None of these genes was expressed differently in the three strains containing an expression cassette in IS/ compared to the reference strain. The modest effect of gene expression in the IS/ region after integration of an expression cassette is in agreement with the finding that the presence of a lacZ expression cassette in IS/ in a strain does not influence the growth rate. Based on the combined results of the experiments described above, we conclude that the integration site IS/ is useful for integrating novel genes for their further characterization.

**Construction of a flexible USER vector set for gene analysis of Aspergillus nidulans.** To facilitate exploiting IS/ as a convenient expression platform for foreign genes, we constructed a flexible vector set which allows for easy construction of gene-targeting substrates for integration of your favorite gene (YFG) into IS/ by taking advantage of the DNA ligase-free improved USER cloning system (22). So far, the set comprises six vectors (Table 2), allowing for integration of YFG into IS/ under the control of either inducible PalcA or constitutive PgdA by using either the selectable marker argB, ble, or pyrG. The latter marker can be recycled after transformation and used in subsequent experiments, since it is flanked by direct repeats that allow pyrG to be eliminated by popout recombination (20). In all vectors, the terminator TtrpC is present downstream of the YFG integration site.

**TABLE 2. Names and descriptions of one-step USER vectors for inserting YFG into IS/**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Marker</th>
<th>Promoter</th>
<th>Terminator</th>
<th>USER cassette/ clonal cassette</th>
<th>Integration site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pU1111-1</td>
<td>argB</td>
<td>PgdA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
<tr>
<td>pU1211-1</td>
<td>argB</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
<tr>
<td>pU2111-1</td>
<td>pyrG</td>
<td>PgdA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
<tr>
<td>pU2211-1</td>
<td>pyrG</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
<tr>
<td>pU3111-1</td>
<td>blea</td>
<td>PgdA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
<tr>
<td>pU3211-1</td>
<td>blec</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsiSI/Nb.BstI</td>
<td>IS/</td>
</tr>
</tbody>
</table>

*a* ble confers resistance to the genotoxin bleomycin.
By using one (or more) of the vectors in our vector set, YFG can rapidly be introduced into IS1 in A. nidulans by performing four simple steps (Fig. 3). In step 1, the gene of interest is PCR amplified with primers containing the appropriate tails for USER cloning into the AsiSI/Nb.BtsI USER cassette. In step 2, the PCR fragment is USER cloned into the appropriate vector; in step 3 the completed gene-targeting substrate containing YFG is released from this vector by restriction enzyme digestion or by PCR. In step 4, the gene-targeting substrate is used for transformation of A. nidulans protoplasts. In our hands, this can be done in less than 48 h, and A. nidulans transformants are obtained a few days later.

The vector set is designed for optimal flexibility. Hence, in case other markers, promoters, terminators, or other integration sites are preferred, the present repertoire of vectors can easily be expanded, since all parts can be replaced in simple USER cloning-based reactions. As a guideline for future vector construction, we present the strategy for building pU2111-1 as a model (see Fig. S5 and, for details, Fig. S1, S2, S3, and S4 in the supplemental material).

Cloning of mpaC from Penicillium brevicompactum and transformation into A. nidulans. To demonstrate the potential of the presented USER vector set for heterologous expression and characterization of YFG in A. nidulans, we decided to investigate a recently discovered gene cluster from Penicillium brevicompactum that has been proposed to encode the enzymes required for MPA biosynthesis (2). The large size of mpaC makes it well suited to test the robustness of our USER cloning-based system for cloning and inserting YFG into IS1. The large mpaC gene was PCR amplified as three fragments, which were subsequently fused by taking advantage of the USER fusion technique (10), which allows several PCR fragments to be merged in a single cloning step. Using this principle, the entire mpaC gene was readily inserted into the argB gene containing vectors pU1111-1 and pU1211-1, hence equipping mpaC with the constitutive PgpdA and inducible PalcA promoters, respectively. In both cases more than 50% of the colonies had mpaC inserted between the promoter and terminator, demonstrating the efficiency of the construction part of the system. The entire mpaC gene was sequenced for both constructs, and no PCR-generated errors were observed. Using the argB marker for selection, the two constructs were used for transformation of A. nidulans IBT28738 protoplasts. This strain allows for efficient integration of gene-targeting substrates by homologous recombination, since the competing pathway for genomic DNA integration, nonhomologous end joining, has been transiently eliminated due to a pyrG insertion in the nkuA locus (17). In agreement with this, a PCR test demonstrated that the mpaC expression cassette was integrated into IS1 in all transformants analyzed (data not shown). Finally, selected transformants were grown on medium containing 5-FOA to reconstitute nkuA to avoid any influence of a defective nkuA gene on further strain characterization (see reference 17 for details).
Introduction of a point mutation in the DSL motif in the ACP domain of mpaC. Detailed characterization of a gene product requires simple means to introduce genetic modifications, like deletions and point mutations. Use of the present commercially available techniques for introduction of such modifications in large vectors, like those containing an entire gene-targeting substrate, constitutes a challenging and tedious task. By making a slight modification to the USER fusion technique described above, point mutations and deletions can easily be introduced in YFG. Hence, if the primer tails used to merge individual segments of YFG contain the desired point mutation, defined sequence modifications can be inserted anywhere in a gene (Fig. 4). Similarly, deletions can be introduced by designing matching USER primer tails, which at the fusion point will bridge two noncontinuous, but successive, sections of YFG. Here we have demonstrated the principle of introducing point mutations into mpaC by using USER cloning to independently introduce two alanine substitutions, D1622A and S1623A, in the conserved DSL motif in the ACP domain of MpaC. Both substitutions are predicted to impair polyketide synthase activity. It is known that the phosphopantetheine moiety of coenzyme A binds to the serine in the DSL domain of PKSs (6), and therefore an S1623A substitution in MpaC is likely to prevent the phosphopantetheine moiety of coenzyme A from binding to the serine in the DSL domain. Accordingly, MpaC cannot be converted from the inactive apo form to the active holo form in this mutant protein. From studies on other PKSs it has been found that the negatively charged aspartate in the DSL motif in the acyl carrier protein domain creates a salt bridge to the acyltransferase domain, ensuring that these two domains interact properly (5). Therefore, the D1622A mutation in MpaC is predicted to disrupt MpaC activity. The creation of the two mpaC mutants was as fast and efficient as cloning of the wild-type mpaC described above. Both mpaC variants were verified by sequencing, and no additional PCR-generated errors were observed. We note that the method is applicable for introducing several point mutations in different regions of interest in a single round of cloning, simply by fusing additional PCR fragments. Specifically, simultaneous construction of two mutations require the fusion of three PCR fragments, three mutations require the fusion of four PCR fragments, and so on. To this end, we note that presently up to five fragments have been efficiently fused by USER fusion and that the upper limit has yet not been delineated (10).

Expression of mpaC in A. nidulans results in 5-methylorsellinic acid production. To investigate 5-MOA production in A.
**FIG. 5.** Expression of mpac in *A. nidulans* results in 5-MOA production. (A) UV chromatograms from LC-UV/Vis-HRMS analyses of the standard 3-MOA and strains NID210, NID211, and NID190. An extracted ion chromatogram, showing an *m/z* 181 corresponding to the [M-H]⁻ ion of methylorsellinic acids, is inserted in each chromatogram. (B) UV spectra for 3-MOA (pure compound) and 5-MOA from strain NID211. (C) Overlay UV chromatogram from HPLC-UV/Vis for the extract obtained from strain NID66 with mpac under the control of the inducible alcA promoter. Chromatograms representing inducing (+Thr) and noninducing conditions (-Thr) are indicated by arrows.

In *A. nidulans*, we first analyzed a reference strain, NID210 (in which AR1 contains *argB* integrated at ISI) for its ability to produce 5-MOA. Since 5-MOA is not commercially available, a reference standard of 3-MOA, which is expected to behave very similarly to 5-MOA in LC-HRMS analyses, was analyzed. Since neither 3-MOA nor 5-MOA has previously been reported in *A. nidulans*, we surprisingly identified a compound eluting at 4.09 min with an elementary composition identical to that of both 3- and 5-MOA in the extract (Fig. 5A). However, since both the retention time and the UV spectrum of this compound were identical to that of the 3-MOA standard, it was unambiguously assigned as 3-MOA. Next, a strain containing the PgpDA:mpac expression cassette at ISI (NID211) was analyzed by LC-HRMS. In contrast to the reference strain, NID211 produced a compound eluting as a prominent peak at 3.78 min with the mass expected for 5-MOA (Fig. 5A). This peak contained a unique ion with *m/z* 181.050, corresponding to the [M-H]⁻ ion of C₉H₁₀O₄. Moreover, it produced a UV spectrum (Fig. 5B) that was identical to the previously published UV spectrum for 5-MOA (24). We note that NID211, like the reference strains, also produced the compound eluting at 4.09 min, supporting that this compound was 3-MOA.

Next we addressed whether the unique compound produced by NID211 is due to the mpac gene product or whether it results from the expression of an endogenous *A. nidulans* gene(s) that is accidentally activated by insertion of the PgpDA:mpac expression cassette into ISI. To this end, we first analyzed the two strains expressing PgpDA:mpac-D1622A and PgpDA:mpac-S1623A (NID189 and NID190, respectively). In both cases, no compound eluted at 3.78 min. Moreover, strain NID66, which harbors the expression cassette PalcA:mpac inserted at ISI, produced a compound eluting at 3.78, but only when the strain was grown on medium inducing expression from PalcA (Fig. 5C). Together, the data conclusively demonstrate that mpac encodes a polyketide synthase that produces 5-MOA. In addition, since the mpac gene expressed in *A. nidulans* contained its native *P. brevicompactum* introns, we conclude that these introns were efficiently removed by the splicing apparatus of *A. nidulans*.

**Concluding remarks.** In this report we have presented a simple USER cloning-based system that allows genes to be transferred from organisms of interest into the well-characterized fungal model *A. nidulans* for further characterization. As proof of concept, we firmly demonstrated that mpac from *P. brevicompactum* encodes the PKS responsible for production of 5-MOA, the first intermediate in MPA production. Importantly, since the vector set is constructed in a flexible manner, it can easily be modified to allow specific integration of YPG into other organisms that support efficient gene targeting, if desirable. The strategy for gene characterization presented here is therefore widely applicable and should greatly facilitate assignment of gene functions in organisms where the genetic toolbox is poorly developed.

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