Challenge models for RTFS in rainbow trout fry (Oncorhynchus mykiss)

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Published in:
DAFINET Workshop

Publication date:
2011

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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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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Received 26 July 2010/Accepted 28 February 2011

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 PpgdA 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.
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 first intermediate in mycophenolic acid production, mpaC, which encodes the enzyme mpaC (also known as mpaC) that catalyzes the biosynthetic enzymes for the production of the immunosuppressant mycophenolic acid (5-MOA). 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-methylseleninic acid (5-MOA), the first intermediate in mycophenolic acid production.

MATERIALS AND METHODS

**Strains and media.** The *A. nidulans* strains IBT28738 (argB2 veA1 pyrG89 nkuA-trS [18]) and IBT29539 (argB2 veA1 pyrG89 Deltaa) (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, 1x salt solution (4), and 2% agar for solid media. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), and 4 mM 1-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. PalA induction medium consisted of 100 mM l-threonine, 100 mM glycerol, 10 mM NaNO₃, mineral mix (1x), 2 g agar/liter. PgdPΔlacZ activity was determined on MM supplemented with 0.12 mM 5-bromo-4-chloro-3-indolyl-beta-n-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 PfxX7 (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 AsISI/Nb.BsmI and AsISI/Nb.BstI USER cassettes or PacI for the PacI/Nt.BbvCI USER cassettes 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 U 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 cassettes A and B (see Fig. S3 in the supplemental material) (8), one AsISI/Nb.BsmI USER cassette (see Fig. S1 in the supplemental material), and an AsISI/Nb.BstI USER cassette (see Fig. S2 in the supplemental material). The founder vector used to construct the USER vectors, 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 Swal and NotI 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-LS. Q denotes the marker present in the plasmid for use after *A. nidulans* transformation, with 0 indicating no marker, 1 indicating argB, 2 indicating AfpypG (flanked by direct repeats to allow marker excision), and 3 indicating ble (resistance to the genotoxin bleomycin). X denotes which promoter is present in the plasmid, with 0 indicating no promoter, 1 indicating PgdPΔA, and 2 indicating PalcA. Y denotes which terminator is present in the plasmid, with 0 indicating no terminator and 1 indicating TrpC. Z is defined by the USER cassettes that are present in the plasmid, with 0 indicating no USER cassette, 1 indicating the AsISI/Nb.BstI cassette, 2 indicating PacI/Nt.BbvCI USER cassettes A and B, 3 indicating AsISI/Nb.BsmI cassette and PacI/Nt.BbvCI USER cassettes A and B, and 4 indicating AsISI/Nb.BsmI cassette A and PacI/Nt.BbvCI USER cassettes A and 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 1. Names and descriptions of the strains used in this work.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NID66</td>
<td>argB2 pyrG89 veA1 Deltaa IS1: PalcA-mpaC-3-Trpc-AFpyrG</td>
</tr>
<tr>
<td>NID127</td>
<td>pyrG89 veA1</td>
</tr>
<tr>
<td>NID189</td>
<td>pyrG89 veA1 IS1: PgdA-mpaC-3-Trpc-argB</td>
</tr>
<tr>
<td>NID190</td>
<td>pyrG89 veA1 IS1: PgdA-mpaC-3-Trpc-argB</td>
</tr>
<tr>
<td>NID192</td>
<td>pyrG89 veA1 IS1: PgdA-lacZ-Trpc-argB</td>
</tr>
<tr>
<td>NID210</td>
<td>pyrG89 veA1 IS1: PgdA-Trpc-argB</td>
</tr>
<tr>
<td>NID211</td>
<td>pyrG89 veA1 IS1: PgdA-mpaC-3-Trpc-argB</td>
</tr>
<tr>
<td>NID389</td>
<td>pyrG89 veA1 IS1: PgdA-RFP-Trpc-argB</td>
</tr>
</tbody>
</table>
USER cloning of lacZ, RFP, and mpcC into USER vectors. E. coli lacZ was amplified from pW1042 (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. brevicompactum mpcC coding sequence, including introns, was amplified from BAC1E13 (25) using the primers BGHA296 and BGHA297. Purified PCR products were USER cloned into the AsBHI/Nb.BstI USER cassette in pU2111-1, pU2121-1, pU1111-1, and pU1211-1. PCR-generated sections of plasmids were sequenced (StarSeq, Germany) to confirm that no mutations were introduced during PCR errors.

*A. nidulans* strain constructions. Protoplasting and gene-targeting procedures were performed as described previously (11, 20). Five micrograms of plasmid was digested either with NotI (argB- and ble-containing constructs) or with StwI (pyrG-containing constructs) to liberate the gene-targeting substrate, which was used for transformation of IBT28738 (using argB as the selection marker) or IBT29539 (using pyrG as the selection marker). Streak-purified transformants were grown on 5-FOA medium to select for recombinants where the *nka4* locus was restored to wild type, as described previously (17). All gene-targeting events were verified by analytical PCR using *Tag* polymerase (Sigma-Alrich) and genomic DNA obtained from individual transformants. A list of primers for verifying transformants in *Isf* can be found in Table S3 of the supplemental material. The region upstream of the integration events was tested using primer *BGHA* and a set of primers which were in the upstream of these sequences, and a putative anneal to the inserted promoter (BGHA502 for *PgdA* and BGHA267 for *PalcA*) (see Fig. S6 in the supplemental material). Similarly, the downstream region of the integration event was tested using primer *BGHA*162, which anneals downstream of the *TS2* sequence, and one that anneals to the selectable marker *BGHA*98 for argB and *BGHA*182 for *AfpyrG*.

Creation of point mutations in *mpcC*. Point mutations in the DSL motif of the *acr* carrier protein (ACP) domain in *mpcC* were created by using a variation of the USER fusion method previously described (10). The DSL-to-ASL mutation was obtained by USER fusing of two PCR fragments generated by primer pairs (BGHA456 with BGHA297 and BGHA457 with BGHA296, respectively), using *mpcC* as template. Simultaneously, the fusion fragment 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 BGHA458 with BGHA297 and BGHA459 with BGHA296. The two mutated *mpcC* genes were inserted into *Isf* 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 inoculated with spores suspended in 0.9% NaCl to a concentration of 1.5 × 10⁹/liter. Twelve hours after inoculation, the stirring rate was increased to 350 rpm and the airflow was increased to 1.5 liters/min and kept at that level for the remainder of the process. Statistical analysis of growth rates was performed using simple linear regression for multipletiplicate data on natural logarithmic-transformed data, and comparisons are based on Student’s *t* test, adopted for linear regression data.

Determination of β-galactosidase activity. Assays for β-galactosidase activity were performed using *Escherichia coli* harvested from batch fermentations in the stationary phase (48 h after inoculation) containing 5 g (dry weight) of biomass/liter. Quantification was performed using the *o*-nitrophenyl-β-D-galactopyranoside method (15) with the following modifications. Mycelia from 1 ml of fermentation were transferred to a 2-ml screw-cap tube containing 250 µl glass beads (0.5-mm diameter), 250 µl ice cold Z-buffer (26), and 12.5 µl of a 100 mM 4-(2-amino-ethyl)-benzenesulfonyl fluoride hydrochloride solution. The samples were homogenized by shaking for 30 s at maximum speed in a Fastprep FP120 (Bio 101 Savant). The extraction volumes were increased by addition of 250 µl ice-cold Z-buffer and cleared for cell debris by centrifugation at 10,000 × g for 30 min at 4°C. Total protein content in the cleared extract was determined using the Quant-IT protein assay kit (Invitrogen) with the Qubit fluorimeter (Invitrogen) according to the instructions of the manufacturer.

Fluorescence microscopy. Microscopy was essentially performed as previously described (23). Images were captured with a cooled Evolution Q5i monochrome digital camera (Medica Cybernics Inc.) mounted on a Nikon Eclipse E1000 camera (Nikon).

RNA isolation and qRT-PCR. *A. nidulans* total RNA, from four strains grown under identical conditions (NID127, NID210, NID211, and NID257), was isolated with the Qiagen plant RNeasy kit. Ten micrograms of RNA was DNase I (Qiagen)-treated prior to cDNA amplification of 1 µg of DNase I-treated RNA samples by using a Phusion RT-PCR kit (Finzymes) according to the manufacturer's protocol. The subsequent quantitative reverse transcription-PCR (qRT-PCR) was performed in a Chromo 4 detector/PTC-200 apparatus (MJ Research) by using SYBR Green JumpStart Taq ReadyMix (Stratagene). The *A. nidulans* actin gene, *actA* AN6542, was the internal standard for normalization of expression levels. The primers combination AN6635-F/AN6635-R was used for AN6635, AN6639-F/AN6639-R for AN6639, AN6640-F/AN6640-R for AN6640, AN6636-F/AN6636-R for AN6636, ANActA-F/ANActA-R for *actA*, and AN10837-F/AN10837-R for AN10837. All primers are listed in Table S3 of the supplemental material. Two types of control samples were included for the qPCR: a DNase-treated RNA sample and a template-free reaction mixture, to test for the primer-dimer influence on overall fluorescence. The individual cDNA samples were run both as concentrated samples (1/10 of the cDNA prep) and 10⁻xdiluted samples (1/100 x). Samples were run in triplicates. The program was 94°C for 2 min and 40 cycles of 94°C for 10 s, 60°C for 15 s, and 72°C for 30 s. A melting curve from 65°C to 95°C with reads every 0.2 min ended the program to evaluate the purity of the reaction products. The fluorescence threshold values (Ct) were determined by using the OpticonMonitor 3.1 software (MJ Research). The relative expression levels were approximated based on 2⁻¹(Ct,target gene)⁻¹(Ct,actA), where 2⁻¹(Ct,actA)−1(Ct,target gene)−1(Ct,reference strain)−1(Ct,target gene)−1(Ct,reference strain). The Ct values were those for the reference strain, NID127.

Chemical characterization of mutants. Three 6-mm-diameter plugs were taken from each strain and grown as three-point inoculations in the dark at 25°C for 7 days on YES medium (27). The plugs were transferred to a 2 ml vial, and 1 ml of acetone (ACN) was added. The plugs were placed in an ultrasonication bath for 60 min. The ACN was filtered and transferred to a new vial, in which the organic phase was evaporated to dryness by applying nitrogen airflow at 30°C. The residues were redissolved ultrasonically for 10 min in 150 µl of an ACN/H₂O (1:1, vol/vol) mixture. Samples of 1 to 5 µl were analyzed by high-performance liquid chromatography (HPLC)–UV/Vis by using 15 to 100% ACN in 20 min on a Luna C18 column (19). 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 C18 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 ESI⁻ and ESI⁺ modes. For compound identification, each peak was matched against an internal reference standard database (~800 compounds) (18). 3-Methylsollisellinic acid (Ambinter, Paris, France) and orsellinic acid (Apin 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 metabolome database reported in Anonymous 2009 (12). Here UV/Vis data and fragmentation, ionization efficiencies in ESI⁻ versus ESI⁺, 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, *Isf*, 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 *Isf* 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 *Isf*...
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 0.2 μmol/min/mg of total protein. This level of activity is similar to what was obtained by Lubertozzi and Keasling, who inserted Pgpda::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 IS1 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 IS1 in a tissue-independent manner. We also investigated whether inserting an expression cassette into IS1 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 Pgpda/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 Pgpda, 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 IS1, 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 IS1 compared to the reference strain. The modest effect of gene expression in the IS1 region after integration of an expression cassette is in agreement with the finding that the presence of a lacZ expression cassette in IS1 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 IS1 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 IS1 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 IS1 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 IS1 under the control of either inducible PalcA or constitutive Pgpda 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 IS1**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Marker</th>
<th>Promoter</th>
<th>Terminator</th>
<th>USER cassette/ cloning cassette</th>
<th>Integration site</th>
</tr>
</thead>
<tbody>
<tr>
<td>pU1111-1</td>
<td>argB</td>
<td>PgpdaA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
<tr>
<td>pU1211-1</td>
<td>argB</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
<tr>
<td>pU2111-1</td>
<td>pyrG</td>
<td>PgpdaA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
<tr>
<td>pU2211-1</td>
<td>pyrG</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
<tr>
<td>pU3111-1</td>
<td>ble&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PgpdaA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
<tr>
<td>pU3211-1</td>
<td>ble&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PalcA</td>
<td>TtrpC</td>
<td>AsIS/Nb.BstI</td>
<td>IS1</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 production (25). 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. 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. nidulans results in 5-methylorsellinic acid production. To investigate 5-MOA production in A.
<p>For the extract obtained from strain NID66 with strain NID211. (C) Overlay UV chromatogram from HPLC-UV/Vis gram. (B) UV spectra for 3-MOA (pure compound) and 5-MOA from arrows. Thr) and noninducing conditions (-Thr) are indicated by /H11001 control of the inducible alcA promoter. Chromatograms representing induction (+Thr) and noninducing conditions (-Thr) are indicated by arrows.</p>

**FIG. 5.** Expression of mpaC in <i>A. nidulans</i> 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.</p>

<i>A. nidulans</i>, 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 <i>A. nidulans</i>, 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 <i>A. nidulans</i> 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 <i>A. nidulans</i> contained its native P. brevicompactum introns, we conclude that these introns were efficiently removed by the splicing apparatus of <i>A. nidulans</i>.

**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 <i>A. nidulans</i> for further characterization. As proof of concept, we firmly demonstrated that mpaC from <i>P. brevicompactum</i> 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 YFG 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.

**ACKNOWLEDGMENTS**

We thank Dorte Marie Koefoed Holm for assisting in identification of ISI. We thank Martin Engelhard Kornholt for valuable technical assistance in the laboratory.

The work was supported by grants number 09-064967 and 09-064240 from the The Danish Council for Independent Research, Technology and Production Sciences to K.R.P. and U.H.M. J.N. was supported by the Chalmers Foundation.

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