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Synthetic Biology Tools for Bioprospecting of Natural Products in Eukaryotes

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SUMMARY

Filamentous fungi have the capacity to produce a battery of natural products of often unknown function, synthesized by complex metabolic pathways. Unfortunately, most of these pathways appear silent, many in intractable organisms, and their products consequently unidentified. One basic challenge is the difficulty of expressing a biosynthesis pathway for a complex natural product in a heterologous eukaryotic host. Here, we provide a proof-of concept solution to this challenge and describe how the entire penicillin biosynthesis pathway can be expressed in a heterologous host. The method takes advantage of a combination of improved yeast in vivo cloning technology, generation of polycistronic mRNA for the gene cluster under study, and an amenable and easily manipulated fungal host, i.e., *Aspergillus nidulans*. We achieve expression from a single promoter of the pathway genes to yield a large polycistronic mRNA by using viral 2A peptide sequences to direct successful cotranslational cleavage of pathway enzymes.

INTRODUCTION

Synthetic biology has led to increasingly successful manipulation of biochemical pathways of biotechnological interest in model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae*. However, both bacteria and yeast suffer the disadvantage that they do not, or do not efficiently, process intron-containing genes, making construction of pathways from most eukaryotes for expression in these hosts time consuming and onerous.

Viral 2A peptides have been shown to promote cotranslational cleavage of eukaryotic polycistronic mRNA (Kim et al., 2011; Sharma et al., 2012). 2A peptide activity has been demonstrated in many eukaryotes, principally by the successful cleavage of reporter proteins, as well as proteins involved in complexes such as the T cell receptor-CD3 complex. As synthetic tools, viral 2A peptides provide an opportunity to express pathways as polycistronic genes in eukaryotes. In this study, we show that it is possible to express an entire complex pathway in a heterologous eukaryotic host polycistronically.

We chose the important model filamentous fungus *Aspergillus nidulans* as the expression host, since this organism, in contrast to *E. coli* and yeast, is a producer of natural products and should, a priori, be more likely to contain assistant genes not directly involved in biosynthesis. An obvious example in this regard is 4′-phosphopantetheinyl transferase, which is responsible for posttranslational activation of the large nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) proteins (Walsh et al., 1997, Gidijala et al., 2009), often the key biosynthetic enzymes of secondary metabolite pathways. Also, in common with most other filamentous fungi, *A. nidulans* genes possess multiple introns, and the organism can process intron-containing genes from other fungi (Unkles, 1992) and, potentially, other eukaryotes. Therefore, technically complicated strategies to prepare cDNA of frequently massive NRPS or PKS genes for expression in *E. coli* and yeast are circumvented. Finally, well-characterized and routinely used transformation selection systems are available for *A. nidulans* (Riach and Kinghorn, 1995).

Natural products, also called secondary metabolites, low-molecular-weight molecules often of novel and unusual chemical structure, can be of enormous benefit to humans—examples being antibiotics (e.g., penicillins and cephalosporins), immunosuppressants (e.g., cyclosporins), and statins (e.g., lovastatin) (Sanchez et al., 2012; Brakhage, 2013; Ozcengiz and Demain, 2013)—and are produced by certain prokaryotes and several eukaryotic groups including plants, fungi, and slime molds. Fungal secondary metabolites are arranged in gene clusters recognized by the inclusion of a hallmark gene that encodes usually either NRPS or PKS (Evans et al., 2011). Genome sequencing projects have shown that fungi commonly possess 25–70 secondary metabolite pathways, but for most of these pathways, the signals required for their activation remain a mystery (Brakhage, 2013). Therefore, fungi represent a rich mine of unknown and potentially useful bioactive compounds. Exploitation of such biosynthetic potential, however, is frustrated by the presence of these pathways in species that often have very slow growth rates or are recalcitrant to laboratory culture, as well as the fact that most secondary metabolite pathways are composed of multiple genes.

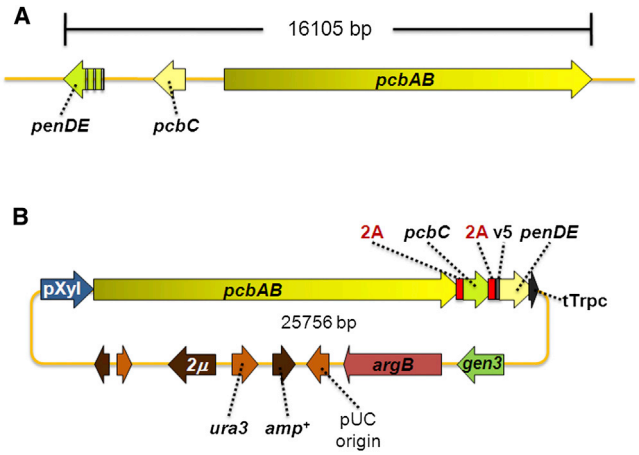

Figure 1. Construct for Heterologous Expression

(A) The penicillin biosynthesis gene cluster in *Penicillium chrysogenum* showing the size (in kilobases) of the region and the relative positions of the genes on the chromosome. The positions of three introns in *penDE* are shown by breaks in the arrow.

(B) Final plasmid p331 for expression in *A. nidulans*. Gene coding regions are shown by solid yellow arrows. Red blocks indicate the position of the 2A sequences and the gray block, the sequence encoding the V5 epitope tag. Other pertinent parts of the plasmid indicated by arrows include the promoter of the *P. chrysogenum xylP* gene (pXyl), the terminator of the *A. nidulans trpC* gene (tTrpc), the *argB* selectable marker for the host *A. nidulans*, and the components of the backbone pYES2 vector relevant to replication and selection in yeast and *E. coli*. Gen3, geneticin resistance gene.

A major technical difficulty in the transfer of such pathway genes to an amenable fungal host is that the NRPS and PKS genes are often massive, leading to cloning and gene transfer difficulties by conventional means. Established methods of heterologous expression require introduction of each gene individually, using a dedicated transformation marker selection system, and such distinct markers are somewhat limited in number. Moreover, a strong and/or inducible, well-characterized promoter is preferable to the natural promoter (with unknown regulators), and so a new DNA promoter sequence is required to be inserted 5′ to the coding region of each individual gene in the cluster. Finally, about half of the gene clusters do not encode transcription factors, making pathway overexpression using transcription factors impossible (Bergmann et al., 2007; Brakhage, 2013). Our interest in novel secondary metabolite discovery led us to test the synthetic approach described here, thus avoiding these technical drawbacks.

RESULTS

In Vivo Recombination of Synthetic Penicillin Biosynthesis Genes in *Saccharomyces cerevisiae*

As proof of concept of the potential of the combination of synthetic genes, PCR technology, in vivo recombination, and the generation of polycistronic mRNAs, we report the heterologous expression of an entire pathway of biotechnological relevance, the penicillin biosynthesis gene cluster of *Penicillium chrysogenum* (Figure 1A), in *A. nidulans* from a polycistronic gene. A major technique to provide such large constructs is based on in vivo recombination in *S. cerevisiae* (Figure S1 available online). The *P. chrysogenum pcbC* and *penDE* genes were synthesized according to the genome sequence of strain WIS 54-1255, with N-terminal porcine teschovirus 2A sequences (Kim et al., 2011; Sharma et al., 2012) and a sequence encoding an N-terminal V5 epitope tag added to *penDE* (between the 2A sequence and the coding region). In contrast, *pcbAB* from strain WIS54-1255 was amplified by PCR and cloned into plasmid pJET1.2. DNA fragments were assembled by in vivo recombination in *S. cerevisiae* as shown in Figure S1 to yield plasmid p331 harboring the polycistronic gene under transcriptional control of a xylose-inducible promoter (Zadra et al., 2000) (Figure 1B).

Heterologous Expression of the Synthetic Polycistronic Penicillin Biosynthesis Gene Cluster in *A. nidulans*

For expression of the synthetic gene cluster, we used as the host *A. nidulans* strain SAA248 (*argB2*, *npeA0049*), which is an arginine auxotrophic strain with a 20 kb deletion of the endogenous penicillin biosynthesis gene cluster (MacCabe et al., 1990). Thus, this strain allowed the assessment of whether the construct containing a *P. chrysogenum pcbAB-pcbC-penDE* polycistronic gene was functional because, after transformation of strain SAA248, the transformant strains should be able to produce penicillin.

A. nidulans strain SAA248 was transformed with plasmid p331, and 20 arginine prototrophic transformants were obtained. As a transformation control, plasmid p62 (Figure S1), identical to p331 except lacking the polycistronic gene, was used. In an initial screen of the p331 transformants, seven were found to produce a substance with antibacterial activity as judged initially by the presence of zones of clearing (or halos), indicating bacterial inhibition around fungal colonies grown at 27°C on *Aspergillus* minimal medium (AMM) agar containing 2% xylose as the sole carbon (inducing conditions) and 5 mM ammonium tartrate (equivalent to 10 mM ammonium) as the sole nitrogen source (Figure 2A).

The seven transformants exhibiting antibacterial activity were purified to single colonies and studied further by growing cells of purified strains in *A. nidulans* liquid AMM containing 2% xylose and 5 mM ammonium tartrate at 27°C. The culture filtrates were bioassayed after 24, 48, and 72 hr. From the results, five antibacterial activity-producing transformant strains (designated 331-T1, 331-T3, 331-T12, 331-T25, and 331-T26) were identified. Transformants 331-T3 and 331-T25 appeared to have marginally higher antibacterial activity as judged by halo diameter and were analyzed further. Southern blots showed that 331-T3 appeared to contain a single copy ectopically integrated, while 331-T25 was multicopy (Figure S2). A time course experiment of 331-T25 indicated that no antibacterial activity was observed at 18 hr, followed by considerable activity at 24 hr, after which values decreased at 48 hr and remained similar at 72 hr and 96 hr (Figure 2B). In contrast, no antibacterial activity was observed in the control transformant 62-C1 without the polycistronic gene (or the wild-type strain 0760) over this time period; neither was antibacterial activity, as judged by halo production, observed in culture filtrate from glucose-grown transformant 331-T25 (Figure S3) or 331-T3. Finally, other routinely used nitrogen sources such as nitrate or urea did not markedly alter antibacterial activity profiles of cultures when grown under xylose-induced conditions (Figure S3). Individual transcript sizes for the genes of this cluster would be around 11.5 kb for *pcbAB*,

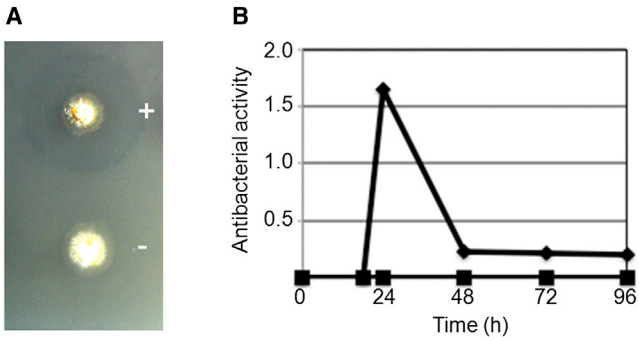

Figure 2. Heterologous Expression of a Natural Product Gene Cluster

(A) Example of preliminary screening of *A. nidulans* transformants. Following selection for arginine prototrophy, transformant colonies were grown for 24 hr at 27°C on AMM agar containing ammonium tartrate as the sole nitrogen source and either glucose or xylose as the sole carbon source. Plates were overlaid with molten medium containing the indicator bacterium, *B. calidolactis*, and further incubated at 50°C overnight. The halo (indicated by +) around the fungal colony reveals the production of an antibacterial substance compared to a nonproducer (−).

(B) Representative time course comparison of antibacterial production in transformants 331-T25 (◆) and 62-C1 (■) grown in liquid AMM containing 2% xylose and 5 mM ammonium tartrate. Antibacterial activity values are arbitrary units derived from the diameter of the inhibition halo (including the 1 cm well) per milligram dry weight of the 20 ml culture biomass. Three independent growth experiments were carried out with the SD being no more that 10%.

1.0 kb for *pcbC*, and 1.1 kb for *penDE*. Northern blot analysis, using a probe for the *P. chrysogenum pcbAB* gene, detected a much larger hybridizing band of around 15.5 kb (shown by the arrow in Figure 3A), indicating that the cluster was indeed produced as a polycistronic mRNA. Moreover, the northern blot analysis revealed that strain 331-T25, which contains the plasmid integrated in multicopy, expressed two polycistronic types of mRNAs with different sizes; one of these transcripts showed the expected size (Figure 3A). The third enzyme of the pathway, isopenicillin N acyltransferase (AT) encoded by the *P. chrysogenum penDE* gene, should possess an N-terminal V5 epitope tag following faithful cleavage at the upstream 2A sequence during translation of the large transcript. Anti-V5 antibody revealed a major protein band of ~41.5 kDa (the expected size of V5-AT) in western blots of protein prepared from both 331-T3 and 331-T25 transformants grown under induction with xylose but not noninduction with glucose conditions (Figure 3B). Attempts to detect unprocessed polyprotein using alternative electrophoresis conditions for high-molecular-size proteins (described in Experimental Procedures) were unsuccessful, indicating either the technical difficulty of detection of a 515 kDa protein or that the cotranslational cleavage at the 2A sequences was very efficient. As expected, no specific cross-reactivity of the antibody was observed to protein derived from wild-type or 62-C1 control cells grown under the same conditions with either xylose or glucose as carbon source. Minor cross-reacting bands between 12 and 35 kDa may represent proteolytic degradation during preparation, but the smallest band of around 13 kDa could be the correctly processed form of AT, which forms a heterodimer of autocatalytically derived N-terminal 11 kDa (13 kDa including the V5 tag) and C-terminal 29 kDa fragments (Whiteman et al., 1990). This autocatalysis per se in *A. nidulans* has, however, been shown to be inefficient, and the primary 40 kDa (size of AT without the V5 tag) product is the major functional form of *A. nidulans* AT (Fernández et al., 2003).

Identification of Penicillin as the Pathway Product

Transformants 331-T3 and 331-T25 (arginine prototrophs harboring the *P. chrysogenum pcbAB-pcbC-penDE* polycistronic gene), the negative control, transformant 62-C1 (arginine prototroph without the *P. chrysogenum pcbAB-pcbC-penDE* polycistronic gene), and the wild-type strain 0760 (containing the endogenous *A. nidulans* penicillin biosynthesis cluster) were grown for 48 hr in shake flask liquid AMM, under conditions to induce expression of the polycistronic gene, i.e., with xylose as the sole carbon source, as well as 5 mM ammonium tartrate as the sole nitrogen source. Chemical examination of the culture filtrate extracts from 331-T25 (Figure 4A, upper panel) and 331-T3 revealed the presence of a molecule not detectable in the medium from the p62-C1 strain (Figure 4A, lower panel) or the wild-type, while mass spectrometry (MS) fragmentation patterns confirmed the product identity as penicillin K (**1**) (Figure 4B). Also, to further verify that penicillin was produced, cultures were fed with phenoxyacetic acid, which results in the production of penicillin V (**2**) (Brakhage et al., 1992). Using a known penicillin V standard, we could confirm that the polycistronic cluster was fully functional in the recipient strain (Figure 4C). Finally, antibacterial activity in culture filtrates from transformants 331-T25 (Figure S2) and 331-T3 was abolished after *Bacillus cereus* penicillinase treatment, confirming that the antibacterial activity was due to a beta-lactam.

To quantify the amount of penicillin produced, the transformant strains were cultivated in penicillin fermentation medium with lactose as the carbon source and phenoxyacetic acid. Induction of the *xyl* promoter was achieved by addition of 2% (w/v) xylose solution to the cultures after 48 hr incubation with 250 rpm at 27°C, and cultures were incubated under the same conditions for another 24 hr. Penicillin V titers were determined as described elsewhere (Brakhage et al., 1992). As shown in Table 1, both transformant strains tested, i.e., 331-T3 and 331-T25, produced more penicillin than the wild-type expressing the endogenous penicillin biosynthesis gene cluster. Further proof of the experimental system was the fact that the wild-type only produced penicillin with lactose as the carbon source but not when xylose was present. By contrast, in the transformant strains, xylose induced the expression of the heterologous penicillin biosynthesis gene cluster, and only under these conditions was penicillin detectable in the supernatant. As a control, strain 62-C1 did not produce penicillin under either conditions because the strain does not encode a penicillin biosynthesis gene cluster. It is interesting that strain 331-T25, carrying several copies of the heterologous gene cluster, produced about three times more penicillin than the wild-type strain, indicating that the expression of a gene cluster using polycistronic mRNA can promote increased yield of the product.

DISCUSSION

The synthetic biology tools presented here circumvent the technical problems of heterologous expression of eukaryotic

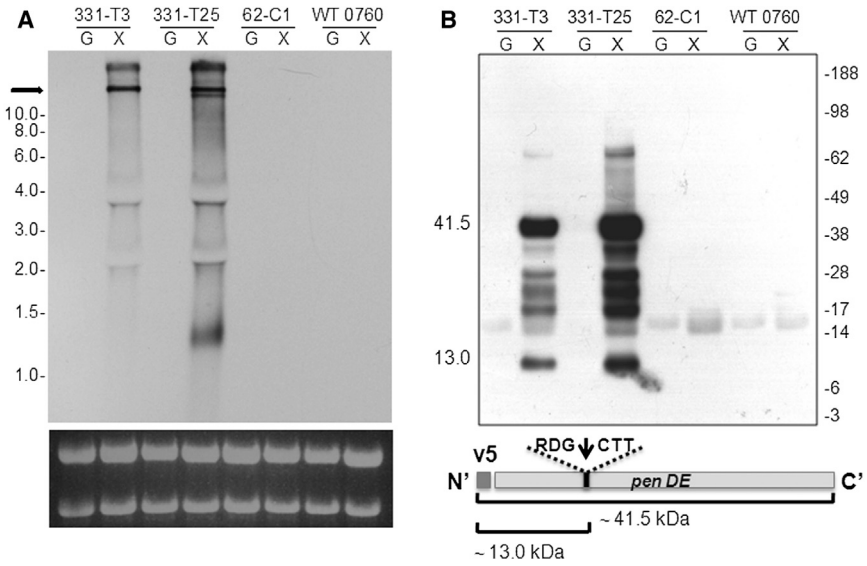

Figure 3. Molecular Analysis of Expression

(A) Northern blot of total RNA isolated from transformants 331-T3, 331-T25, 62-C1, and wild-type 0760 grown under noninducing (glucose [G]) or inducing (xylose [X]) conditions. The upper panel shows the blot probed with a 700-base-pair fragment of the *pcbAB* gene and hybridization visualized as detailed in the Experimental Procedures. The positions of the bands of the RNA size ladder are shown on the left. The lower panel shows a section of the ethidium bromide stained gel prior to blotting, with the ribosomal RNA bands indicating approximate equality of RNA loading. The polycistronic transcript is indicated by the arrow.

(B) Immunoblot analysis of transformants 331-T3, 331-T25, 62-C1, and wild-type 0760. Proteins were prepared from strains grown under non-inducing (glucose [G]) or inducing (xylose [X]) conditions. The V5-tagged protein was detected by anti-V5 HRP-conjugated antibody. Molecular size markers (in kilodaltons) are shown at the right.

pathways comprising several genes and push the boundaries of what has been previously reported for 2A "stop-carry on" translation by demonstrating that cotranslational cleavage occurs even after translation of a massive NRPS protein (Figure 5). In addition, double selection (geneticin resistance and repair of uracil auxotrophy) to force plasmid construct recombination in yeast between fragments with as short as 30-nt overlaps has dramatically increased the ease and efficiency of the procedure while simultaneously reducing the cost and the time required for construct generation.

Using heterologous production of penicillin, a well-known metabolite, as an example, we have shown here that it is possible to express a synthetic biosynthesis pathway to a level that allows further study of the biological and chemical nature of the product. The production of penicillin has been carried out here successfully without using prior knowledge regarding (1) additional metabolites such as a side chain (or side chains), often added to the culture; (2) the need to develop new and specific media recipes; (3) the natural location of the enzymes; or (4) the regulatory circuits involved. Penicillin K, shown to be the product of this engineered pathway, was produced in AMM by young cultures using molecules solely of endogenous origin, remarkably unlike the conditions normally used for wild-type penicillin production. By extrapolation, other unknown metabolites should be discernable in the same way. However, liquid chromatography-MS (LC-MS) analysis of culture supernatants and also of mycelia may be needed to identify novel masses and, thus, compounds.

While efficient systems for expression of prokaryotic secondary metabolite pathways exist, with this methodology, we have demonstrated that *A. nidulans* presents a convenient host for the expression of heterologous eukaryotic secondary metabolite pathways, accommodating and processing a novel transcript of over 15 kb. Rapid and efficient construct assembly in yeast, followed by expression in *A. nidulans*, opens the door to the investigation of more inscrutable pathways from other organisms, including other fungi and perhaps even plants. As such, we now have the means to reveal hitherto undiscovered molecules that may be the key to developing new medical or agricultural applications. Our study also demonstrates that the expression of a gene cluster using polycistronic mRNA potentially leads to increased yield of the product.

Questions still remain as to what extent does the relative amount of proteins of a cluster influence the product formation. Member genes of a cluster can be regulated by promoters with different strength, thus leading to potentially different amounts of the various proteins of a cluster. By expression of polycistronic mRNA, an equal amount of proteins of a gene cluster will be produced, which could lead to an unbalanced flux and also to the accumulation of possible toxic intermediates. Furthermore, it remains to be shown how large these constructs can become or how many genes can be included before 2A processing becomes too inefficient for expression. Since most natural product pathways are predicted to comprise more than three genes, with some containing 20 or more, it may be necessary to generate three or even four large constructs to cover all the genes of a pathway. Fortunately, auxotrophic markers for transformant selection in *A. nidulans* are sufficiently numerous, and the generation of recipient strains with multiple markers is relatively simple (Todd et al., 2007) to allow such manipulation.

SIGNIFICANCE

Synthetic biology holds promise for the discovery and design of novel metabolites by microbes. However, the presence of introns, the large size of some genes, and the number of genes involved form a major stumbling block for expression of many eukaryotic genes and pathways in amenable hosts. This is particularly true for filamentous fungi, which have the capacity to produce a multitude of natural products, most of them having unknown function. Moreover, the majority of these secondary metabolites are produced by complex pathways. Unfortunately, most of these pathways appear silent, many are present in

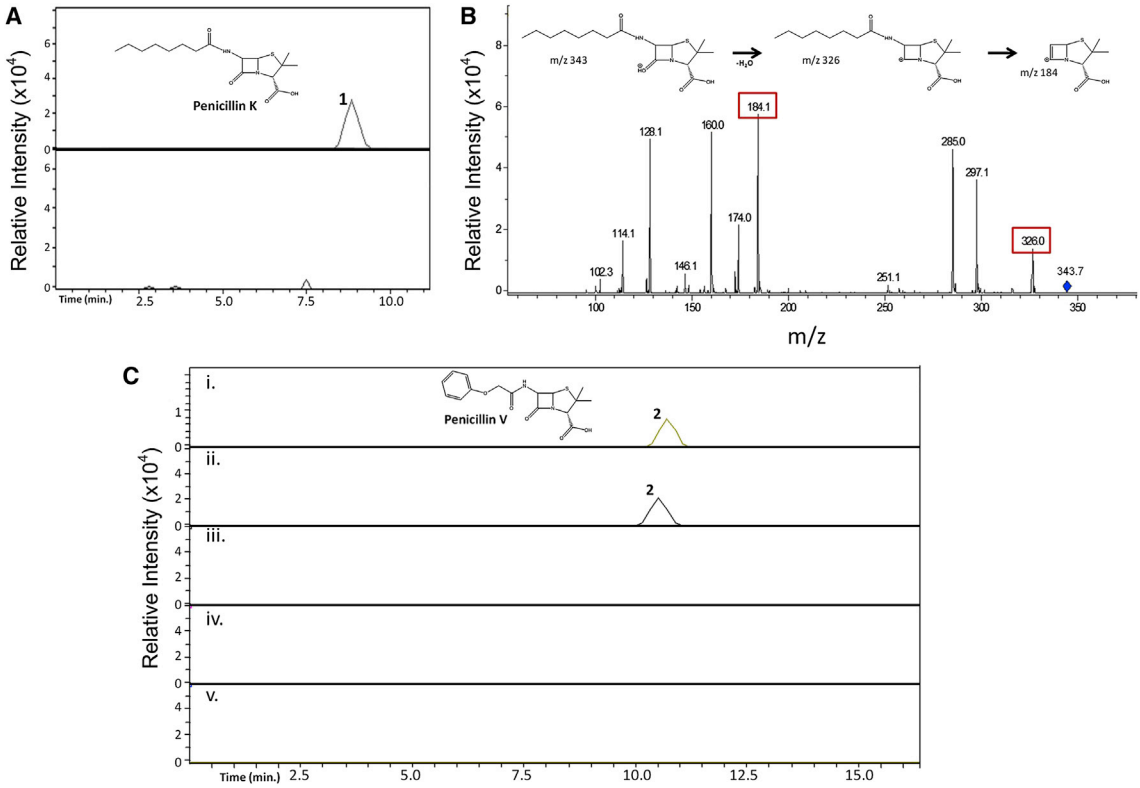

Figure 4. Chemical Analysis of Expression

(A) Extracted ion chromatogram (EIC) mass-to-charge ratio (m/z) 343 $[M + H]^+$ profiles for penicillin K (**1**). Electrospray ionization-MS (ESI-MS) comparing extracts from transformant 331-T25 (top) to control tranformant 62-C1 (bottom) grown under inducing conditions.

(B) Tandem MS m/z 343.7 $[M + H]^+$ spectrum of penicillin K **(1)** (ESI-MS, positive mode).

(C) EIC m/z 350 $[M-H]^-$ profiles for penicillin V (**2**) (ESI-MS, negative mode): Penicillin V standard (i.); transformant 331-T25 with phenoxyacetic acid added to media (ii.); transformant 331-T25 without phenoxyacetic acid (iii.); wild-type strain *Aspergillus nidulans* 0760 (iv.); and control tranformant 62-C1(v.).

intractable organisms, and their products consequently remain unidentified. In this study, we show that, in combination with significant improvements of another synthetic biology tool—yeast in vivo cloning that is necessary to generate the very large constructs required—it is possible to express a biosynthesis pathway for a complex natural product in a heterologous eukaryotic host. As a proof of concept, we describe how the entire penicillin biosynthesis pathway can be produced in a heterologous host. The increased in vivo cloning efficiency has facilitated generation of the large constructs required for expression of a nonribosomal peptide synthetase and additional enzymes leading to production of penicillin. As we show here, expression from a single promoter of all the pathway genes to yield a large polycistronic mRNA has been made possible using viral 2A peptide sequences to direct successful cotranslational cleavage of pathway enzymes in *A. nidulans*. The methodology raises the prospect of discovery of novel secondary metabolite molecules in a convenient eukaryotic host. In a wider application, this synthetic biology approach permits incorporation of genes from different eukaryotes as a single polycistronic mRNA to form entirely novel, artificial pathways.

EXPERIMENTAL PROCEDURES

Microbial Strains and Penicillin Bioassay

Aspergillus nidulans strain SAA248 *npeA0049 argB2* and strain 0760, wild-type, with regard to penicillin production, were used. The penicillin biosynthesis genes of *Penicillium chrysogenum* strain WIS 54-1255, the

Table 1. Amount of Penicillin V Produced by the Transformant Strains 331-T3 and 331-T25 Compared to the Wild-Type Strain and the Deletion Mutant 62-C1

Carbon Source	Lac	Lac + Xyl	Lac	Lac + Xyl	Lac	Lac + Xyl	Lac	Lac + Xyl
Strain	wt 0760	wt 0760	62-C1	62-C1	331-T3	331-T3	331-T25	331-T25
Penicillin V produced (ng/ml)	239 ± 34	nd	nd	nd	nd	281 ± 23	nd	687 ± 74

Means ± SEM are reported. Lac, lactose; Xyl, xylose; nd, not detectable.

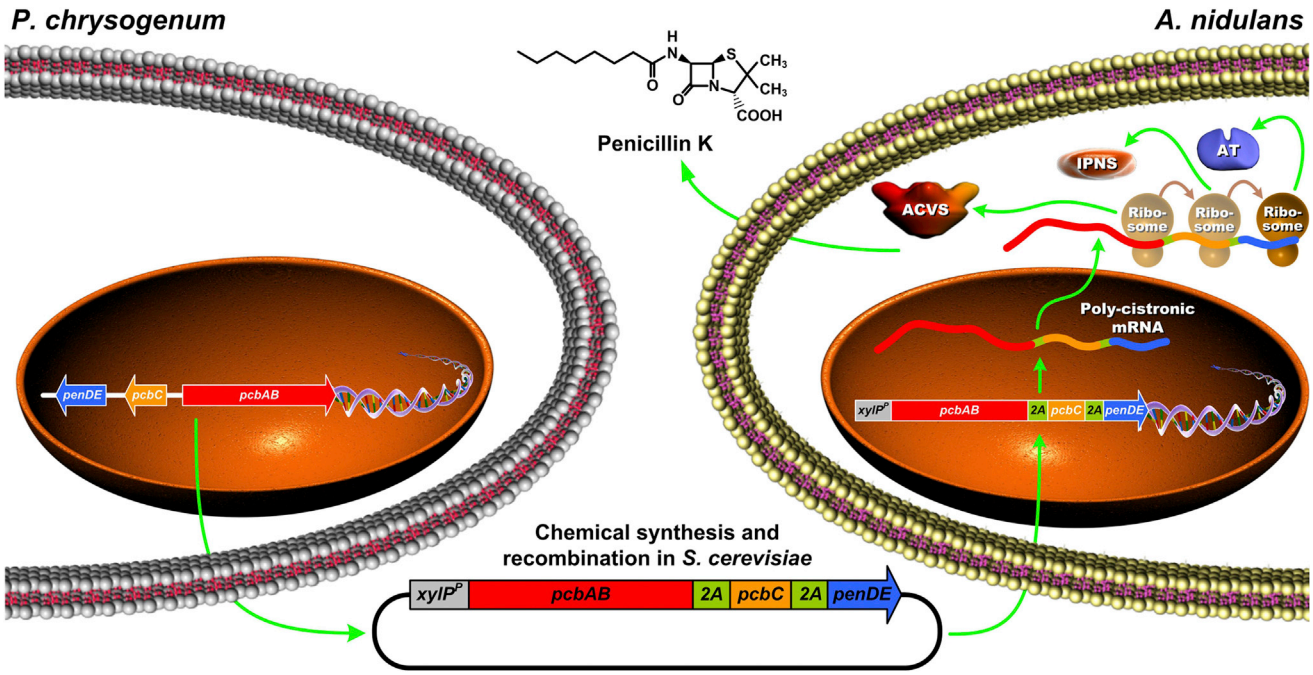

Figure 5. Summary of Heterologous Production of Natural Product Biosynthesis Pathways Using Virus 2A Peptide
Polycistronic mRNA is translated by ribosomes and the nascent peptide chain cotranslationally cleaved to the single biosynthetic proteins.

genome sequence of which has been determined (van den Berg et al., 2008), were used as the source for expression in *A. nidulans*. In vivo recombination for construct generation was carried out in *Saccharomyces cerevisiae* strain BY4741. *Bacillus calidolactis* C953 was used for bioassay analysis as described by Brakhage et al. (1992). *Escherichia coli* strain DH5α was used for standard molecular procedures. For penicillin V production, media with 4% (w/v) lactose were supplemented with 0.5 g phenoxyacetic acid per liter (Brakhage et al., 1992). For induction of the *xyl* promoter, 2% (w/v) xylose was added to the culture broth.

Preparation of Gene Constructs

The *P. chrysogenum pcbC* and *penDE* genes (accession numbers gi|255956720 and gi|255956718, respectively) were synthesized by Biomatik (http://www.biomatik.com) according to the genome sequence of strain WIS 54-1255 with N-terminal porcine teschovirus 2A sequences (Kim et al., 2011; Sharma et al., 2012), and a sequence encoding an N-terminal V5 epitope tag was added to *penDE* (between the 2A sequence and the coding region). In contrast, *pcbAB* from strain WIS54-1299 (accession number gi|255956722) was amplified by PCR and cloned into pJET1.2 (http://www.thermoscientificbio.com), and the DNA was sequenced to check for fidelity. Primers used for PCR amplification are given in Table S1. Fragments were assembled by in vivo recombination in *S. cerevisiae* as detailed in the Supplemental Experimental Procedures and Figure S1.

Southern and Northern Blots

For Southern blot analysis, cultures were grown for 24 hr at 27°C in liquid AMM containing 1% (w/v) glucose (Brakhage et al., 1992). DNA was extracted using the Master Pure Yeast DNA Purification Kit (http://www.epibio.com) according to the manufacturer's instructions. For northern blot analysis, cultures were grown for 24 hr at 27°C in liquid AMM containing either 1% (w/v) glucose or 2% (w/v) xylose as carbon source. Total RNA was prepared using the GeneMATRIX Universal RNA Purification Kit (http://www.roboklon.de). Probe labeling, hybridization, and detection were performed using the DIG Labeling Mix, DIG Easy Hyb, and the CDP-Star ready-to-use kit, respectively, according to the instructions of the manufacturer (http://www.roche-applied-science.com). Primers used for preparing labeled probes are reported in Table S1.

Fungal Transformation

The *A. nidulans* transformation procedure used was essentially that carried out as described elsewhere (reviewed in Riach and Kinghorn, 1995 and references therein). Selection of transformants was based on arginine prototrophy.

Protein Production Analysis

For immunological detection, cultures were grown for 48 hr at 27°C in liquid AMM containing 5 mM ammonium tartrate as nitrogen source and either 1% (w/v) glucose (noninducing conditions) or 2% (w/v) xylose (inducing conditions) as carbon source. Around 100 mg pressed wet weight of mycelium was ground in liquid nitrogen, and the fine powder was suspended in 500 μl extraction buffer consisting of 10 mM sodium orthophosphate, 200 mM sodium chloride, 10% (v/v) glycerol, pH 7.0, to which was routinely added fresh 1 mM phenylmethanesulfonylfluoride. After 3 min agitation, samples were centrifuged at 16,000 × g for 15 min at room temperature to yield the filtrate fraction. Protein concentration was estimated using Bradford's assay with BSA as the standard, and 1.5 μg protein samples were run on a 4%–12% NuPAGE gel (http://www.lifetechnologies.com), blotted on to Immobilon-P membrane (http://www.millipore.com), as described by the manufacturer. Protein was detected using V5-specific horseradish peroxidase (HRP)-conjugated antibody (http://www.lifetechnologies.com) and Immobilon Western Chemiluminescent HRP Substrate (http://www.millipore.com).

Extraction and LC-MS Analysis of Molecules with Antibacterial Activity

The filtrates of the fungal cultures were extracted exhaustively with an equal volume of ethyl acetate, dried with sodium sulfate, and concentrated with a rotary evaporator. The material was redissolved in 1 ml of methanol and filtered through a 0.2 μm PTFE filter (http://www.carlroth.com). Twenty microliters were then injected on to an Agilent 1100 series LC/MSD Trap LC-MS system equipped with an electrospray ion source using an Agilent XDB-C8 (5 μm, 4.6 × 150 mm) column. The gradient consisted of acetonitrile/0.1% (v/v) HCOOH (H_2O) 10/90 in 15 min to 90/10, then 90/10 to 100/0 in 0.5 min and 100% acetonitrile for 5.5 min; flow rate, 1 ml/min^{-1}.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.02.010.

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