Eighty Years after Its Discovery, Fleming’s Penicillium Strain Discloses the Secret of Its Sex

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Eighty years ago, Alexander Fleming discovered antibacterial activity in the asexual mold Penicillium, and the strain he studied later was replaced by an overproducing isolate still used for penicillin production today. Using a heterologous PCR approach, we show that these strains are of opposite mating types and that both have retained transcriptionally expressed pheromone and pheromone receptor genes required for sexual reproduction. This discovery extends options for industrial strain improvement programs using conventional genetical approaches.

The ability to mate fungi under controlled laboratory conditions is a valuable tool for genetic analysis and classical strain improvement (29). In ascomycetes, mating typically occurs between morphologically identical partners that are distinguished by their mating type. In most cases a single mating-type (MAT) locus conferring mating behavior consists of dissimilar DNA sequences in the mating partners, termed the MAT1 and MAT2 idiomorphs. MAT1 contains a gene encoding a protein with an alpha-box domain, whereas MAT2 carries a gene encoding a protein with a high-mobility group (HMG) domain. In addition to these genes, other genes may also be present at the MAT locus (6, 10, 29, 37, 38). In contrast to self-sterile (heterothallic) fungi, self-fertile (homothallic) filamentous ascomycetes contain genes indicative of both mating types in the same genome, either linked or unlinked (17, 28, 32, 34, 40).

The ubiquitous genus Penicillium consists of numerous important, apparently asexual species. Unfortunately, due to its asexuality, Penicillium is difficult to improve for penicillin production by conventional genetics approaches. However, analysis of the complete genome sequences of the asexual human pathogens Aspergillus fumigatus and Penicillium marneffei, relatives of P. chrysogenum, revealed the presence of genes associated with sexual reproduction, including mating-type genes and genes for pheromone production and detection (17, 25, 27, 39).

Here we present the discovery of transcriptionally expressed mating-type genes in P. chrysogenum, the industrial producer of the β-lactam antibiotic penicillin. Moreover, we find homologs of pheromone and pheromone receptor genes that are known to function in mating and signaling in sexually reproducing filamentous ascomycetes. To the best of our knowledge, P. chrysogenum is the first industrial fungus for which transcriptionally expressed mating-type genes were discovered. Our findings open up the possibility of inducing mating and sexual reproduction for alternative strain improvement strategies.

MATERIALS AND METHODS

Fungal strains, growth conditions, and preparation of nucleic acids. A list of all P. chrysogenum isolates used, with source details, is provided in Table 1. Strains were routinely maintained at 27°C on CCM complete culture medium (0.3% [wt/vol] sucrose, 0.05% [wt/vol] NaCl, 0.05% [wt/vol] K2HPO4, 0.05%...
sequence was determined (MWG-Biotech, Germany). The primers were used in a heterologous PCR screen of 12 isolates (Table 1) with 50-μg aliquots of total RNA were treated with DNase I (Invitrogen, Germany) according to the manufacturer’s recommendations. Reverse transcription (RT) was performed with 400 U Superscript II (Invitrogen, Germany) and deoxynucleoside triphosphates at a concentration of 0.33 mM. As a control for successful DNase treatment, each reverse transcription was carried out twice, once with and once without reverse transcriptase. All samples were used as templates for the expression analysis of the MAT-1 and MAT-2 genes and the pheromone precursor and receptor genes Pcppg1, Pcppg2 and Pcppg3 using the following primers: (i) for the MAT-1 gene primers, MAT-1-f (5'-CTCTGCTCAATGGACACTGTCGTC-3') and MAT-1-r (5'-ATGAACTAGCTGGCACTGTCGTC-3') were designed based on conserved regions of the A. fumigatus APN2 and SLA2 genes, respectively. 

PCR amplification and cloning of the A. fumigatus MAT-1 or MAT-2 idiomorph. Primers Apa2-nf (5'-ACATTATATGGGCTAGGATGGAAAC-3') and Abi2-r (5'-CTGACACTGCCGCTGGAGAGAGCAT-3') were designed based on conserved regions of the A. fumigatus APN2 and SLA2 genes, respectively. The primers were used in a heterologous PCR screen of 12 P. chrysogenum isolates (Table 1) with 50-μl reaction mixtures containing 250 μg genomic DNA, 10 pmol of each primer, 1 mM of each deoxynucleoside triphosphate, and 5 U HotMaster Taq polymerase (5' Prime, Germany). The resulting amplicons were sequenced (MWG-Biotech, Germany) and deoxynucleoside triphosphates at a concentration of 0.33 mM. As a control for successful DNase treatment, each reverse transcription was carried out twice, once with and once without reverse transcriptase. All samples were used as templates for the expression analysis of the MAT-1 and MAT-2 genes and the pheromone precursor and receptor genes Pcppg1, Pcppg2, and Pcppg3 using the following primers: (i) for the MAT-1 gene primers, MAT-1-f (5'-CTCTGCTCAATGGACACTGTCGTC-3') and MAT-1-r (5'-ATGAACTAGCTGGCACTGTCGTC-3') were designed based on conserved regions of the A. fumigatus APN2 and SLA2 genes, respectively. 

RESULTS AND DISCUSSION 

Identification of opposite mating-type loci in Fleming’s and penicillin production strains. The APN2 gene, encoding a putative DNA lyase, and the SLA2 gene, encoding a cytoskeleton assembly control factor, have been found neighboring MAT loci in A. fumigatus and many other ascomycetes (14, 16, 17). Therefore, to determine whether mating-type genes are also present in

| Table 1: Strains used in this study including mating types, descriptions, and sources |
|---------------------------------|-----------------|-----------------------------|-----------------|
| Strain or isolate | Mating type | Locus of isolation or description | Source or reference |
|-------------------|-------------|-------------------------------|------------------|
| NRRL1951 | MAT-1 | Moldy cantaloupe fruit; United States | ARS culture collection (NRRL), Peoria, IL Sandoz GmbH, Kundl, Austria |
| ATCC 28089, ATCC 16521, WisQ-176 | MAT-2 | Derivatives of strain NRRL15951, obtained by random mutagenesis | CBS fungus database, The Netherlands |
| NRRL832 | MAT-1 | Contamination of must; Belgium | CBS fungus database, The Netherlands |
| NRRL807 | MAT-1 | Contamination from cheese; United States | CBS fungus database, The Netherlands |
| DAOM193710 | MAT-1 | Contamination from cheese; United States | CBS fungus database, The Netherlands |
| DAOM155627 | MAT-1 | Contamination from paper; Canada | CBS fungus database, The Netherlands |
| NRRL1249B21 | MAT-2 | Fleming strain (George A. Harrop) | DMSZ, Germany |
| NRRL824 | MAT-2 | Fleming strain (Charles Thom collection) | DMSZ, Germany |
| DSM6288 | MAT-2 | Contamination from optical glass | DMSZ, Germany |
| DAOM155628 | MAT-2 | Contamination from paper; Canada | DMSZ, Germany |
| DAOM216701 | MAT-2 | Semenitus indescum; Korea | DMSZ, Germany |
| DAOM59494C | MAT-2 | Substrate unknown; Honduras | DMSZ, Germany |

FIG. 1. Genealogy of P. chrysogenum strains containing either the MAT1 or MAT2 idiomorph. Strains used in this study are boxed.
the industrially relevant fungus *P. chrysogenum*, we designed primers corresponding to conserved regions of the *A. fumigatus* APN2 and SLA2 genes, respectively. Genomic DNA from Fleming’s original strain, NRRL1249B21, and from 11 other isolates, including ATCC 28089, a derivative of the cantaloupe strain NRRL1951 (Table 1), was used. This strain most probably belongs to the same taxa as the original Fleming isolate, since both strains contain completely identical internal transcribed spacer 2 sequences (data not shown). This sequence is highly suitable for evaluating whether the taxa of a species can potentially interbreed and was already used with numerous eukaryotes (8). Two different types of amplicons of 3.6 and 3.7 kb were obtained. Six isolates, including the cantaloupe strain NRRL1951, generated the larger amplicon, while the other six, including the Fleming strain NRRL1249B21, generated the smaller one. Sequencing of the larger amplicon of strain ATCC 28089 revealed the presence of a putative 1,077-bp MAT-1 gene whose open reading frame (ORF) is interrupted by a 48-bp intron; splicing of this intron was confirmed by RT-PCR (Fig. 2A and C). The putative MAT-1 gene encodes a predicted protein of 342 amino acid residues harboring a conserved alpha-box domain (Fig. 3A). Surprisingly, sequencing of the smaller amplicon of the Fleming strain (NRRL1249B21) revealed a homology to other fungal MAT2 loci. This identified MAT2 locus is 1,136 bp in size and contains a single ORF interrupted by two introns of 53 bp and 50 bp, splicing of which was also confirmed by means of RT-PCR (Fig. 2B and C). The ORF encodes a protein of 303 amino acid residues with a conserved HMG-DNA-binding domain with an amino acid identity of 37 to 59% to HMG mating-type proteins from other ascomycetes. The second intron of the *P. chrysogenum* MAT-2 gene is located at a conserved position in a serine (S) codon of the HMG domain (Fig. 3B) and thus is at the same position as in all of the ascomycete HMG mating-type genes known to date (11). Similar to the structural organization of mating-type loci in many sexually reproducing filamentous ascomycetes, highly conserved flanking regions (95.8% identical nucleotides within a 1,318-bp upstream and 1,217-bp downstream flanking region) were found upstream and downstream of the MAT idiromorphs (Fig. 2A and B). Both *P. chrysogenum* mating-type loci are directly flanked by APN2 and SLA2. This is in accordance with the mating-type organization of *A. fumigatus* and other asexual species of the genus *Aspergillus* (14, 25) but clearly different from those of the recently characterized mating-type idiromorphs of the human pathogens *Histoplasma capsulatum*, *Coccidioides immitis*, and

FIG. 2. Mating-type locus of *Penicillium chrysogenum*. (A and B) Schematic illustration of the mating-type idiromorphs and their flanking regions. Idiomorphs are presented as gray boxes and their flanking regions as white boxes. The positioning and transcriptional direction of the mating-type genes in each idiromorph are indicated by an arrow, and introns are shown in black. (A) MAT1 idiromorph of strain ATCC 28089 (accession no. AM904544). The relative position of the alpha-box domain of MAT-1 is indicated. (B) MAT2 idiromorph of strain NRRL1249B21 (accession no. AM904545). The relative position of the HMG domain of MAT-2 is indicated. (C) Expression of mating-type genes and genes involved in sexual reproduction in *P. chrysogenum*. RNA transcripts (cDNA) either with (+) or without (−) reverse transcriptase were amplified by RT-PCR. PCR amplification of genomic DNA (gen. DNA) was used as a positive control. The strain designation and mating type are indicated above each lane; sizes of cDNA and genomic DNA amplicons are given on the right.
Coccidioides posadasii. In *H. capsulatum*, the cytochrome c oxidase subunit VIa gene *COX13* instead of the *APN2* gene is located upstream of the mating-type locus, whereas in the Onygenales members *C. immitis* and *C. posadasii*, the dissimilarity between the two idiomorphs expands beyond the HMG and alpha-box regions and encompasses the adjacent *APN2* and *COX13* genes (16, 21). In contrast to the case with *A. fumigatus*, *C. posadasii*, and *P. chrysogenum*, no additional ORFs could be identified within the mating-type idiomorphs of *C. immitis*.

**Equal distribution of MAT1 and MAT2 loci in geographically separated *P. chrysogenum* isolates.** To test whether the remaining 10 strains carry either the *MAT1* or *MAT2* locus, specific primer pairs amplifying either the HMG domain or the conserved alpha-box domain were designed. The detection of six *MAT1* strains and six *MAT2* strains confirmed the presence of both mating types in equal proportions (Table 1). A one-to-one distribution of *MAT1:MAT2* strains indicates that occasionally sexual reproduction occurs in *P. chrysogenum*. Similar results were also obtained for the pathogens *A. fumigatus* (25) and *Coccidioides* (21). Moreover, investigation of the alpha-box and HMG domain genes provided no evidence for loss-of-function mutations. Further RT-PCR analyses showed that both genes are expressed (Fig. 2C). Taken together, these data suggest that *P. chrysogenum* has the potential to reproduce sexually. This finding prompted us to search for homologs of pheromone and pheromone receptor genes that function in mating and pheromone signaling in sexually reproducing filamentous ascomycetes (19, 23, 26, 30, 36).

**Both mating-type strains carry transcriptionally expressed pheromone and pheromone receptor genes.** Two different classes of pheromones are known to be involved in sexual reproduction in outbreeding and in self-sterile filamentous ascomycetes (9, 19, 23, 26, 30, 36). One class of genes encodes peptide pheromone precursors that contain multiple copies of the mature peptides flanked by protease cleavage sites, while the other class of pheromone genes encodes a small protein with a CAAX motif at the C terminus. This motif is expected to produce a mature lipopeptide pheromone with a C-terminal carboxy methyl isoprenylated cysteine (7). To identify any putative pheromone precursors encoded by the *P. chrysogenum* genome, primers based on conserved-region sequences of the peptide pheromone precursor gene *ppgA* of *A. fumigatus* were used for PCR. Sequencing of the obtained amplicon of 214 bp revealed a similarity of 57.1% with the *ppgA* gene of *A. fumigatus*. Subsequently, a screen of a *P. chrysogenum* cDNA library led to the isolation of a putative *P. chrysogenum* gene encoding a 111-amino-acid peptide pheromone precursor. The identified *P. chrysogenum* gene was named *PcPPG1*. Within the polypeptide encoded by this gene, two identical repeats could be identified. The two repeats encode the decapetide amino acid sequence KWCGHIGQGC (Fig. 4). The decapetide sequence bears strong similarity to pheromones from other filamentous ascomycetes (31). Interestingly, a hydrophobic signal sequence was detected within the N terminus of *P. chrysogenum*.
num PPG1. Therefore, the putative pheromone is most likely secreted from the cell via the classical secretion pathway. In A. fumigatus and other species of the genus Aspergillus, extensive TBLASTN analysis failed to identify a homolog encoding a lipopeptide pheromone. We therefore did not try to identify a P. chrysogenum lipopeptide pheromone gene by means of heterologous PCR. However, using the same strategy as for the isolation of the Pcp1g1 gene, we were able to identify two pheromone receptor genes from P. chrysogenum. The gene products of Pcp1p and Pcp2p are predicted to have seven transmembrane spanning domains and displayed a high level of amino acid identity with the α-factor receptor Ste3p and the α-factor receptor Ste2p of Saccharomyces cerevisiae, respectively, as well as with pheromone receptors of filamentous ascomycetes (see Fig. S1 and S2 in the supplemental material). Using the RT-PCR approach, we demonstrated that both the pheromone and receptor genes are expressed in strains of both mating types (Fig. 2C), an observation similar to that with A. fumigatus (25).

Attempts to mate strains with opposite mating-type loci. The results of our transcriptional expression data suggest the existence of a heterothallic sexual cycle in P. chrysogenum. This finding opens up the possibility of inducing mating and sexual reproduction in P. chrysogenum, as was previously shown for the typical “asexual” yeast, Candida albicans (3). While in C. albicans the discovery of mating-type genes led to the development of procedures for the mating of strains with opposite mating-type loci, similar attempts failed so far with the filamentous fungus A. fumigatus. We similarly tried to mate P. chrysogenum strains with opposite mating types (B. Hoff et al., unpublished data). This was done by combining all strains listed in Table 1 under different physiological conditions. For example, the plates were incubated in the dark as well as in light, and the cultures were grown on different rich media as well as minimal media. Moreover, we used sealed or unsealed plates of P. chrysogenum, conditions known to promote or prevent sexual differentiation in A. nidulans (4). However, microscopic analyses of plates after more than 10 weeks of incubation did not indicate the generation of cleistothecia. In contrast, telemorphs of Penicillium belonging to the genus Eupenicillium (13) generated cleistothecia and ascospores on complete medium in the dark under limited air exchange after 2 weeks of incubation. At this point, it should be noted that all P. chrysogenum strains were derived from type culture collections and might have lost fertility during long storage. For the heterothallic H. capsulatum, it has been reported that fertility is rapidly lost during laboratory passage, and it has been suggested that selective pressures may serve to maintain fertility in the environment (16, 20). Moreover, despite molecular verification, mating compatibility tests of H. capsulatum strains of different mating types did not result in the formation of an ascocarp and ascospores (5). Thus, it will be more promising to repeat the P. chrysogenum experiments with strains directly isolated from nature.

Furthermore, we tested whether penicillin production of a given strain was changed in the presence of the opposite mating-type partners. Since only a high-producer strain and derivatives thereof with identical mating-type loci (MAT1) were available (Table 1), we had to use wild-type strains for these experiments. MAT2 strains, however, produce only low levels of penicillin. Therefore, moderate changes in antibiotic production were hard to detect by bioassays or high-performance liquid chromatography analysis. Further experiments will be required to demonstrate sexual reproduction in P. chrysogenum. A successful outcome of these attempts will extend the options for manipulating P. chrysogenum genetically and should ultimately provide the opportunity to generate genetically engineered penicillin production strains with novel metabolic properties.

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