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Identification of a Polyketide Synthase Involved in Sorbicillin Biosynthesis by *Penicillium chrysogenum*

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ABSTRACT

Secondary metabolism in *Penicillium chrysogenum* was intensively subjected to classical strain improvement (CSI), the resulting industrial strains producing high levels of β-lactams. During this process, the production of yellow pigments, including sorbicillinoids, was eliminated as part of a strategy to enable the rapid purification of β-lactams. Here we report the identification of the polyketide synthase (PKS) gene essential for sorbicillinoid biosynthesis in *P. chrysogenum*. We demonstrate that the production of polyketide precursors like sorbicillinol and dihydrosorbicillinol as well as their derivatives bisorbicillinoids requires the function of a highly reducing PKS encoded by the gene *Pc*21g05080 (*pks13*). This gene belongs to the cluster that was mutated and transcriptionally silenced during the strain improvement program. Using an improved β-lactam-producing strain, repair of the mutation in *pks13* led to the restoration of sorbicillinoid production. This now enables genetic studies on the mechanism of sorbicillinoid biosynthesis in *P. chrysogenum* and opens new perspectives for pathway engineering.

IMPORTANCE

Sorbicillinoids are secondary metabolites with antiviral, anti-inflammatory, and antimicrobial activities produced by filamentous fungi. This study identified the gene cluster responsible for sorbicillinoid formation in *Penicillium chrysogenum*, which now allows engineering of this diverse group of compounds.

Sorbicillinoids are the diverse group of yellow pigments produced by *Trichoderma* (1), *Aspergillus* (2), *Verticillium* (3), *Streptomyces* (4), and *Penicillium* (5) species. Sorbicillin (Fig. 1, compound 1) was the first characterized sorbicillinoid, initially isolated from *Penicillium notatum* as a contaminant during the production of clinical penicillins (5). The typical hexaketide structure of this molecule is a core scaffold for more than 30 monomeric and dimeric derivatives isolated from different environments (6). The oxidative dimerization of sorbicillinol (compound 2), a hydroxylated derivative of sorbicillin (7), can be achieved via Diels-Alder or Michael type oxidative dimerization reactions (8), leading to structural diverse bioactive compounds with interesting bioactive properties. For instance, radical scavenging properties have been assigned to the bisorbicillinoids like bisorbicillinol (compound 5), bisvertinoquinol (compound 9), and bisorbibutenolide (compound 10) (9). The group of trichodimerols (compound 11) shows antiviral and anti-inflammatory activities by inhibiting the prostaglandin H synthase 2 and tumor necrosis factor alpha (TNF-α) in human peripheral blood monocytes (8). Bisvertinols (compound 12) are equipped with antimicrobial activity through inhibition of 1,6-glucan biosynthesis in the plant pathogen *Phytophthora capsici* (10). Recently, a sponge-associated *Penicillium chrysogenum* E01-10/3 strain was isolated that showed under optimized cultivation conditions the production of large quantities of sorbicilactone A/B (compounds 13 and 14). These have anti-HIV properties and show cytotoxic effect against L5178y leukemic cells (11, 12).

The remarkable bioactive potential of sorbicillinoids has raised interest in their biosynthetic origin (13). Feeding experiments with radiolabeled acetate indicate that hexaketide molecules act as precursors for the corresponding sorbicilactones (11). For the biosynthesis of these precursors, the involvement of a highly reducing (HR) polyketide synthase (PKS) enzyme and a nonreducing (NR) PKS enzyme was proposed, and a putative biosynthetic gene cluster was suggested for *Penicillium* E01-10/3 (Fig. 2A) (14, 15). An orthologous gene cluster exists in *P. chrysogenum* and consists of seven genes encoding two fungus-specific transcriptional factors (Orf1 and Orf5), an oxidase (Orf7), two oppositely transcribed NR and HR PKS enzymes (SorB and SorA), a transporter (Orf6), and a monooxygenase (SorC). The latter enzyme of *Penicillium* E01-10/3 was expressed in *Escherichia coli* and shown to catalyze the hydroxylation of the sorbicillin and dihydrosorbicillin, yielding sorbicillinol and dihydrosorbicillinol, respectively (7). The proposed hypothetical biosynthesis pathway suggests that a triacetic product of the HR PKS serves as the substrate for the starter unit acyltransferase (SAT) domain of the second NR PKS enzyme. Upon three iterative malonyl coenzyme A (malonyl-
CoA) extensions, the methylated hexaketide might be reductively released from the PKS as an aldehyde, and upon cyclization, sorbicillin and/or dihydrosorbicillin are formed. The latter intermediate is presumably derived from a triketide precursor, wherein the first enoyl reduction during chain extension by the HR PKS is omitted (Fig. 2B). Although these studies provide a glimpse at the possible mechanism of sorbicillinoid biosynthesis, the direct involvement of the PKS enzymes has not been demonstrated.

Unlike natural isolates of *P. chrysogenum*, strains with an improved penicillin production, like Wisconsin 54-1255 and its derivatives, are not capable of sorbicillinoid production. Transcriptional profiling of secondary metabolite genes in related strains of a lineage of improved β-lactam producers indicate the presence of a PKS gene cluster that was silenced during classical strain improvement (CSI) (log$_2$ fold change, −4.4 and −4.7 for *pks12* and *pks13*, respectively) (16). In contrast, the progenitor strain NRRL1951, which still produces sorbicillinoids, exhibits the highest transcriptional level of the corresponding gene cluster (16). In addition, mutations emerged in each of the putative PKS enzymes that likely led to an inactivation. These findings suggest a complex mechanism for the elimination of the biosynthetic pathway of sorbicillinoids during the strain improvement program. Here we report on the identification of the PKS-encoding gene *pks13*, which is essential for sorbicillinoid biosynthesis, using the natural producer strain *P. chrysogenum* NRRL1951. Repair of the native nucleotide sequence of this gene in a strain that was derived from a high-β-lactam-producing strain resulted in the restoration of sorbicillinoid biosynthesis. This now allows the study of sorbicillinoid biosynthesis using standard molecular techniques, which was previously restricted due to the natural genetic background of sorbicillinoid-producing isolates.

**MATERIALS AND METHODS**

**Strains, media, and growth conditions.** The parental *P. chrysogenum* isolate NRRL1951 and its derivative penicillin gene cluster-free strain DS68530 were kindly provided by DSM Anti-Infectives (Delft, The Netherlands). YGG medium (18) was used to grow the fungus for genomic DNA (gDNA) extraction and protoplasting. Secondary metabolite production (SMP) medium (17) was used for secondary metabolite analysis. All growth experiments were performed in shaken flasks at 25°C and 200 rpm. Positive selection of the transformants was performed on acetaldehyde (AMDS) agar medium supplemented with acetamide as the nitrogen source. Solid SMP medium supplemented with agar was used for the rapid selection of the pigment-producing fungal colonies. R-agar sporulation medium was used for purification of the transformants and preparation of the rice batches for the long-term storage of the conidia (18).
Construction of the Pc21g05080 gene inactivation strain. A deletion plasmid for the Pc21g05080 gene was constructed using the modified Gateway cloning protocol (Invitrogen). 3' and 5' homologous regions for the deletion cassette were amplified with the PCR master mix with the primers 1 to 4 listed in Table 1 using genomic DNA of strain DS68530 as a template. The obtained fragments were cloned into the corresponding donor vectors pDONR P4-P1R and pDONR P2R-P3 with BP clonase II enzyme mix (Invitrogen). The resulting plasmids were isolated from kanamycin-resistant E. coli DH5α/H9251 transformants. Constructs were further used for an in vitro recombination reaction with the destination vector pDEST-amdS (LR clonase II enzyme mix) resulting in the isolation of the final pKO13 deletion plasmid from ampicillin-resistant E. coli transformants. Before transformation into protoplasts of P. chrysogenum NRRL1951, the deletion cassette was linearized with the AjiI and SapI restriction nucleases (Thermo Scientific). For the targeted integration of the amdS (acetamidase-encoding gene) marker into the locus of the pks17 (Pc21g16000) gene, plasmid pKO17 (O. Salo, unpublished data) was used as the template for the amplification of the cassette using primer pair 9/10 (Table 1). Transformation and screening. Protoplastation and transformation of the fungal mycelia were done as described previously (18). Transformants were obtained by inoculation of single conidia on AMDS selective medium followed by a sporulation step on R-agar plates. After two sporulation phases, fungi were grown on rice for inoculation and long-term storage of the conidia. The correct knockout strain was selected by colony PCR analysis using Phire Hot Start II DNA polymerase (Thermo Scientific).

TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Primer sequence (5’→3’)</th>
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<tbody>
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</tr>
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<td>2</td>
<td>attB1RPc21g05080</td>
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</tr>
<tr>
<td>3</td>
<td>attB2FPc21g05080</td>
<td>GGGGACAGCTTTCTTGTACAAAGTGGGGTCATGTCCGAGAAGCTGTC</td>
</tr>
<tr>
<td>4</td>
<td>attB3RPc21g05080</td>
<td>GGGGACAACTTTGTATAATAAAGTTGCGCCCTTGTTGAAAGGCTCC</td>
</tr>
<tr>
<td>5</td>
<td>pks13F</td>
<td>GGCCGCCATGACAGACTCAGAC</td>
</tr>
<tr>
<td>6</td>
<td>pks13R</td>
<td>CACCGGTCACTGTACAGAGCAGCTCG</td>
</tr>
<tr>
<td>7</td>
<td>Probe 13 F</td>
<td>GTTCATGTCCGAGAACGCTG</td>
</tr>
<tr>
<td>8</td>
<td>Probe 13 R</td>
<td>CGCCCTTTGTTGAAAGGCTCC</td>
</tr>
<tr>
<td>9</td>
<td>pks17F</td>
<td>AATTGATACCTTTGATGATATTTCATCCTCACC</td>
</tr>
<tr>
<td>10</td>
<td>pks17R</td>
<td>ATTTGGCCGCCCGAGAATGGAGACT</td>
</tr>
<tr>
<td>11</td>
<td>Fw-NRRLpks12</td>
<td>GCACGTGCTGATATTCAATG</td>
</tr>
<tr>
<td>12</td>
<td>RV-NRRLpks13</td>
<td>CTTGGTTGAGCATCGATTC</td>
</tr>
</tbody>
</table>
The mycelium of the transformants was homogenized in 20 μl of Milli-Q water, and 2 μl of the cell suspension was used immediately for PCR validation with the forward primer (primer 5) that anneals outside the recombination region, while the reverse primer (primer 6) was amds marker specific (Table 1). The presence of an expected 1.6-kb PCR product was used to select the correct homologous recombinants.

Southern analysis. The downstream region of the Pc21g05080 gene was used as a probe and amplified by PCR with primer set 7/8 (Table 1). The probe was labeled with digoxigenin (DIG) using the HighPrime kit (Roche Applied Sciences, The Netherlands). gDNA (10 μg) was digested with the NdeI (Thermo Scientific) restriction enzyme and separated on 0.8% agarose gel. After equilibration in 20X SSC buffer (3 M sodium chloride, 0.3 M sodium citrate), the DNA was transferred overnight onto Zeta-probe positively charged nylon membranes (Bio-Rad). Blots were treated with anti-DIG–alkaline phosphatase antibodies (Sigma) and supplemented with CDP-star (Roche Applied Science, The Netherlands). The fluorescence signal was measured with a LumI Imager (Roche Applied Science, The Netherlands).

Restoration of pks12 and -13 genes. To repair the mutations in the Pc21g05050 and Pc21g05080 genes in strain D68530, a DNA fragment of 9,769 bp covering the two point mutations in these genes was amplified by PCR from genomic DNA from strain NRRL1951 using the primer set 11/12 listed in Table 1. The DNA fragment was cloned in the vector pJE1.2/blunt (Thermo Scientific CloneJET PCR cloning kit) according to the manufacturer’s instructions. The obtained plasmid was purified from ampicillin-resistant E. coli DH5α and used as a new DNA template with the primers described above. The amplified DNA fragment was used along with the amds selection marker in a cotransformation of protoplast isolated from P. chrysogenum D68530 using a standard protocol (18). The amds gene was under the control of the gpDA promoter of Aspergillus nidulans. This cassette marker was amplified from plasmid pKO17 by PCR using the primer set listed in Table 1, yielding a 1,000-bp overhang size in the two flanks of the fragment to target the polyketide synthase gene Pc21g16000, which is responsible for green pigment formation. The screening for transformants was performed on plates supplemented with acetamide as a unique nitrogen source among the mutants that had lost green pigmentation. Individual colonies were grown on solid SMP medium and examined for the formation of a yellow halo. Positive colonies were grown in 10 ml SMP using shaken flasks at 25°C and 200 rpm. After 5 days of shaking, the flasks were supplemented with 8 ml with fresh SMP, and growth was continued for 4 days, whereupon yellow pigment formation was verified visually and by liquid chromatography-mass spectrometry (LC-MS) as described below. Restoration of the mutations in Pc21g05050 and Pc21g05070 was confirmed by sequencing of the gDNA of the clones, using gDNA from strain D68530 as a control.

Metabolite analysis. Spent medium of fungal cultures on SMP medium was collected after 3, 5, and 7 days of growth and subjected to secondary metabolite analysis. Samples were filtered with a 2-μm-pore polytetrafluoroethylene (PTFE) syringe filter and stored at −80°C. LC-MS analysis was performed using Accela1250 high-performance liquid chromatography (HPLC) system coupled with the benchtop ES-MS Orbitrap Exactive (Thermo Fisher Scientific, San Jose, CA). A sample of 5 μl was injected into Shim-pack XR-ODS C18 column (3.0 by 75 mm, 2.2 μm) (Shimadzu, Japan) operating at 40°C and a flow rate of 300 μl/min. The linear gradient began with 90% of solvent A (100% water) and 5% of solvent C (100% acetonitrile) starting after 5 min of isocratic flow. The first linear gradient reached 60% of C at 30 min and the second 95% of C at 35 min. The washing step for 10 min at 90% of solvent C was followed by the column equilibration for 15 min at initial isocratic conditions. Solvent D (2% formic acid) was continuously used to maintain the final 0.1% of formic acid in the system. The column flux was directed to the Exactive ES-MS Orbitrap operating at the scan range (m/z 80 to 1,600 Da) and switching between positive and negative modes. Voltage parameters for the positive mode were 4.2 kV for spray, 87.5 V for capillary, and 120 V for tube lens. Voltage parameters for negative mode were 3 kV for spray, −50 V for capillary, and −150 V for tube lens. The capillary temperature of 325°C and a sheath gas flow of 60 arbitrary units (AU) was used. The auxiliary gas was off to maintain a high detection sensitivity for both positive and negative modes during analysis. The differential analysis of the LC-MS samples was performed using the Thermo Scientific SIEVE software.

NMR. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 700-MHz or 600-MHz spectrometer. Sample temperatures ranged from 250 to 300 K. The assignments were achieved by means of one-dimensional (1D) 13C correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) spectra. Samples were dissolved in CDCl3, and in CDCl3, with one drop of pyridine to neutralize acidic impurities in the chloroform.

Nucleotide sequence accession number. The nucleotide sequence of pks12 and pks13 in the strain D685630 has been submitted to the GenBank database under accession no. KU955361.

RESULTS

Deletion of pks13 in P. chrysogenum NRRL1951. P. chrysogenum NRRL1951 is a low-β-lactam-producing strain that has not been subjected to extensive classical strain improvement (CSI) and that secretes yellow pigments into the medium. These pigments originate from the hexaketide sorbicillin, but the polyketide synthase responsible for its production has not been identified. Previously, we demonstrated that sorbicillin-related metabolites are produced early during fermentation, which is accompanied with the expression of a PKS gene cluster of unknown function (16). Protein BLAST analysis indicated that the particular cluster consists of the two PKS genes (Pc21g05070 [pks12] and Pc21g05080 [pks13]), two transcription factor genes (Pc21g05050 [reg50] and Pc21g05090 [reg90]), a monooxygenase gene (Pc21g05060 [mox60]), a transporter gene (Pc21g05100 [mfs100]), and an oxidoreductase gene (Pc21g05110 [ox110]) (Fig. 2A). The predicted product of pks12 is a 2,581-amino-acid-long nonreducing iterative type 1 polyketide synthase showing the highest (64%) identity to an unknown PKS gene of Trichoderma reesei and 43% identity to citrinin biosynthesis gene of Monascus purpureus (19). The neighboring pks13 gene encodes a 2,664-amino-acid-long highly reducing polyketide synthase with 65% identity to an unknown PKS of T. reesei and 48% identity to the lovastatin diketide synthase LovF of Aspergillus terreus (19). In the derived strains P. chrysogenum Wisconsin 54–1255 and DS17690, the two PKS genes within the aforementioned cluster acquired mutations during CSI, and this may have led to their functional inactivation. In the nonreducing PKS12, the isolectine at position 2210 located at the interdomain region of methyltransferase (MT) and thioesterase (TE) domains is substituted for an arginine. The second mutation is present within the ketosynthase domain of highly reducing PKS13, causing a leucine-to-phenylalanine substitution at position 146 (Fig. 2A; see Fig. S1 in the supplemental material). Both mutations occurred early during the CSI and have been inherited by the Wisconsin 54–1255 strain and thus also by later improved β-lactam producers (16). Since the CSI-derived strains of P. chrysogenum are not able to produce yellow pigments, the natural isolate NRRL1951 was chosen as the host in our study. To functionally characterize the aforementioned cluster, the PKS-encoding gene pks13 was targeted for inactivation by homologous recombination by replacing it with the amds marker for acetamide selection. Gene targeting in the NRRL1951 strain occurs with a very low efficiency due to the predominance of nonhomologous
The empirical formulas of these compounds were calculated based on the detected accurate mass (<2 ppm). The major compound produced after 3 days of growth has a retention time (RT) of 21.15 min and m/z [H]+ of 249.11, with the calculated empirical formula C14H16O4, which corresponds to sorbicillinol (compound 2). The second compound with an RT of 23.47 min and an m/z [H]+ of 251.13 has the empirical formula C14H18O4 and corresponds to dihydrosorbicillinol (compound 3). Both masses were found to be part of the tandem mass spectrometry (MS/MS) fragmentation pattern of compounds 4, 6, and 5, respectively, while compound 2 shows a corresponding fragmentation pattern overlapping with compound 3. This indicates that compound 3 is composed of compounds 1 and 2. A complete list of the unique masses related to the pks13 deletion is shown in Table 2. To confirm that the compounds eliminated from secondary metabolism of the NRRL1951 Δpks13 mutant indeed belong to the class of sorbicillinoids, metabolite 5 was isolated by means of preparative HPLC, and its structure was verified by NMR. The isolated fraction was dissolved in CDCl3, as well as in CD3CN with one drop of pyridine-d5 with the purpose to neutralize traces of acid. In the presence of acid, metabolite 5 occurred in two tautomeric forms and suffered from a slow degradation, which was not the case in the neutralized NMR sample. 2D spectra were recorded from both NMR samples, and careful interpretation led to the conclusion that metabolite 5 corresponds to bisorbicillinol. All 1H- and 13C-NMR chemical shifts agree very well with the data reported previously (9). In addition, these authors also observed the presence of the tautomeric equilibrium, albeit in a slightly different way. They observed two compounds after derivatization with diazomethane and concluded that this was the result of a tautomeric equilibrium. More details of the assignment and NMR data are given in the supplemental material.

Overall, these results indicate that pks13 is essential for the production of the polyketide precursors for the biosynthesis of sorbicillinoids and their derivatives by the P. chrysogenum strain NRRL1951.

Recovery of sorbicillinoid biosynthesis in the improved β-lactam-producing strain DS86830. In order to define the functional role of the mutations accumulated by the improved penicillin-producing strains during the CSI, we aimed to restore the native amino acid sequence of the PKS enzymes mutated during the CSI in the industrially improved strain DS86830. Strain DS86830 is a derivative of strain DS17690 in which multiple β-lactam gene clusters have been removed genetically (20). This ensures a secondary metabolite profile that is not further dominated by the presence of β-lactams. The nucleotide sequence of pks12 and pks13 in the improved strain DS86830 (GenBank accession no. KU955361) is identical to that in NRRL1951 (GenBank accession no. KU955360), except for the aforementioned mutations in the structural genes. A DNA fragment of the 9.7-kb region of the oppositely oriented pks12 and pks13 genes was amplified from the genomic DNA of the parental strain NRRL1951. This allowed the recovery of the native nucleotide sequence of the mutated pks13 by a homologous recombination event. For positive selection of transformants, the amdS selection marker was cotransformed and targeted to the open reading frame of the naphthapyrone synthase gene (Pc21g16000) that is essential for the green conidial pigment biosynthesis in P. chrysogenum (Salo et al., unpublished). The albino phenotype of the amdS-carrying mutants provided an additional control over the purity of the trans-
formants grown on the media without selective pressure. Mutants able to grow on acetamide-supplemented medium and deficient in green coloring of the conidia were selected after three rounds of sporulation on R-agar and selection on AMDS medium. The mycelium of the obtained candidates was inoculated on SMP-agar medium to perform a qualitative selection of the yellow pigment-producing clones. The 15-kb genome region carrying both clustered $pks12$ and $pks13$ genes was amplified by PCR and sequenced, and this confirmed the correct reversion of the mutation in the $pks13$ gene. The intergenic region between two PKS-encoding genes was amplified by PCR and sequenced, and this confirmed the correct reversion of the mutation in the $pks13$ gene.

**TABLE 2** Comparative metabolite profiling for sorbicillin-related compounds in the culture broth of strains NRRL1951, NRRL1951 $\Delta pks13$, DS68530, and DS68530 Res13$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Formula</th>
<th>Acquired [M+H]$^+$</th>
<th>RT (min)</th>
<th>Expression of compound by$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sorbicillin</td>
<td>C$<em>{14}$H$</em>{16}$O$_3$</td>
<td>233.12</td>
<td>30.80</td>
<td>NRRL1951</td>
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<tr>
<td>2</td>
<td>Sorbicillinol</td>
<td>C$<em>{14}$H$</em>{18}$O$_4$</td>
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<td>21.15</td>
<td>0.88</td>
</tr>
<tr>
<td>3</td>
<td>Dihydrosorbicillinol</td>
<td>C$<em>{14}$H$</em>{17}$O$_4$</td>
<td>251.13</td>
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<tr>
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<td>C$<em>{14}$H$</em>{16}$O$_5$</td>
<td>265.11</td>
<td>26.90</td>
<td>13.93</td>
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<td>5</td>
<td>Bisorbicillinol</td>
<td>C$<em>{12}$H$</em>{16}$O$_6$</td>
<td>497.22</td>
<td>29.43</td>
<td>0.32</td>
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<td>Bisvertinolone</td>
<td>C$<em>{12}$H$</em>{18}$O$_8$</td>
<td>513.21</td>
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<td>7</td>
<td>Dihydrobisvertinolone</td>
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<td>515.23</td>
<td>35.62</td>
<td>12.18</td>
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<td>Tetrahydrobisvertinolone</td>
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<td>34.14</td>
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</table>

$^a$ The retention time (RT) on LC-MS and the calculated empirical formula are indicated.

$^b$ The data in these columns are response ratios (see reference 17). --, not found.
The corresponding DS68530 Res13 strain was used for the secondary metabolite production analysis. LC-MS analysis of SMP medium-grown cultures revealed the accumulation of novel metabolites in the culture medium of strain DS68530 Res13 (Fig. 4B). These were sorbicillin (compound 1), sorbichenin (compound 2), dihydrosorbicillinol (compound 3), oxosorbicillinol (compound 4), bisorbenolin (compound 5), bisvertinolone (compound 6), tetrahydrosvertinolone (compound 7), and dihydrosverbiniolone compound 8 (Table 2). In addition, a set of new compounds, 15 to 21, that were previously found in the culture broth of NRR1951 strain were also observed in the medium of the DS68530 mutant. The fragmentation pattern shows that they are related to the sorbicillinoids, but their structures are unknown. These data demonstrate that the reversion of the mutation in pks13 suffices to restore sorbicillinoid production in a classical strain improvement-selected P. chrysogenum strain.

**DISCUSSION**

The yellow pigments sorbicillinoids are a large group of structurally related metabolites produced by many fungi (1, 3, 5, 11, 21–24). The polyketide origin of these compounds was demonstrated by radiolabeled acetate feeding experiments (11), but the genes involved in the biosynthesis of these secondary metabolites remained unknown. Here we deleted the pks13 (Pc21g05080) gene located in a highly expressed gene cluster of P. chrysogenum NRR1951 to elucidate its role in secondary metabolism of this fungus. pks13 belongs to a cluster of seven genes, among which is a second PKS-encoding gene that is oppositely transcribed, namely, pks12 (Pc21g05070) (Fig. 2A). A related gene cluster was implicated in the biosynthesis of sorbicillactone A/B in the marine isolate E01-10/3 (7, 11), but direct evidence for the involvement of the PKS enzymes was not demonstrated. As shown in our previous work (16), this complete gene cluster is highly expressed in the parental strain NRR1951 and is transcriptionally silenced in the improved β-lactam producer Wisconsin 54-1255 and its derivatives. The mechanism of the transcriptional silencing of this gene cluster is unknown, although the CSI-improved strains also accumulated mutations in the Velvet complex (16) that may have impacted the expression of these secondary metabolite genes. However, the functional inactivation in the CSI-improved P. chrysogenum strains can be assigned to mutagenesis events at the early stages of CSI. Each of the PKS-encoded genes carries a single nucleotide polymorphism that causes amino acid substitution: i.e., at the intradomain region of PKS12 and within the KS domain of the PKS13, respectively. No additional mutations were detected within the intergenic region of the clustered PKS genes or the entire genomic region of the corresponding gene cluster. Importantly, the leucine residue at position 146 that was substituted for a phenylalanine during the CSI is conserved within the KS domain. pks13 is processed or is not reverse the mutation in pks13. Possibly, the DNA fragment that covered the relevant parts of pks12 and pks13 is processed or fragmented during the transformation of the fungal protoplasts. Nevertheless, the reversal of the mutation in PKS13 sufficed to restore sorbicillinoid biosynthesis. In the culture broth of the restored strain, sorbicillin (compound 2) and dihydrosorbicillinol (compound 3) as well as further derived sorbicillinoids were readily detected. This includes compounds 1 and 4 to 8 as well as structurally unknown intermediates (compounds 15 to 22) (Table 2) that, based on the fragmentation patterns in LC-MS/MS, can be classified as sorbicillinoids. Importantly, the concentration of the sorbicillinoids in the culture medium of the DS68530 Res13 strain is significantly lower than that in the NRR1951 wild type (Table 2), and this likely relates to the lower expression of these genes in DS68530 Res13 and/or a reduced activity of PPS12 that still carries the reductive release of hexaketide precursors as in aldehydes, and this is essential for the formation of sorbicillin/dehydrosorbi- cillin that occurs through cyclization (7, 14). Further reduction to sorbicillin/dihydrosorbicillin and oxidative dimerization leads to the formation of the vast group of sorbicillinoid derivatives.

In conclusion, our work identified pks13 (Pc21g05080) as a key gene in the biosynthesis of sorbicillinoids and derivatives in P. chrysogenum. We restored the production of the sorbicillin-related metabolites in a genetic background of an improved penicillin-producing strain that is adapted for growth under industrial conditions and for which a genetic toolbox is available. This will simplify the application of the molecular cloning techniques for studying of sorbicillinoid biosynthesis and opens new perspectives to engineer this pathway for the production of individual sorbicillinoids.
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