Heterologous Reconstitution of the Intact Geodin Gene Cluster in Aspergillus nidulans through a Simple and Versatile PCR Based Approach

Nielsen, Morten Thrane; Hoof, Jakob Blæsbjerg; Anyaogu, Dianna Chinyere; Holm, Dorte Koefoed; Nielsen, Kristian Fog; Larsen, Thomas Ostenfeld; Mortensen, Uffe Hasbro

Published in:
P L o S One

Link to article, DOI:
10.1371/journal.pone.0072871

Publication date:
2013

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

Link back to DTU Orbit

Citation (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Heterologous Reconstitution of the Intact Geodin Gene Cluster in *Aspergillus nidulans* through a Simple and Versatile PCR Based Approach

Morten Thrane Nielsen*, Jakob Blæsbjerg Nielsen*, Dianna Chinyere Anyaogu*, Dorte Koefoed Holm, Kristian Fog Nielsen, Thomas Ostenfeld Larsen*, Uffe Hasbro Mortensen*

Department of Systems Biology, Technical University of Denmark, Kgs. Lyngby, Denmark

**Abstract**

Fungal natural products are a rich resource for bioactive molecules. To fully exploit this potential it is necessary to link genes to metabolites. Genetic information for numerous putative biosynthetic pathways has become available in recent years through genome sequencing. However, the lack of solid methodology for genetic manipulation of most species severely hampers pathway characterization. Here we present a simple PCR based approach for heterologous reconstitution of intact gene clusters. Specifically, the putative gene cluster responsible for geodin production from *Aspergillus terreus* was transferred in a two step procedure to an expression platform in *A. nidulans*. The individual cluster fragments were generated by PCR and assembled via efficient USER fusion prior to transformation and integration via re-iterative gene targeting. A total of 13 open reading frames contained in 25 kb of DNA were successfully transferred between the two species enabling geodin synthesis in *A. nidulans*. Subsequently, functions of three genes in the cluster were validated by genetic and chemical analyses. Specifically, ATEG_08451 (gedc) encodes a polyketide synthase, ATEG_08453 (gedl) encodes a halogenase that catalyzes conversion of sulochrin to dihydrogeodin. We expect that our approach for transferring intact biosynthetic pathways to a fungus with a well developed genetic toolbox will be instrumental in characterizing the many exciting pathways for secondary metabolite production that are currently being uncovered by the fungal genome sequencing projects.


**Editor:** Marie-Joelle Virolle, University Paris South, France

**Received:** November 15, 2012; **Accepted:** July 19, 2013; **Published:** August 23, 2013

**Copyright:** © 2013 Nielsen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the Danish Research Agency for Technology and Production, grant 09-064967. The PhD studies of which this work was part of was funded by the Research School for Biotechnology at the faculty of Life Sciences, University of Copenhagen. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: toll@bio.dtu.dk (TOL); um@bio.dtu.dk (UHM)

¢ Current address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Harsholm, Denmark

¤ These authors contributed equally to this work.

**Introduction**

Fungal natural products constitute a rich resource for bioactive secondary metabolites [1,2]. To fully exploit this potential, it is essential to identify the genes required for the biosynthesis of these compounds. This process is becoming progressively easier due to the rapidly increasing number of fungal genomes that have been fully sequenced; and since the genes involved in the production of a given secondary metabolite often cluster together in the same region of a chromosome [1–3]. Importantly, the genome sequencing projects have revealed that the number of putative gene clusters for secondary metabolite production greatly exceeds the number of known natural products in a given fungus, hence, indicating that most fungal compounds are yet to be discovered.

The prerequisite for genetic exploration of the huge reservoir of undiscovered biosynthetic pathways is solid methodologies for cultivating, propagating and genetically manipulating the producing species. However, the vast majority of newly sequenced organisms fail to meet these requirements, hence, hampering pathway elucidation and exploitation. An attractive solution to this problem is to transfer pathways into another fungus where the methodology is well established. This approach has been used successfully to investigate several gene clusters [4–6]. All of these studies apply a strategy where individual genes in a cluster are PCR amplified, cloned, and integrated sequentially into either a random or defined locus. One advantage of this strategy is that it allows easy engineering of individual genes prior to integration in the host strain. In addition, it is possible to insert the foreign gene(s) into a well characterized locus that supports high expression levels [7]. Inserting the genes into a known locus also simplifies strain validation. In a recent example of this strategy, Itoh et al. [6] transferred five genes from *Aspergillus fumigatus* to *A. oryzae* allowing the authors to deduce the biosynthetic route for the meroterpenoid pyropyripene A. However, the strategy may be limited to reconstitution of simple pathways depending on only a small number of genes since assembly of multistep pathways will require several rounds of tedious iterative integration steps or enough genetic markers.
For multistep pathways it is therefore desirable to transfer entire gene clusters from the natural producer to the expression host in one or a few steps. This requires a host, which can efficiently express all genes in the cluster and correctly splice the resulting transcripts. Assembly of multiple genes and PCR fragments can efficiently be performed in S. cerevisiae by recombination based methods [8]. However, the demand for splicing disfavors S. cerevisiae as expression host for this gene transfer strategy as only little splicing occurs in yeast. In contrast, correct and efficient splicing of heterologous transcripts by another filamentous fungus is likely. Activation of the cluster in the new host requires as a minimum that the chromatin structure at the insertion site is in an open configuration and that transcription factors exist that recognize the individual promoters in the cluster. The latter may be facilitated by the fact that many clusters appear to contain one or more genes that encode transcription factors. In a pioneering study, Bergmann et al. showed that expression of such a gene activated the entire aspyridone gene cluster in A. nidulans [9]. The potential of transferring entire clusters to an expression host has been demonstrated by Sakai et al. who managed to produce citrinin in A. oryzae [10]. To obtain this feat, they isolated and transformed a cosmid from a Monascus purpureus library containing all six genes required for production of citrinin into this production host. Here constitutive expression of the citrinin pathway regulator encoded by ctnA dramatically increased citrinin production in the heterologous host. However, construction and screening of cosmid libraries is not simple and a versatile PCR based method that facilitates the transfer of entire gene clusters from the natural producer to the expression host is desirable.

We have previously developed a versatile PCR based expression platform that can be used for heterologous expression in A. nidulans of one or a pair of genes from the defined locus IS1, which supports a high level of gene expression [4,7]. Here, we demonstrate how this platform can be expanded to allow transfer of an entire gene cluster from another fungus into IS1.

Results and Discussion

2.1 Method for PCR based reconstruction of fungal gene clusters in a heterologous host

Our method for transfer of large DNA fragments relies on successive gene targeting events that introduce ~15 kb fragments into a defined locus. In the example of the method presented here, we transfer a gene cluster into IS1 taking advantage of a vector set we have previously developed for this purpose [7]. In our method, fragments covering the entire gene cluster are PCR amplified, combined via USER fusion into ~15 kb fragments, and inserted into the integration vector by USER cloning (Figure 1A) [11,12]. The first fragment to be integrated is assembled in the vector and integrated into IS1 as we have described previously for integration of single genes [7]. The following fragments are integrated as an extension of the previous one by using one of two different markers, argB and pyrG, for selection. In principle, an indefinite number of integrations can be done, since the marker from the previous integration is excised as the new fragment with the other marker integrates (Figure 1B). This principle is referred to as iterative gene targeting [13]. Importantly, marker replacements allows for a simple selection scheme for identification of correctly targeted strains. If, as in our case, the pyrG marker is flanked by a direct repeat, we recommend to use the pyrG marker in the last integration step, as the pyrG marker subsequently can be removed by direct repeat recombination if desirable [14]. In this manner both markers are available in the finalized strain providing a marker repertoire for additional genetic engineering.

In the present study we demonstrate the potential of our method by transferring the geodin gene cluster from A. terreus to A. nidulans. This cluster was chosen, firstly, because it contains a gene encoding a putative transcription factor, which potentially could facilitate activation of the other genes in the cluster. Secondly, the biosynthetic pathway for geodin production is partially characterized (Figure 2) [15–21], which simplifies the delineation of the cluster size. Thirdly, the geodin pathway shares several steps with the monodictyphenone pathway including production of several common intermediates/products e.g. emodin [22]. We therefore envisioned, that the chance of producing geodin in A. nidulans would be increased, as the corresponding endogenous enzymes could complement geodin enzymes that might not be functional. Moreover, shared intermediates would likely be non-toxic to the host.

2.2 Delineation of the putative geodin producing gene cluster in A. terreus

Three enzymes involved in geodin production have previously been linked to genes. Specifically, dihydrogeodin oxidase, a polyketide synthase, and a thioesterase are encoded by ATEG_08458 and ATEG_08450, respectively, see Aspergillus Comparative Sequencing Project database [Broad Institute of Harvard and MIT, http://www.broadinstitute.org/]. As indicated by the gene numbers these genes localize in close proximity to each other strongly suggesting that a gene cluster responsible for production of geodin exists. Three additional enzymes required for geodin biosynthesis have been characterized biochemically in vivo: emodin anthrone oxygenase [15], emodin O-methyltransferase [16] and questin oxygenase [17]. Moreover, the occurrence of chlorine atoms in geodin suggests the involvement of a halogenase.

To explore the possibility that genes encoding these four enzymatic activities were also present in this region, we examined all annotated open reading frames (ORFs) positioned between ATEG_08458 and ATEG_08450, as well as 20 kb upstream of ATEG_08458 and downstream of ATEG_08450. Among the ORFs in this region, none had a functional annotation corresponding to these enzymatic activities. We therefore subjected all annotated ORFs in this regions to a functional prediction using the BLAST algorithm [23] from NCBI and the HHpred software [24]. This analysis uncovered three genes that could encode putative methyltransferases (ATEG_08449, ATEG_08452 and ATEG_08456), one ORF that may encode an oxygenase (ATEG _08460), see Table 1.

Unexpectedly, none of the annotated ORFs were found to encode the emodin anthrone oxygenase (Figure 2). To investigate this apparent dilemma, we searched the literature for other oxygenases catalyzing a similar reaction. Via this effort, we found an oxygenase that catalyzes conversion of norsolorinic acid to norsolorinic acid, a step towards aflatoxin production in A. flavus [25]. This recently identified enzyme is encoded by the gene hypC. Inspired by these findings, we used the sequence of HypC to conduct pair-wise alignments to putative proteins encoded by alternative ORFs in the proposed geodin gene cluster. One short putative ORF encodes a protein of 150 amino acid residues with an overall identity of 34% with the 210 residues of HypC. Moreover, the conserved amino acid residues were primarily positioned in catalytic regions or conserved domains (Figure S1, [25]). Interestingly, the putative ORF is oriented in the opposite direction of ATEG_08457. This strongly indicates that the region at ATEG_08457 is wrongly annotated and contains two separate ORFs that we now denote ATEG_08457-1 (the originally
Figure 1. Schematic overview of the PCR based USER cloning strategy for transfer of entire gene clusters from one fungus to another. In the illustrated case, the geodin gene cluster in *A. terreus* is PCR amplified, cloned, and integrated into the *IS1* locus in *A. nidulans*. A) ORFs GedA-GedL are depicted as arrows. The yellow and green arrows represent the ORFs encoding the transcription factor and the PKS, respectively. Remaining ORFs are represented by red arrows. Arrow size is proportional to ORF length and arrow direction indicates genomic orientation. Numbers above the gene cluster specify sequence in base pairs. Genomic DNA fragments and cloning vectors are amplified as PCR products using primers extended with uracil-containing tails. The tails contain matching sequences (indicated by identical colors) allowing for PCR product assembly in a single USER Fusion reaction. For the geodin cluster, all putative ORFs are fused into two fragments, which are individually inserted into a vector prepared for gene targeting. Blue boxes labeled up (upstream) and dw (downstream) represent targeting sequences for homologous recombination into *IS1* in the first gene-targeting event. The targeting sequences in the second integration event are represented in gray and blue and consist of the overlapping region between Fragment 1 and 2 and the downstream part of *IS1*, respectively. Genetic markers used for selection are depicted in orange (*argB*) and purple (*AFpyrG*). The sizes of uracil-containing tails, vector elements and PgpdA fragment are not drawn to scale. B) The first gene-targeting event introduces the first fragment into *IS1* by homologous recombination between *IS1* up and down-sequences as indicated. The second gene-targeting event introduces the second fragment using the overlapping region of the Fragment 1 and 2 (gray) and the downstream section of *IS1* as targeting sequences. Note that additional DNA can be inserted in subsequent gene-targeting events. For example, a third fragment can be inserted by using the downstream end of fragment 2 and the downstream region of *IS1* as targeting sequences. See text for details concerning use and recycling of markers.

doi:10.1371/journal.pone.0072871.g001

Figure 2. Proposed pathway for geodin production. The PKS (ACTS, [20]), thioesterase (ACTE, [20]) and dihydrogeodin oxidase previously linked to genes as well as the sulochrin halogenase identified in this study (highlighted in bold) are denoted by their *ged*-annotation. Enzymatic reactions for which the enzyme has been characterized but the gene not identified are marked in bold as EOX = emodin anthrone oxygenase, EOM = emodin-O-methyltransferase and QO = questin oxygenase. Reactions involving compounds 8-10 shown in brackets are inferred reactions proposed by Henry and Townsend based on a similar intra-molecular rearrangement in aflatoxin biosynthesis [26,27].

doi:10.1371/journal.pone.0072871.g002
annotated ATEG_08457-1 and ATEG_08457-2 (the new putative HypC homolog). Specifically, we suggest that ATEG_08457-2 and ATEG_08457-1 are positioned on A. terreus supercontig 12, base pairs 1307175–1307627 and 1308053–1308540, respectively.

Finally, we inspected the remaining ORFs in the region for activities relevant for production of geodin. Among these, one (ATEG_08454) was functionally annotated as a glutathione-S-transferase and two ORFs (ATEG_08455 and ATEG_08457-1) uncovered by the BLAST analysis displayed similarity to oxidoreductases and MdpH, respectively. The latter is a protein of unknown function required for emodin synthesis in A. nidulans [22]. To substantiate our predictions of the involvement of these genes, we conferred literature on similar biosynthetic pathways. The requirement for an oxidoreductase in geodin, we noticed the presence of a gene, ATEG_08453, which encodes a putative transcription factor. The position of this gene upstream of ATEG_08453, which encodes the putative transcription factor described above, with the intention that this modification would activate transcription of all genes in the geodin cluster after its integration into IS1. Importantly, a fragment of the geodin cluster (2 kb) is included in both constructs to serve as the upstream targeting sequence for the second gene-targeting event as illustrated in Figure 1B.

2.4 Production of geodin in A. nidulans

Using the two vectors constructed above, the putative geodin gene cluster (ged) was successfully transferred to an A. nidulans reference strain as well as to a strain where the entire monodictyphenone gene cluster (mdpA-L) had been deleted. Geodin production in the mdpA-L strains would indicate that all genes in the geodin cluster are functionally expressed, while geodin formation in the reference strain could be mediated via metabolites produced in the monodictyphenone pathway. The ability of the recombinant strains to produce geodin on minimal medium was analyzed by UHPLC-HRMS. The presence of geodin in fungal extracts was identified by comparison of retention time, accurate mass spectra, and isotope ratio to an authentic geodin standard. These analyses demonstrated that both ged+ and ged mdpA-L produced geodin (Figure 3A). Consequently, we suggest that the putative transcription factor, ATEG_08453, is renamed gedR and that the enzymes in the cluster (ATEG_08449-08452 + ATEG_08454-08460) are renamed gedA-L. In different experiments, we observed that the amount of geodin is reproducibly higher in the ged+ strain (40–70 μg/plate) as compared to the amount in the ged+ mdpA-L strain (2–4 μg/plate), which does not produce emodin via the mdp cluster. We therefore speculate that natively produced emodin, or other intermediates towards emodin production, could be converted into geodin in the ged+ strains.

2.5 Genetic characterization of the geodin cluster in A. nidulans

One reason for transferring a gene cluster into a host with a well-developed genetic toolbox is the possibility for further
Figure 3. Production of geodin in *A. nidulans* ged\(^+\) strains. A) Left panels depict extracted ion chromatograms (ESI-) of geodin m/z 396.9876 ± 0.005 amu from fungal extracts of ged\(^+\), ged\(^+\) mdpA-L\(\Delta\) and reference strains (Bruker maXis system). An authentic geodin standard is included for comparison. The mass spectra of the putative geodin peak in ged\(^+\) and the authentic geodin standard are depicted in panels to the right. B) and C) ESI chromatograms of geodin m/z 396.9876 ± 0.005 amu (B) and sulochrin m/z 331.0812 ± 0.005 amu (C) extracted from ged\(^+\) mdpA-L\(\Delta\) (grey), ged\(^+\) mdpA-L\(\Delta\) gedL\(\Delta\), (blue), ged\(^+\) mdpA-L\(\Delta\) gedC\(\Delta\) (purple) and ged\(^+\) mdpA-L\(\Delta\) gedR\(\Delta\) (red).

doi:10.1371/journal.pone.0072871.g003
characterization of the cluster. To demonstrate this possibility, we decided to investigate the functionality of three key genes in the cluster, gedC, gedR and gedL encoding the PKS, the putative regulator, and the putative halogenase, respectively. We focused our efforts on the ged" mdpA-LΔ strains, as they provide a genetic background with no risk of complementation by mdp enzymes. UHPLC-HRMS analysis of strains grown on minimal medium revealed that all three deletion strains were unable to synthesize geodin (Figure 3B), thereby confirming that geodin is indeed produced from the reconstituted cluster and that the corresponding proteins of all three genes were functional in A. nidulans and play a role in geodin biosynthesis. We note the presence of a co-eluting isobaric compound, seen as the broad peak (6.7–7.3 min) in Figure 3B. However, this compound is not geodin as it does not contain a chlorine isotopic pattern. In agreement with previous analyses [19–21], no intermediates of the proposed geodin pathway (Figure 2) accumulated in the ged" gedLΔ mdpA-LΔ strain, which is expected, as the PKS responsible for geodin formation is absent.

According to the proposed biosynthetic route for geodin production, the halogenase accepts sulochrin as substrate and adds two chlorine atoms to form dihydrogeodin (Figure 2). Consistent with the hypothesis that gedL encodes the sulochrin halogenase, sulochrin accumulated significantly in the ged" gedLΔ mdpA-LΔ strain (1.2 – 1.8 μg/plate), but was undetectable in the gedR or gedC deletion strains (Figure 3C). To confirm that this lack of halogenase activity was due to the gedL deletion we reintroduced the gedL ORF at another ectopic site, IS3, which is a site located on a chromosome different from the one harboring IS1, see Figure S2. Surprisingly, no production of geodin was observed in this strain (Figure S3A). This prompted us to perform a BLAST search of the GenBank database [23] using the amino acid sequence of the current ATEG_08460.1 gene model as query. Strikingly, the majority of the best hits were enzymes that contain additional 49 amino acid residues in their N-terminus, including a conserved MSIP/MSVP motif at the very N-terminal end, see Figure S4A. Interestingly, intron prediction based on Augustus [29] predicts an intron just upstream of the AUG predicted by the current gene model (ATEG_08460.1). Taking this into account and by using an ATG further upstream in the gedL gene, a very similar extension can be generated for GedL, see Figure S4B and C. We therefore inserted a larger fragment of the gedL locus that includes this new ATG as well as its native UTR sequence into IS2 [4] in the ged" gedLΔ mdpA-LΔ strain. In this strain, geodin was produced in ample amounts (4.0 – 6.8 μg/plate) strongly suggesting that gedL indeed encodes the sulochrin halogenase. Interestingly, in this strain, targeted analysis of the UHPLC-HRMS data and comparison to an in-house metabolite database [30], revealed 0.04 – 0.06 μg/plate of sulochrin and trace amounts of monochlor-sulochrin indicating that chlorine is added in two

0.04 – 0.06 comparison to an in-house metabolite database [30], revealed and gedL

Table 1. This analysis demonstrated that transcription of all seven selected genes was downregulated in the absence of GedR. Most prominently transcription from four of the genes (gedF, G, H, and K) was reduced to less than 10% of the level obtained in the ged" mdpA-LΔ strain (Figure 4). We note that the de novo annotated candidate gene for the emodin anthrone oxidase, gedH (ATEG_08457-2), is transcribed in both the ged" mdpA-LΔ and ged" mdp" strains. In addition, its expression levels in the two

The fact that geodin production was significantly higher in the ged" than in the ged" mdpA-LΔ strains prompted us to investigate whether GedR could also activate transcription of the mdp cluster. Specifically, we compared transcription from mdpG, encoding the monodictyphenone PKS, in the ged" and the reference strains. In agreement with our hypothesis the mdpG transcript was easily detectable in the ged" strain, but undetectable in the reference, see Figure S6.

2.6 Conservation of gene clusters resembling the geodin cluster in other fungal species

Finally, we speculated whether gene clusters of a similar organization could be found in other sequenced fungal species as emodin is well-known to serve as precursor to a wide range of natural products [31–34]. Comparison of the geodin gene cluster to all Aspergillus genomes available at the Aspergillus Comparative Sequencing Project database (Broad Institute of Harvard and MIT, http://www.broadinstitute.org/) revealed the presence of putative gene clusters in A. fumigatus and A. fischerianus containing putative homologs of 12 of the 13 annotated ORFs in the geodin

Figure 4. The A. terreus transcription factor GedR is important for gene expression in the geodin gene cluster in A. nidulans.

Transcription levels of selected ged-genes in ged" mdpA-LΔ, gedRΔ strains relative to the corresponding levels in ged" mdpA-LΔ strains. doi:10.1371/journal.pone.0072871.g004
cluster (the halogenase, \textit{gedL}, is absent). The internal organization of the putative clusters in \textit{A. fumigatus} (AFu4g14450-14580) and \textit{A. fischerianus} (101790-101920) were identical to the gedoin cluster with the exception of an inversion affecting the five ORFs \textit{gedK-gedL}. Moreover, the amino acid identities between biosynthetic enzymes in \textit{A. terreus} and \textit{A. fumigatus/A. fischerianus} were in average 38% and 60%, respectively. The conservation across these three species further substantiates our delineation of the gedoin cluster and hints that the putative clusters in \textit{A. fumigatus} and \textit{A. fischerianus} may encode the biosynthesis for a similar compound. Both species are known to produce trypacidin [35], which differs from gedoin only by the absence of chlorines and the presence of an additional methyl group [36]. In agreement with the structural differences between gedoin and trypacidin, the putative \textit{A. fumigatus} and \textit{A. fischerianus} gene clusters contain one additional putative methyltransferase, but lack the putative halogenase. Thus, the two putative clusters are candidates for trypacidin gene clusters.

**Materials and Methods**

**Strains and media**

\textit{Escherichia coli} strain DH5\textalpha was used to propagate all plasmids. Genomic DNA from the gedoin producing \textit{A. terreus} IBT15722 strain was used as template for PCR amplification of the gedoin cluster. \textit{A. nidulans} strains are shown in Table S1. \textit{A. nidulans} strains were grown on solid glucose minimal medium (MM) prepared as described by Cove [37], but with 1 % glucose, 10 mM NaNO\textsubscript{3} and 2 % agar. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), and/or 4 mM L-arginine (Arg) when required. Solid plates containing 5-fluoroorotic acid (5-FOA) were made as MM+Uri+Ura medium supplemented with filter sterilized 5-FOA (Sigma-Aldrich) to a final concentration of 1.3 mg/ml.

**Vector construction**

All vectors were made by USER cloning and USER fusion [7,11]. All PCR products were amplified in 35 cycles using proof-reading PhoX polymerase [38]. Next USER fusions of vector and inserts were performed as previously described [12]. Reactions were incubated for 20 min at 37 °C, followed by 20 min at 25 °C before transformation into \textit{E. coli}.

The pU2111-3 vector was constructed by USER fusion of 5 PCR amplified fragments: 1) vector backbone for propagation in \textit{E. coli} (amplified with primers DH110/DH111), 2) US (upstream) targeting sequence for insertion in \textit{IS3} (DH112/DH113), 3) PggAa-S::UCE::TlrC (DH114/DH115), 4) \textit{A. fumigatus} pyrG (marker) (DH116/DH117) and 5) DS (downstream) targeting sequence for insertion in \textit{IS3} (DH118/DH119). UEC: uracil excision cassette. Template for fragments 1 and 3: pU1111, for fragments 2 and 5: \textit{A. nidulans} genomic DNA, and for fragment 4: pDEL2 [13]. p2110-3-lac\textsubscript{Z} was constructed by combing an AsISI and Nb.BstI pU2111-3 vector fragment with a PCR product containing the \textit{E. coli} lac\textsubscript{Z} gene (amplified from pU2110-1-lac\textsubscript{Z} using motn136/motn137 [7] by primers) by USER cloning. The plasmid p2010-3-PgdR-lac\textsubscript{Z} was constructed by USER fusion of 5 PCR amplified fragments: 1) gedR promoter sequence (DH120/DH121, template: NID6472 genomic DNA with), 2) lac\textsubscript{Z}::TlpC::AfpyrG (motn136/DH122, template: p2111-3), 3) vector backbone for propagation in \textit{E. coli} (DH110/DH111 template: pU1111), 4) DS targeting sequence for insertion in \textit{IS3} (DH123/DH119 template: pU2111-3) and 5) US targeting sequence for insertion in \textit{IS3} (DH112/DH113). The vectors pU2110-3-AEG_08460.1 and pU2110-2-gedL were constructed by combining the PCR fragments generated with the primers JBN K35/K36 and JBN W77/W78 with pU2111-3 and pU2111-2 [4] vector fragments, respectively, by USER fusion. Prior to USER fusion both plasmids were digested with AsISI and Nb.BstI to create the vector fragments.

The two integration vectors containing the gedoin gene cluster, pU1110-1-ged1 (containing Fragment 1) and pU2000-ged2 (containing Fragment 2), were made as follows. Primers for generating all PCR fragments for Fragment 1 and 2 assemblies are shown in Table S2, pU11110-1-ged1 was constructed by combining all relevant fragments for Fragment 1 assembly into an AsISI and Nb.BstI pU1111-1 vector fragment by USER fusion. The vector fragment of pU2000-ged2 is based on two PCR fragments generated by using primers 77/422 and 422/710 as well as p204 [7] and pU2111-1, respectively, as templates. These two PCR products and all relevant fragments for Fragment 2 assembly were then combined by USER fusion. The inserts in the two integration vectors were fully sequenced (StarSEQ, Germany).

**Construction of \textit{A. nidulans} strains**

Protoplastation and gene-targeting procedures were performed as described by Nielsen et al [14] using either \textit{argB} or \textit{AFpyrG} as marker. All strains were verified by PCR analysis using spores as the source of DNA. Prior to PCR, the samples were incubated for 25 min at 98 °C to liberate genomic DNA. This treatment was followed by touch-down PCR programs with annealing temperatures ranging from 64–56 °C. Reactions were carried out in 33 cycles using 40 \mu L volume with less than 1000 spore (one light stub in the colony with a pipette tip).

The \textit{gedR} (NID677) and \textit{gedL} \textit{mdpA-ΔA} (NID695) strains were obtained by transformation of the relevant gene targeting substrates into NID74 [39] or NID356, respectively. The gene targeting substrates containing Fragment 1 and Fragment 2 were liberated from pU1111 (NotI) and pU2052 (SwaI), respectively, and gel purified (GFX\textsuperscript{TM}, GE Healthcare) prior to transformation. The resulting strains, NID677 and NID695, were subjected to counter selection on 5-FOA, generating NID802 and NID823, in order to recycle the \textit{AFpyrG} marker, hence, allowing the use of this marker for subsequent gene deletions of \textit{gedC}, \textit{gedR} and \textit{gedL}. These deletions were made as described in [14] using the primers listed in Table S2.

The strains NID1291 and NID1297, expressing lac\textsubscript{Z} under the control of ATEG\textsubscript{08453} promoter was made by transforming a gene targeting substrate liberated from pU2010-3-PgdR-lac\textsubscript{Z} by digestion with SwaI into NID823 and NID925, respectively. A control strain expressing lac\textsubscript{Z} under the constitutive promoter PgdA \textit{NID1278}, was constructed by integrating the gene-targeting substrate liberated from pU2110-3-lac\textsubscript{Z} by SwaI. Both gene-targeting substrates integrate into IS3 located between genes AN4770 and AN4769 on chromosome III, at position 1047840-1051735, by homologous recombination (See Figure S2).

The halogenase complementation strains, ATEG\textsubscript{08460.1} and gedL, were made in the NID1279 background, a pop-out recombinant strain from NID043. Digestion of vector pU2110-3-AEG\textsubscript{08460.1} and pU2110-2-gedL liberated gene-targeting constructs for transformation and integration in IS3 and IS2, respectively.

**Chemical characterization of \textit{A. nidulans} strains**

All strains were grown as three point stab inoculations for 7 days at 37 °C in the dark on solid MM-media. Extraction and analysis of...
metabolites were performed by 2 methods: i) The agar plug extraction method described by Smetsbaard [40], using a total of 1 cm² of a colony, followed by analysis using reversed phase separation UHPLC- UV/VIS-HRMS on a maXis G3 quadrupole time of flight (qTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA), and equipped with a 10 cm Kinetex C18 column (Phenomenex Torrance, CA, USA) running a 10–100% acetonitrile gradient system in 15 min at 40 °C; ii) more concentrated samples were made by extracting metabolites from a total of 15 cm² of a colony using 12 ml solvent (ethyl acetate-dichloromethane-methanol-formic acid 60:30:15:1 v/v/v/v) in a 16-ml vial. The extract was evaporated to dryness with N₂ flow and redissolved in 0.5 ml methanol and analyzed by reversed phase separation an Agilent 1290 UHPLC coupled to an Agilent 6530 qTOF (Santa Clara, CA, USA) equipped with a electrospray source, and equipped with a 25 cm Agilent Poroshell phenyl hexyl running a 10–100% acetonitrile gradient system in 15 min 60 °C. Both MS instruments were mainly operated in ESI+ as geodin and related compounds ionizes best here in this mode [30]. Identification and quantification of geodin and sulochrin (BioAustralis, Smithfield, NSW, Australia) were based on comparison of peak area, retention time, accurate mass (±1.5 ppm), isotopic pattern and adduct pattern to quantitative authentic standards. Non quantitative standards representing 3-O-methylsulochrin; sulochrin-2'-methyl ether; isosulochrin; 3-O-demethylsulochrin; trypaclinid; and emodin were also included in the analyses. Other intermediates were identified by comparison to an internal reference standard database (~1500 compounds) [30]. For the identification of geodin in NID695, high resolution MS (50 000 FWHM) and mass accuracy (< 1.3 ppm) of the maXis G3 was needed to exclude a non chlorine containing co-eluting isobaric compound, seen as the broad peak in Figure 3 (6.7–7.3 min) that impaired the identification of geodin. In the following strains geodin was further verified by better chromatographic separation on a 25 cm phenyl-hexyl column on the Agilent UHPLC-qTOF.

RNA isolation and quantitative RT-PCR

RNA isolation from the A. nidulans strains and subsequent quantitative RT-PCR reactions were done as previously described in [7] except that biomass for RNA isolation was prepared with a Tissue-Lyser LT (Qiagen) by treating samples for 1 min at 45 MHz. The A. nidulans histone 3 encoding gene, bhitA (AN0735) was used as an internal standard for normalization of expression levels. All primers used for quantitative RT-PCR are shown in Table S2.

Bioinformatic analysis of gedL (ATEG_08460.1)

Alignment illustrations and sequence were made in CLC Main Workbench 6.8.4; Alignment parameters Gap open costs = 10, Gap extension cost = 1. All sequences in the alignments can be retrieved from Genbank, by the following references for the putative halogenases: Aspergillus oryzae ref|XP_001818590.1|, Aspergillus terreus ref|XP_001217599.1| and the A. terreus genome sequence resource at the Broad Institute, Bipolaris sorokiniana gb|EM66881.1|, Chaetomium chircisi (Red1) gb|ACM42402.1|, Chaetomium globosum ref|XP_001227515.1|, Pochonia chlamydosporia (Red2) gb|ADB6580.1| [39] and Talatomyces stipitatus ref|XP_002486044.1| [39]. Intron prediction of the A. terreus ATEG_08460.1 locus was done based on the Augustus gene prediction resource [29].

Concluding Remarks

We have described the complete and targeted transfer of all 13 genes of the geodin gene cluster from A. terreus to A. nidulans through a sequential integration approach enabling A. nidulans to synthesize geodin. In principle, this strategy can be used to reconstitute gene clusters of any size as the sequential integrations are based on marker recycling. In addition, defined promoters can easily be introduced in front of relevant genes in the cluster of interest. Importantly, we demonstrate that the cluster can be genetically dissected for clarification of its biochemical potential. We therefore envision that our method will significantly speed up the uncovering of biochemical pathways in fungi where the genome has been sequenced.

Supporting Information

Figure S1 Identification of putative HypC homolog encoded by gedH (ATEG_08457-2) in the A. terreus geodin gene cluster. Pairwise alignment of putative emodin anthrone oxidase, GedH (ATEG_08457-2), from A. terreus and norsolanic anthrone oxidase, HypC, from A. flavus. The conserved DUF-1772 domain and putative catalytic regions proposed by Ehrlich et al [25] are highlighted in green and blue, respectively. (TIF)

Figure S2 Schematic overview of the integration of a gene-expression cassette into the integration site, IS3, by homologous recombination. IS3 is located between genes AN4770 and AN4769 on chromosome III. The cassette consists of six parts: upstream targeting sequence (US), promoter (P, in this case 0.5 kb PgdId), your favorite gene (YFG), terminator (T, TtrpC), marker (in this case Afpgr) flanked by direct, and the downstream targeting sequence (DS). The orientations of the genes AN4770 and AN4769 are indicated by green arrows. The sizes of US, DS and the intergenic region are 1984 bp, 1911 bp, and 3007 bp, respectively. (TIF)

Figure S3 Complementation of halogenase deficiency. A: Left panel: detection of sulochrin (-ESI, EIC(m/z 331.0812)); right panel: detection of geodin (-ESI, EIC(m/z 396.9876)). Strains for halogenase analysis, from top to bottom: NID843 (gedLA); NID1280 (gedLA, IS3::gedL-ATEG_08460.1); and NID1306 (gedLA, IS2::gedL). B: Ratio of geodin, monochlor-sulochrin, and sulochrin in the NID1306 strain, including ESI+MS spectrum of monochlor-sulochrin showing the isotopic pattern and the mass deviations relative to the theoretical masses. Reference standards of geodin and sulochrin were included in all runs (data not shown). (TIF)

Figure S4 Identification of the likely start codon of gedL. A: Alignment of the top hits in a BLAST search for ATEG_08460.1 homologs shows that they contain a very conserved 48 amino acid residue addition in the N-terminus. Amongst the homologs, Rd2, has been characterized as a halogenase by [39] MLAS is the predicted N-terminus of ATEG_08460.1. Drawing is not to scale. B: The position of putative exons and intron in the 5’end of gedL as predicted by the Augustus software [29]. The predicted protein sequence encoded by exon 1 and by the first section of exon 2 is indicated. C: Full alignment of the halogenase homologs and GedL based on the GedL sequence derived from the new start codon. (TIF)

Figure S5 Expression of lacZ under the control of the gedR promoter (PgedR). Left panel: the positions of the strains...
on the plate are shown in the right panel, NID823 (godd mdpA-ΔA) is the reference strain without the ΔA. NID1276 is a control strain containing the PgdR-lacZ construct in ΔS3. The NID1291 (godd mdpA-ΔA PgdR-lacZ) strain carries PgdR-lacZ in ΔS3. The strains were stabbed on MM containing X-gal and incubated three days at 37 °C in the dark before photography.

Figure S6 Constitutive expression of gedR induces transcription of the A. nidulans gene mdpG. mdpG mRNA levels in reference (NID1) and in the godd strain (NID677) were evaluated by quantitative RT-PCR. For each strain, RNA was extracted as described in Materials and Method and the RNA samples analyzed in triplicate by quantitative RT-PCR. The samples were loaded and analyzed by 1% agarose gel-electrophoresis as indicated in the figure.

Table S1 Strain genotypes. *= For reference see Nielsen et al ([13]).

References

Table S2 Primers list. The sequence of PCR products #1-3 corresponds to superconjug 12: 1302695–1315163 (ATEG_08434-08440) in the genome sequenced isolate NIH2624 (IBT28053). Similarly, PCR products #4-6 and 8 corresponds to superconjug 12: 1298727–1304484 (ATEG_08449-08454) in NIH2624 (IBT28053). *= For reference see Hansen et al ([7]).

Acknowledgments
We would like to acknowledge Bjarne Gram Hansen for fruitful discussions throughout the project and Martin Engellard Kogle for technical assistance.

Author Contributions
Conceived and designed the experiments: MTN JBN KFN DCA DKH. Analyzed the data: MTN JBN KFN DCA DKH. Contributed reagents/materials/analysis tools: MTN JBN KFN DCA TOU LHM DKH. Wrote the paper: MTN JBN UHM.


