Eukaryotic Cell

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***Eukaryotic Cell*** **2008, 7(3):465. DOI: 10.1128/EC.00430-07.**
**Published Ahead of Print 25 January 2008.**

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EUKARYOTIC CELL, Mar. 2008, p. 465–470 Vol. 7, No. 3
1535-9778/08/$08.00+0 doi:10.1128/EC.00430-07
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# Eighty Years after Its Discovery, Fleming's *Penicillium* Strain Discloses the Secret of Its Sex▽†

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Received 28 November 2007/Accepted 16 January 2008

**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 observation that a mold could inhibit bacterial growth is perhaps the most important breakthrough in the history of therapeutic medicine. In 1928, Alexander Fleming noticed that the growth of staphylococcus colonies growing near a contaminating mold colony was disrupted. The following year, he published a paper describing the mold as *Penicillium notatum* and terming the active compound penicillin (15). The finding that penicillin was not toxic to animals only increased interest, and this was followed by the attempts of H. W. Florey, N. G. Heatley, and E. B. Chain, working at Oxford University, to purify penicillin. Later, D. C. Hodgkin and B. Low went on to determine its β-lactam structure using X-ray crystallography. However, it was not until 1941 that the first clinical trials with penicillin were undertaken (1), and since chemical synthesis was not found to be possible, fermentation became the commercial source of penicillin. Unfortunately, Fleming's original *P. notatum* strain (NRRL1249B21) produces only low levels of penicillin, so that strain improvement and large-scale production became essential. However, British industry was unable to produce the large amounts of required penicillin due to the restraints on resources caused by World War II. Therefore, Florey and Heatley went to the United States to raise the interest of the American pharmaceutical industry and U.S. Department of Agriculture in penicillin production. Here the problem of large-scale penicillin production was solved when *Penicillium chrysogenum* (Thom) strain NRRL1951 was isolated from a moldy cantaloupe obtained in a Peoria, IL, market (33). Strain WisQ-176, a derivative of strain NRRL1951, was obtained by conventional strain improvement programs and is the ancestor of all strains used today for biotechnical penicillin production (12, 18), with a total world market of about $8 billion (2) (Fig. 1).

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† Supplemental material for this article may be found at http://ec.asm.org/.

▽ Published ahead of print on 25 January 2008.

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] $K_2HPO_4$, 0.05%



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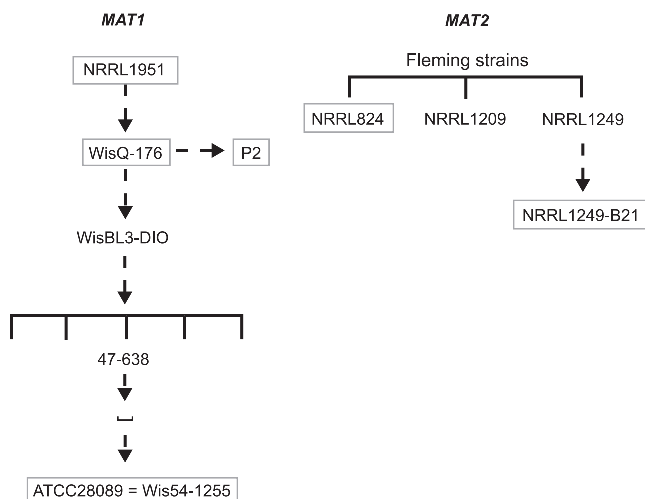


FIG. 1. Genealogy of *P. chrysogenum* strains containing either the *MAT1* or *MAT2* idiomorph. Strains used in this study are boxed.

[wt/vol] $MgSO_4$, 0.001% [wt/vol] $FeSO_4$, 0.5% [wt/vol] tryptic soy broth, 0.1% [wt/vol] yeast extract, 0.1% [wt/vol] meat extract, 1.5% [wt/vol] dextrine, pH 6.9 to 7.1). For DNA and RNA extraction, *P. chrysogenum* strains were grown at 27°C and 130 rpm for 4 days in liquid CCM medium. After growth, resulting mycelia were removed, flash frozen, and ground under liquid nitrogen prior to DNA and RNA extraction. Fungal genomic DNA and RNA were extracted with phenol-chloroform and chloroform/isoamyl alcohol methods as previously described (24).

**PCR amplification and cloning of *P. chrysogenum* genes.** In an attempt to detect *P. chrysogenum* isolates containing a *MAT1* or *MAT2* idiomorph, primers Afapn2-f (5′-ACATTTATATGGGCTAGCGATTGGAAC-3′) and Afsla2-r (5′-CGTCAACGCCT TGGAGAGATGCGC-3′) were designed based on conserved region sequences 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). Cycle parameters were 2 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 55°C, and 5 min at 68°C, and a final step of 15 min at 25°C. Amplicons of strains ATCC 28089 and NRRL1249B21 of predicted sizes for the idiomorphs were cloned into the vector pDrive (Qiagen, Germany) according to the manufacturer's recommendation, and the insert sequence was determined (MWG-Biotech, Germany).

Extracted genomic DNA of *P. chrysogenum* was further used as a template for the amplification of a pheromone precursor gene using primers Appg1-f (5′-G GCTGCCGCC GCCGTCCAGGC-3′) and Appg1-r (5′-CGCTTGGCCTTGGC ACAACCCTGGCC-3′) based on sequence comparison of the *ppg1* genes of *Aspergillus nidulans*, *Aspergillus terreus*, and *A. fumigatus*. In order to amplify pheromone receptor genes from *P. chrysogenum*, primers were designed according to conserved regions of the *A. fumigatus preA* gene (AfpreA-f [5′-TGGCC CACGGACGATGTGGATTCATGGTGG-3′] and AfpreA-r [5′-AAGGAAAA ATAGACGCAGGAATCTGGACTT-3′]) and the *preB* gene (AfpreB-f [5′-TG GCTAGAGAGTGCCGCCACGAATATC-3′] and AfpreB-r [5′-TGGGATGG TCA TAGTTTGGCAACCCAT-3′]), respectively. PCR was conducted using a total volume of 50 μl containing 250 μg genomic DNA, 10 pmol of each primer, 1 mM of each deoxynucleoside triphosphate, and 5 U *Taq* polymerase (5′ Prime, Germany). PCR conditions were as follows: 2 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 48 to 50°C, and 1.5 min at 72°C, and a final step of 15 min at 25°C. The resulting amplicons were sequenced (MWG-Biotech, Germany), and the arising data were analyzed using the BLAST program.

The full-length sequences of the genes were derived from cDNA clones detected in a *P. chrysogenum* cDNA library (Sandoz GmbH, Kundl, Austria) that was screened according to previously published procedures (22).

**Semiquantitative RT-PCR for expression analyses.** For use in semiquantitative RT-PCR, cDNA synthesis was carried out as previously described (24) with the following modifications: 2-μg aliquots of total RNA were treated with DNase I (Invitrogen, Germany) according to the manufacturer's recommendations, and 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 Pc*ppg1*, Pc*pre1*, and Pc*pre2* using the following primers: (i) for the *MAT-1* gene primers, MAT-1-f (5′-CTTCGT CCATTGAACTCTTTTATG-3′) and MAT-1-r (5′-ATCCCAAC CAGCCATC CTGAGATA-3′); (ii) for the *MAT-2* gene primers, MAT-2-f (5′-CCAAGT CTATCCACGAGGCTG-3′) and MAT-2-r (5′-GCAGGCAGTTGGCACGGG AAC-3′); (iii) for the Pc*ppg1* gene primers, Pcppg1-f (5′-TTACTCGTCATCC TCTTCCTCGA-3′) and Pcppg1-r (5′-ATGAAGTTCACCTCCGTCGTC-3′); (iv) for the Pc*pre1* gene primers, Pcpre1-f (5′-GTTCTGCGTGGCTGTTCCAG T-3′) and Pcpre1-r (5′-GGGATGCCAGG GGAGGGAGAGCAT-3′); and (v) for the Pc*pre2* gene primers, Pcpre2-f (5′-GTCACTAAG CTCGGATTCGCC-3′) and Pcpre2-r (5′-CGATTGAATTGTTCCTTTGAAC-3′). RT-PCR was performed in a volume of 50 μl with HotMaster *Taq* polymerase (5′ Prime, Germany) according to the manufacturer's recommendations. PCR conditions were as follows: 2 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 53 to 55°C, and 5 min at 68°C, and a final step of 15 min 25°C.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the mating-type genes and the pheromone precursor and receptor genes have been deposited in the EMBL database under the accession numbers indicated: *MAT-1*, AM904544; *MAT-2*, AM904545; Pc*ppg1*, AM904541; Pc*pre1*, AM904542; and Pc*pre2*, AM904543.

## 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 |
| ATCC 28089, ATCC 16521, WisQ-176 P2 | *MAT-1* | Derivatives of strain NRRL1951, obtained by random mutagenesis | Sandoz GmbH, Kundl, Austria |
| 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 | 35 |
| DAOM155627 | *MAT-1* | Contamination from paper; Canada | 35 |
| NRRL1249B21 | *MAT-2* | Fleming strain (George A. Harrop) | DSMZ, Germany |
| NRRL824 | *MAT-2* | Fleming strain (Charles Thom collection) | CBS fungus database, The Netherlands |
| DSM62858 | *MAT-2* | Contamination from optical glass | DSMZ, Germany |
| DAOM155628 | *MAT-2* | Contamination from paper; Canada | 35 |
| DAOM216701 | *MAT-2* | *Sesanum indicum*; Korea | 35 |
| DAOM59494C | *MAT-2* | Substrate unknown; Honduras | 35 |

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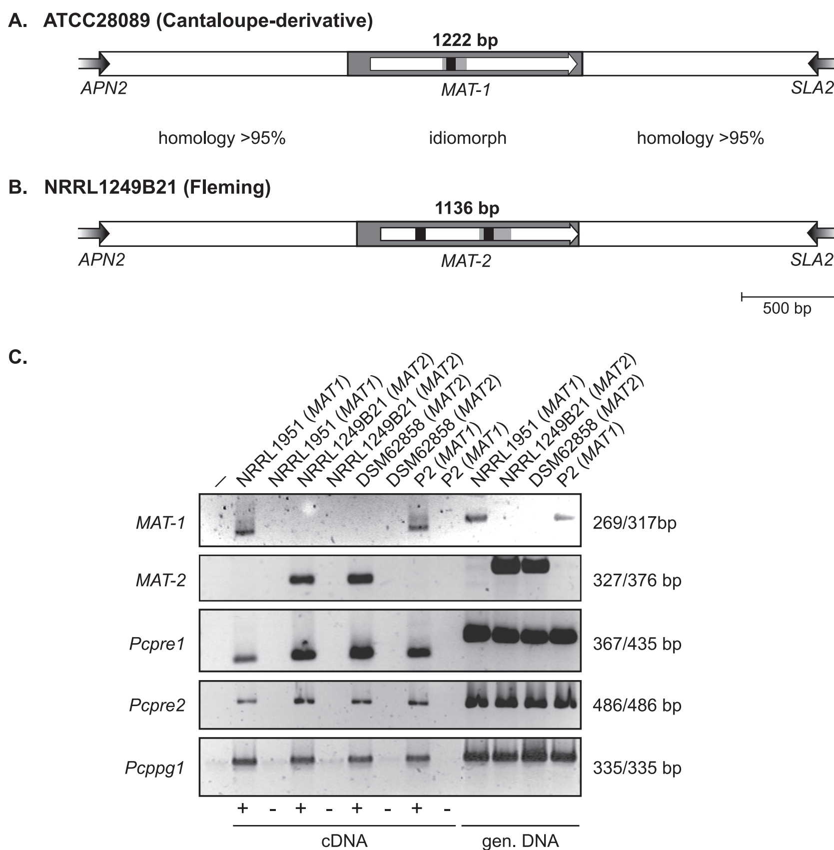


FIG. 2. Mating-type locus of *Penicillium chrysogenum*. (A and B) Schematic illustration of the mating-type idiomorphs and their flanking regions. Idiomorphs are presented as gray boxes and their flanking regions as white boxes. The positioning and transcriptional direction of the mating-type genes in each idiomorph are indicated by an arrow, and introns are shown in black. (A) *MAT1* idiomorph of strain ATCC 28089 (accession no. AM904544). The relative position of the alpha-box domain of *MAT-1* is indicated. (B) *MAT2* idiomorph 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.

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* idiomorphs (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 idiomorphs of the human pathogens *Histoplasma capsulatum*, *Coccidioides immitis*, and

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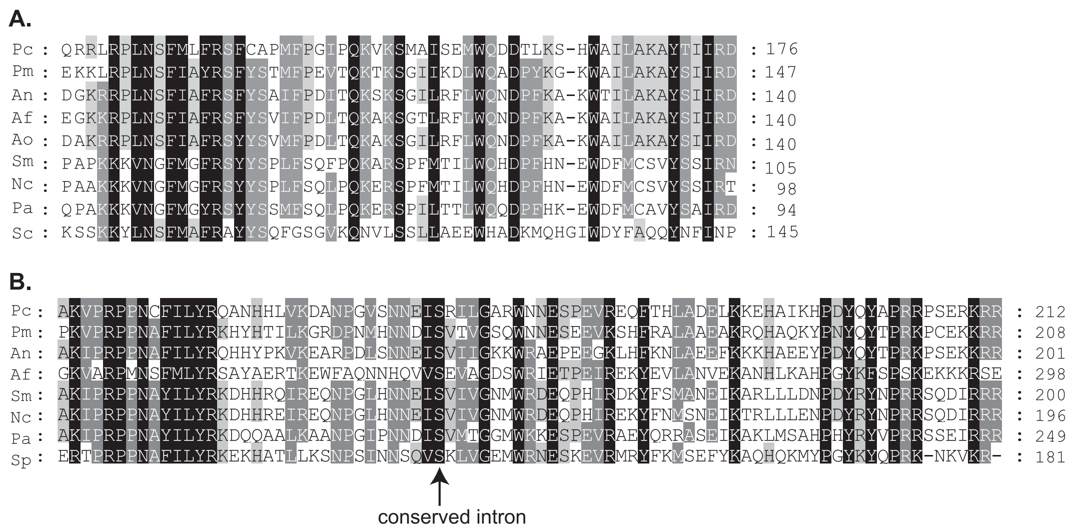


FIG. 3. Conserved domains of mating-type proteins from *P. chrysogenum*. (A) Multiple alignment of the alpha-box region of the *P. chrysogenum* MAT1 protein with alpha-box proteins from other ascomycetes. Abbreviations and accession numbers (Ac.) are as follows: Pc, *Penicillium chrysogenum* (Ac. AM904544); Pm, *Penicillium marneffei* (Ac. Q1A3S7); An, *Aspergillus nidulans* (Ac. Q7Z896); Af, *Aspergillus fumigatus* (Ac. AAX83123.1); Ao, *Aspergillus oryzae* (Ac. Q2U537); Sm, *Sordaria macrospora* (Ac. O42837); Nc, *Neurospora crassa* (Ac. P19392); Pa, *Podospora anserina* (Ac. P35692); Sc, *Saccharomyces cerevisiae* (Ac. YP_087100.1). (B) Multiple alignment of the HMG domain of the *P. chrysogenum* MAT2 protein with HMG mating-type proteins from other ascomycetes. For abbreviations and accession numbers, see panel A; also, for Pc,Ac. AM904545; for Pm, Ac. ABC68485.1; for An, Ac. Q7Z8M2; for Af, Ac. XP_751590.1); for Sm, Ac. CAA71624.1; for Nc, Ac. P36981; for Pa, Ac. P35693; for Sp (*Schizosaccharomyces pombe*), Ac. P10840. The position of a conserved intron is indicated by an arrow.

*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*, *P. marneffei*, *C. immitis*, and *C. posadasii* (16, 21, 39), no additional ORFs could be identified within the mating-type idiomorphs of *P. chrysogenum*.

**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, 25, 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, 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 Pc*ppg1*. Within the polypeptide encoded by this gene, two identical repeats could be identified. The two repeats encode the decapeptide amino acid sequence KWCGHIGQGC (Fig. 4). The decapeptide sequence bears strong similarity to pheromones from other filamentous ascomycetes (31). Interestingly, a hydrophobic signal sequence was detected within the N terminus of *P. chrysoge-*

***MKFTSVVVAVIAAGTVQA***AALAPSETLPKWCGHIGQGC**KR**

TTDASLDVKRSADALAEAMAGGLPLVLQKWCGHIGQGCYK

A**KR**AADAVDEV**KR**TSDALARAFAALEEEDDE

FIG. 4. Amino acid sequence of the peptide pheromone from *P. chrysogenum* (accession no. AM904541). Repeats are shown in white and boxed in black, Kex2 processing sites (KR) indicated in bold, putative STE13 processing sites are underlined, and the hydrophobic leader sequence is indicated in bold italics.

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*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 Pc*ppg1* gene, we were able to identify two pheromone receptor genes from *P. chrysogenum*. The gene products of Pc*pre1* and Pc*pre2* are predicted to have seven transmembrane spanning domains and displayed a high level of amino acid identity with the a-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, teleomorphs 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.

## ACKNOWLEDGMENTS

We thank Ingeborg Godehardt, Kerstin Kalkreuter, and Stefanie Mertens for technical assistance and Hubert Kürnsteiner, Thomas Specht, and Ivo Zadra (Kundl, Austria) for helpful discussion.

We acknowledge financial support from Sandoz GmbH (Kundl, Austria) and from the Christian Doppler Society (Vienna, Austria).

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