Pathway for the Biosynthesis of the Pigment Chrysogine by *Penicillium chrysogenum*

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ABSTRACT Chrysogine is a yellow pigment produced by *Penicillium chrysogenum* and other filamentous fungi. Although the pigment was first isolated in 1973, its biosynthetic pathway has so far not been resolved. Here, we show that deletion of the highly expressed nonribosomal peptide synthetase (NRPS) gene *Pc21g12630* (*chyA*) resulted in a decrease in the production of chrysogine and 13 related compounds in the culture broth of *P. chrysogenum*. Each of the genes of the *chyA*-containing gene cluster was individually deleted, and corresponding mutants were examined by metabolic profiling in order to elucidate their function. The data suggest that the NRPS ChyA mediates the condensation of anthranilic acid and alanine into the intermediate 2-(2-aminopropanamido)benzoic acid, which was verified by feeding experiments of a Δ*chyA* strain with the chemically synthesized product. The remainder of the pathway is highly branched, yielding at least 13 chrysogine-related compounds.

IMPORTANCE *Penicillium chrysogenum* is used in industry for the production of β-lactams, but also produces several other secondary metabolites. The yellow pigment chrysogine is one of the most abundant metabolites in the culture broth, next to β-lactams. Here, we have characterized the biosynthetic gene cluster involved in chrysogine production and elucidated a complex and highly branched biosynthetic pathway, assigning each of the chrysogine cluster genes to biosynthetic steps and metabolic intermediates. The work further unlocks the metabolic potential of filamentous fungi and the complexity of secondary metabolite pathways.

KEYWORDS *Penicillium chrysogenum*, chrysogine, filamentous fungi, pigment, secondary metabolites

*P*enicillium chrysogenum* and several other filamentous fungi produce the yellow pigment chrysogine (1, 2). Pigments are known to protect microorganisms against adverse environmental conditions, such as ultraviolet (UV) radiation, and often these compounds also exhibit antimicrobial activity (3). The function of chrysogine has not been extensively investigated, but unlike many other pigments it lacks antimicrobial or anticancer activity (4). *N*-pyruvoylanthranilamide [2-(2-oxopropanamido)benzamide], a related compound produced by *P. chrysogenum* (5) and also identified in *Colletotrichum lagenarium*, is equipped with antiauxin activity (6).

Chrysogine was first isolated in 1973 by Hikino et al. (5), who observed increased

TABLE 1 Production of chrysogine and related metabolites from strain DS68530[a]

compound number	compound name	formula	acquired [M+H]+	RT (min)	DS68530 48 h	96 h
1	chrysogine	$C_{10}H_{10}O_2N_2$	191.08	12.02	185,02	236,37
2	2-(2-acetamidopropanamido)benzamide	$C_{12}H_{15}O_3N_3$	250.12	10.77	19,85	53,92
3	2-(2-oxopropanamido)benzamide	$C_{10}H_{10}O_3N_2$	207.08	10.32	0,65	11,26
4	3-((1-((2-carbamoylphenyl)amino)-1-oxopropan-2-yl)amino)-3-oxopropanoic acid	$C_{13}H_{15}O_5N_3$	294.11	10.62	24,84	152,64
5	(1-((2-carbamoylphenyl)amino)-1-oxopropan-2-yl)glutamine	$C_{15}H_{20}O_5N_4$	337.15	8.60	4,92	0
6	chrysogine related	$C_{15}H_{18}O_6N_3$	336.12	9.02	0	2,44
7	chrysogine related	$C_{13}H_{12}O_5N_2$	277.08	11.06	0	0,82
8	2-(2-(2-carboxyacetamido)propanamido)benzoic acid	$C_{13}H_{14}O_6N_2$	295.09	15.20	0	1,12
9	chrysogine related	$C_{20}H_{20}O_6N_4$	413.14	14.95	0,42	0,68
10	chrysogine related	$C_{20}H_{20}O_6N_4$	413.14	15.70	0,42	0,57
12	3-oxo-3-((1-(4-oxo-1,4-dihydroquinazolin-2-yl)ethyl)amino)propanoic acid	$C_{13}H_{13}O_4N_3$	276.10	12.59	0,50	1,10
13	2-(2-((4-amino-1-carboxy-4-oxobutyl)amino)propanamido)benzoic acid	$C_{15}H_{19}O_6N_3$	338.13	12.65	2,91	0,20
14	2-(2-aminopropanamido)benzoic acid	$C_{10}H_{12}O_3N_2$	209.09	6.20	1,15	0,18
15	2-(2-aminopropanamido)benzamide	$C_{10}H_{13}O_2N_3$	208.11	2.47	0,17	0

[a]Numbers in the DS68530 columns represent the peak areas of the compounds corrected for the internal standard reserpine. The culture broth of strain DS68530 was analyzed after 48 and 96 h of growth in an SMP medium.

production upon feeding with anthranilic acid and pyruvic acid. The putative biosynthetic gene cluster has been identified in *P. chrysogenum* (7, 8) and includes a nonribosomal peptide synthetase (NRPS). Recently, Wollenberg et al. showed that a dimodular NRPS is responsible for chrysogine biosynthesis in *Fusarium graminearum* and also suggested a putative cluster (9) homologous to the respective gene cluster of *P. chrysogenum*. However, the actual biosynthetic pathway has remained elusive.

NRPSs are complex multimodular enzymes that use amino acids and carboxylic acids as substrates (10). The genome of *P. chrysogenum* contains 10 genes that encode NRPSs (11). Nonetheless, transcriptomic analysis performed on chemostat cultures of *P. chrysogenum* strain Wisconsin 54-1255 and the industrially improved DS17690 strain showed that only four of these NRPS genes are expressed (11). This set includes three NRPS genes that are involved respectively in the biosynthesis of penicillins (12), roquefortines (13), and hydrophobic cyclic tetrapeptides (14). The fourth highly expressed NRPS gene (7–9) is therefore potentially involved in the biosynthesis of chrysogine, which is among the most abundant secondary metabolites produced by this fungus. Furthermore, five genes flanking the *Pc21g12630* gene are also highly coexpressed, suggesting that they form a gene cluster (11).

Here, by overexpression and deletion of the core NRPS gene of the chrysogine pathway, deletion of the individual pathway gene, and by feeding experiments using chemically synthesized intermediates, we elucidate a complex and branched pathway of at least 13 compounds, assigning a function to each enzyme of the biosynthetic gene cluster.

RESULTS

Identification of chrysogine-related compounds. In order to identify the secondary metabolites produced by the NRPS Pc21g12630, this gene was deleted from *P. chrysogenum* DS68530 by homologous recombination. In this strain, the penicillin cluster is removed (15, 16), facilitating further identification of other secondary metabolites, as the metabolite profile is not dominated by β-lactams. The strain with the *Pc21g12630* gene deleted did not produce chrysogine and 13 other metabolites, here referred to as chrysogine-related compounds (Table 1). This identified Pc21g12630 as the NRPS responsible for chrysogine biosynthesis, and thus this gene was named *chyA*.

Compounds 1, 2, 3, 4, 8, and 13 were isolated by preparative high-performance liquid chromatography (HPLC), and their structures were determined by nuclear magnetic resonance (NMR) (see the supplemental material). Compound 1 was confirmed to be chrysogine, and compound 3 was identified as *N*-pyruvoylanthranilamide [2-(2-oxopropanamido)benzamide]. These compounds were first described in *P. chrysogenum* by Hikino et al. (5). Compound 2 was found to be *N*-acetylalanylanthranilamide
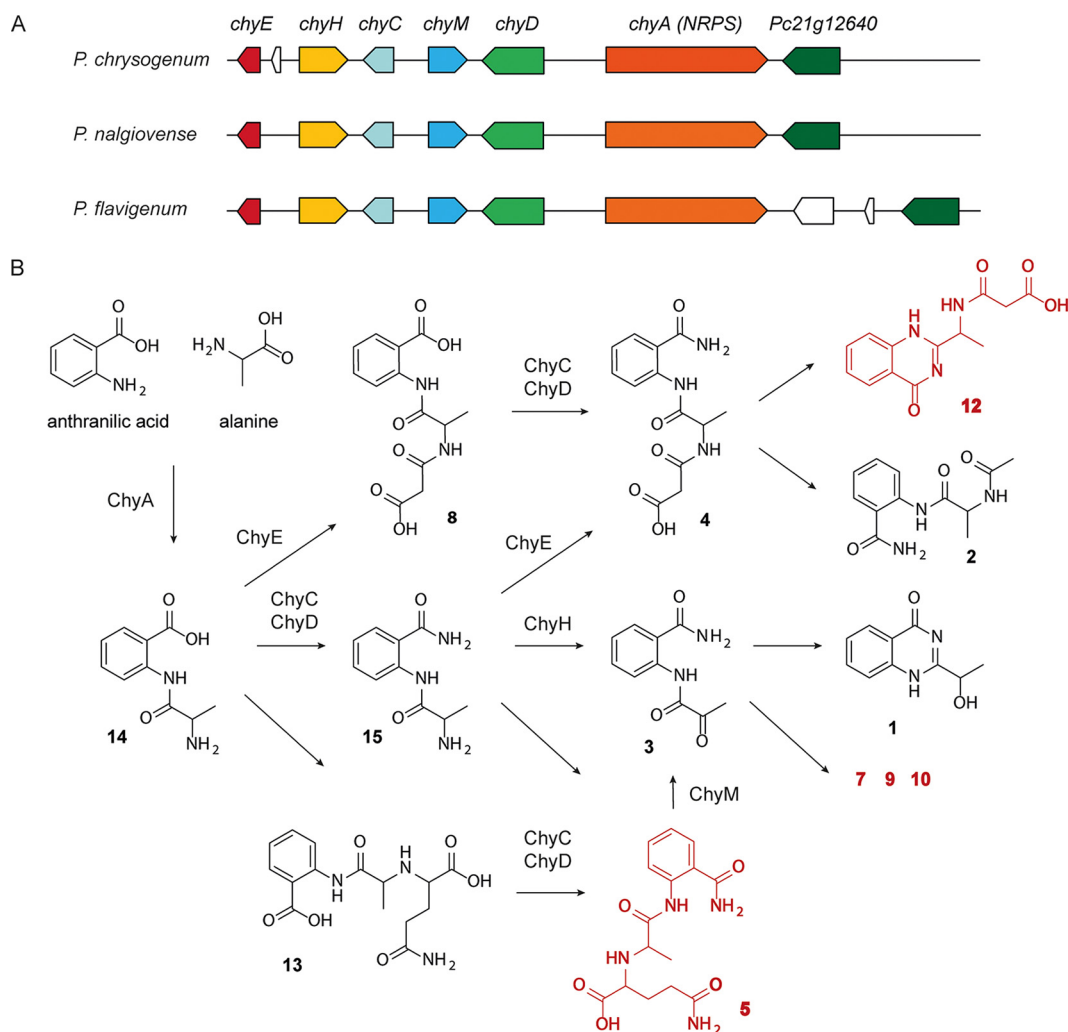
FIG 1 Representation of the chrysogine biosynthetic gene cluster and proposed pathway. The chrysogine biosynthetic gene cluster in *P. chrysogenum* and two other chrysogine-producing species. Genes with the same color have >80% identity. This study identified ChyA as the NRPS, ChyE as malonyl transferase, and ChyD as amidase; ChyC participates in amidation reactions, while ChyH and ChyM are involved in oxidation reactions. The substrates of ChyA and the compounds identified in this study are depicted in black, and the putative structures and uncharacterized compounds are represented in red.

[2-(2-acetamidopropanamido)benzamide], previously isolated from a marine *Penicillium* species (17). Compounds 4, 8, and 13 were identified as novel metabolites that are clearly related to chrysogine. The structures of compounds 14 [2-(2-aminopropanamido)benzoic acid] and 15 [the amidated form of compound 14, 2-(2-aminopropanamido)benzamidine] were further confirmed by the comparison of their HPLC retention times with those of the independently synthesized standards (see the supplemental material). The structures of compounds 5 and 12 were proposed based on their molecular formulas. We could not assign structures to compounds 6, 7, 9, and 10, which could not be isolated due to their low production.

Transcriptomic analysis performed on chemostat cultures of *P. chrysogenum* Wisconsin 54-1255 and the industrially improved DS17690 strain showed that five genes flanking *chyA* (*Pc21g12570*, *Pc21g12590*, *Pc21g12600*, *Pc21g12610*, and *Pc21g12620*) were also highly expressed, indicating that they could be part of the chrysogine gene cluster (11) (Fig. 1). Furthermore, quantitative PCR confirmed the expression of the above-listed genes in the DS68530 strain after 48 h of growth in a secondary metabolite production (SMP) medium (Fig. S2). Therefore, we tentatively assigned these as *chy* genes. *Pc21g12640*, found adjacent to the *chy* genes, exhibits a strong similarity to a
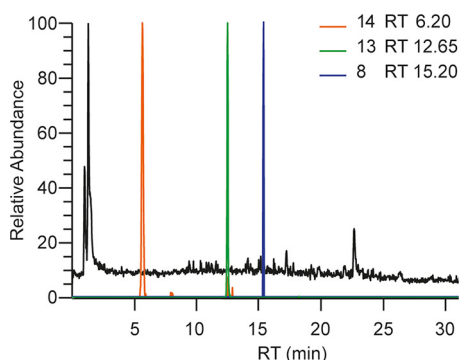
FIG 2 Chromatogram of culture broth from the *chyA*-expressing strain. Total ion chromatogram (TIC; black) and extracted ion chromatograms (EIC; colored) of secondary metabolites produced by the *chyA*-expressing strain after 48 h of growth in an SMP medium.

cutinase transcription factor beta from *Fusarium solani* (11). Although this gene was not significantly expressed in DS68530, the possible role of *Pc21g12640* as regulator of the cluster was also investigated.

Expression of the NRPS gene *chyA* in a chrysogine cluster deletion strain. In order to identify the products of the NRPS gene *chyA*, a chrysogine cluster deletion strain (8) was used to overexpress the *chyA* gene from an episomal AMA1-based plasmid. The *chyA* overexpression strain produced compounds 14, 8, and 13 (Fig. 2). It is likely that compound 14 is the immediate product of the NRPS and that this compound is derived from the condensation of anthranilic acid and alanine. Compounds 8 and 13 could be derived from compound 14 by addition of a malonyl and a glutaminyl group, respectively. Our data suggest an immediate branching of the pathway, where two groups of compounds are derived from compounds 8 and 13.

Metabolite profiles of *chy* gene deletion strains. The expression of *chyA* in a chrysogine cluster deletion strain allowed identification of the product of the NRPS and metabolites produced early in the pathway. To elucidate how the initial products were further modified by the enzymes of the cluster and to resolve the complete pathway, individual *chy* genes knockout strains were made, and metabolite profiling was performed (Table 2).

The deletion of *chyD* led to a depletion of most chrysogine-related metabolites— only compounds 14, 8, and 13 were accumulated during cultivation of this mutant. This suggests that ChyD is an early enzyme in the pathway, being responsible for converting compounds 14, 8, and 13 into downstream compounds. Based on its formula, we propose that compound 14 is converted into compound 15, which is its amidated form.

TABLE 2 Secondary metabolites of the chrysogine pathway in the knockout strains compared to that of the parental strain[a]

compound name	ΔchyC 48 h	ΔchyC 96 h	ΔchyD 48 h	ΔchyD 96 h	ΔchyE 48 h	ΔchyE 96 h	ΔchyH 48 h	ΔchyH 96 h	ΔchyM 48 h	ΔchyM 96 h	ΔPc21g12640 48 h	ΔPc21g12640 96 h
1	0,19	0,37	0	0	0,66	0,73	0,03	0,03	0,01	0,02	0,96	0,83
2	0,16	0,23	0	0	0	0,01	0,86	0,77	1,88	1,89	0,80	0,70
3	0,23	0,21	0	0	0,68	0,58	0	0,02	0	0	0,93	0,82
4	0,19	0,45	0	0	0	0,01	0,84	0,75	1,16	1,31	0,84	0,75
5	0	0	0	0	0,67	0,41	0,66	0	1,44	2,40	0,75	0,04
6	0	0,05	0	0	0	1,03	0	0,51	0	0,42	0	0,58
7	0	0,45	0	0,09	0	0,37	0	0	0	0	0	0,69
8	0	3,86	0,31	48,40	0	0,23	0	1,80	0	1,09	0	0,42
9	0	0	0	0	0,51	0,54	0	0	0	0	0,86	0,50
10	0	0	0	0	0,51	0,92	0	0	0	0	0,86	0,80
12	0,17	0,24	0	0	0	0	0,97	0,48	3,39	3,07	0,93	0,78
13	0	4,09	15,59	12,75	0,52	9,16	0,67	0,22	1,36	40,26	0,81	2,86
14	0,33	15,87	11,56	16,73	0,34	2,34	0,92	0,28	1,98	13,99	0,89	1,24
15	0	0	0	0	0,05	0,01	0,99	0	2,64	0,05	0,69	0

Max — Response ratio — Min

[a]Numbers represent the peak areas of the compounds corrected for the internal standard reserpine and relative to those of the parental strain, DS68530. The culture broth of the strains was analyzed after 48 and 96 h of growth in an SMP medium.

The Δ*chyC* strain showed a metabolite profile similar to that of the Δ*chyD* strain, suggesting that ChyC could be also involved in the conversion of compounds 14, 8, and 13. Nonetheless, downstream compounds were still produced in small amounts in the Δ*chyC* strain.

In the Δ*chyE* strain, compounds 2, 4, 8, and 12 were not detected, or were produced in low concentrations compared to the parental strain, suggesting that these compounds belong to the same initial branch of the pathway. Based on the structures and molecular formulas available, compounds 2, 4, and 12 are derived from compound 8, with compound 4 being most likely spontaneously converted into compounds 2 and 12. Since ChyE affected the production of compound 8 and downstream compounds and accumulated compound 14 after 96 h of growth, we propose that this enzyme converts compound 14 into compound 8.

A trend opposite that of the metabolite profile of the Δ*chyE* strain can be observed in the Δ*chyM* strain. Peak areas of compounds 2, 4, 8, and 12 were comparable to those of the DS68530 strain, while compounds 1, 3, 7, 9, and 10 were absent or detected in small amounts. This indicates that these compounds are part of an independent branch of the pathway and derived from compound 13. The result is confirmed by the accumulation of compounds 14 and 13 in the Δ*chyM* strain. The molecular formula of compound 5 suggests that it is derived from compound 13 and that it is the precursor of compound 3, which is further converted into compounds 1, 7, 9, and 10. Because compound 3 and downstream compounds were not produced in this mutant, we propose that ChyM is responsible for the conversion of compound 5 into 3. Chrysogine (compound 1) is likely formed by a spontaneous ring closure from compound 3. Compounds 9 and 10 are isomers, having the same molecular masses but different retention times (RT) in HPLC.

Finally, the Δ*chyH* strain showed a metabolite profile similar to that of Δ*chyM*, suggesting that both enzymes are needed for the formation of the same compounds. Nonetheless, the Δ*chyH* strain did not accumulate compounds 13 and 5, suggesting that ChyH forms compounds 1, 3, 7, 9, and 10 through an independent path. In the analysis of the mutant strains, we could not assign the position of compound 6 in the pathway. Based on the molecular formula, compound 6 could be an unstable precursor of compound 13.

Metabolite profile and gene expression in a strain with a deletion of a putative transcription factor. *Pc21g12640* encodes a putative transcription factor and, because of its chromosomal location in the vicinity of the chrysogine biosynthetic gene cluster, it would be plausible that it acts as a local regulator of this pathway. Although *Pc21g12640* is not significantly expressed in the DS68530 strain, transcription factors can regulate transcription even when present at very low levels. Therefore, to investigate its possible role as a regulator of the chrysogine cluster, *Pc21g12640* was deleted from strain DS68530. Nonetheless, the Δ*Pc21g12640* strain did not show any significant changes in the chrysogine-related metabolite profiles compared to those of the parental strain (Table 2). Similarly, quantitative PCR (qPCR) indicated that the deletion of *Pc21g12640* did not significantly affect the expression of the genes of the chrysogine cluster (Fig. S2). Thus, *Pc21g12640* is not part of the chrysogine biosynthetic gene cluster.

Feeding of the Δ*chyA* strain with compounds 14 and 15. In order to further investigate the role of compounds 14 and 15 as potential NRPS products, the Δ*chyA* strain was fed with chemically synthesized variants of these. Based on the formula, compound 15 is the amidated form of 14.

We showed above that the expression of *chyA* in the chrysogine cluster deletion strain resulted in the production of compounds 14, 8, and 13. The Δ*chyA* strain fed with compound 14 produced compounds 2, 4, and 8, while 13 and downstream compounds were not detected (Fig. 3A). This result suggests that the conversion of compound 14 into 8 is faster than its conversion into 13. The feeding with compound 15 resulted in the production of metabolites that are derived from compounds 8 (compounds 2, 4, 12)
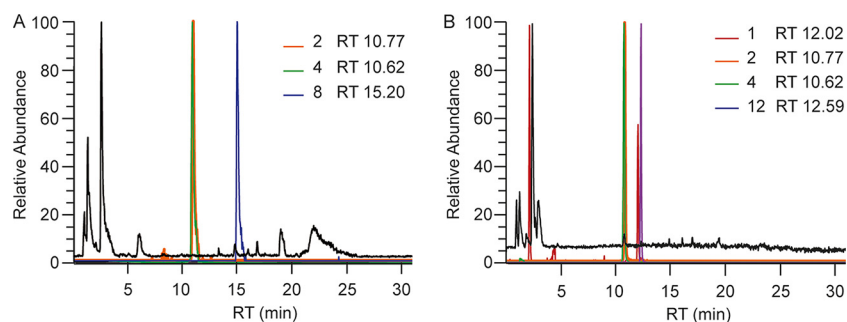
FIG 3 Chromatogram of culture broth from the ΔchyA strain fed with compound 14 or 15. TIC (black) and EIC (colored) of secondary metabolites produced by the ΔchyA strain fed with compound 14 (A) or 15 (B) after 48 h from the feeding.

and 13 (compound 1; Fig. 3B). As compound 15 is very similar to compound 14, we suggest that compound 15 undergoes the same reactions, being converted into compound 4 by ChyE and into compound 5 by a transaminase. Since ΔchyH affected the production of compound 3 and downstream metabolites without any accumulation of compound 5, we propose that ChyH is involved in the biosynthesis of compound 3 from 15. Therefore, the late metabolites can be formed from two different paths.

Distribution and diversity of chrysogine gene clusters in *Penicillium* species. Since the above studies characterized the chrysogine biosynthetic gene cluster, the distribution of this gene cluster in other *Penicillium* species was investigated (Fig. 1). The *chy* genes and *Pc21g12640* from *P. chrysogenum* were subjected to a BLAST analysis against the genomes of two known chrysogine producers (2), *P. nalgiovense* and *P. flavigenum*, recently sequenced by Nielsen et al. (18). These genomes contain a chrysogine gene cluster with a similar gene organization, while a *Pc21g12580* homolog is missing, supporting the notion that this gene is not essential for chrysogine biosynthesis. Interestingly, *P. flavigenum* has two extra genes located near the NRPS gene, suggesting that it may produce additional chrysogine-related metabolites.

DISCUSSION

Chrysogine was isolated from the culture broth of *P. chrysogenum* in 1973 (5) and was found to be produced also by other filamentous fungi (1, 2). Chrysogine biosynthesis is mediated by a dimodular NRPS that we recently identified in *P. chrysogenum* (7, 8) and that was also shown to be responsible for chrysogine biosynthesis in *Fusarium graminearum* (9). Although the biosynthetic gene cluster was suggested, the role of the enzymes in the pathway has thus far not been characterized. In this work, we assigned a function to each enzyme of the cluster and elucidated a complex pathway, validating the compound structures by NMR. The pathway is highly branched, with some enzymes involved in multiple steps of the biosynthesis (Fig. 1).

The NRPS ChyA is a 260-kDa dimodular enzyme which is predicted to contain two adenylation domains. The increased production of chrysogine upon feeding with anthranilic acid and pyruvic acid (5) suggests that these molecules are possible substrates of the NRPS. However, here we identify compound 14 as the direct product of ChyA, showing that the NRPS, in addition to anthranilic acid, utilizes alanine instead of pyruvic acid. However, alanine is readily derived from pyruvic acid by transamination which explains why pyruvic acid stimulates chrysogine production. NRPSsp, NRPSpredictor2, and SEQL-NRPS (19–21) were used for predicting the substrates of ChyA, but results were inconclusive. For the first adenylation domain, phenylalanine, a hydrophobic aliphatic amino acid, 2,3-dihydroxy-benzoic acid, or salicylic acid was predicted, while for the second adenylation domain, proline or a hydrophobic aliphatic amino acid was suggested. This shows that with fungal NRPS genes, predictions can be unreliable, necessitating experimental validation. Compound 14 acts as a substrate for several enzymes, which immediately results in a split in the pathway by forming compounds

8, 13, and 15, the latter being the amidated form of compound 14. Two independent groups of compounds are derived from compounds 8 and 13. Since compound 15 undergoes the same reactions as 14, the more distal metabolites in the pathway can be formed via either of the converging branches.

Transcriptomic data (11) suggested that *chyA* and five flanking genes could form a cluster. These genes are coexpressed under a set of conditions, whereas expression profiles in the flanking regions of the putative gene cluster vary. Metabolic profiling of the mutant strain indicated that ChyE is a malonyl transferase that can convert compounds 14 and 15 into compounds 8 and 4, respectively. Interestingly, the expression of *chyA* in a chrysogine cluster deletion strain showed that compound 14 can be converted into compound 8 without involvement of any of the enzymes of the cluster; this conversion likely involves a transferase. In line with this observation, the deletion of *chyE* did not lead to a complete depletion of compound 8 and downstream metabolites, although it significantly decreased the amounts produced. These data suggest that *chyE* is part of the biosynthetic cluster, as it is coexpressed together with the other genes (11) and its deletion affects chrysogine metabolite production, but one or more other transferases can catalyze the same reactions. The orthologous gene in *Fusarium* species is not involved in chrysogine biosynthesis, showing a different expression pattern compared to the genes of the cluster (9).

In addition, compound 13 was formed by the strain that solely expresses *chyA*, likely through the involvement of a transaminase, whose encoding gene is not part of the gene cluster. Based on sequence alignment, no genes encoding a transaminase have been identified in the immediate vicinity of the chrysogine genes, but the genome contains many transaminases.

Our data indicate that ChyD is an amidase, being responsible for the amidation of the carboxylic acid moiety of compounds 14, 8, and 13, in line with the bioinformatics prediction of ChyD as an asparagine synthetase that amidates aspartate to form asparagine. The ΔchyC strain showed a metabolite profile similar to that of the ΔchyD strain, suggesting that ChyC is involved in the same reactions as ChyD. Indeed, downstream compounds were still produced in small amounts in the ΔchyC strain. For this reason, we speculate that ChyC plays a more minor role in the amidation reactions compared to ChyD, whose deletion completely abolished the production of the late metabolites. Protein alignment does not provide sufficient information to assign a specific function to ChyC. ChyH and ChyM are predicted to be involved in oxidation reactions and to form compound 3 from compounds 15 and 5, respectively. Compound 3 originates from two further branches in the pathways, yielding chrysogine and compounds 7, 9, and 10.

Regulatory genes are usually clustered with secondary metabolite biosynthetic genes (22). Therefore, we hypothesized that the putative transcription factor Pc21g12640 can regulate the expression of the chrysogine genes, since *Pc21g12640* is located downstream of *chyA*. Nonetheless, metabolite profiling and qPCR of the deletion strain gave no indications that Pc21g12640 is involved in the regulation of the *chy* genes. This conclusion is supported by the absence of the transcription factor in *Fusarium* and the other filamentous fungi investigated by Wollenberg et al. (9), although the orthologous gene is present in the genome of other *Penicillium* species (Fig. 1).

As already shown for some other fungal secondary metabolite clusters (22, 23), it is possible that the chrysogine biosynthetic genes are regulated by other transcription factors. Moreover, epigenetic regulation has been suggested for the chrysogine cluster. Shwab et al. (24) first demonstrated that secondary metabolite genes can be regulated by chromatin remodeling, for example, by histone acetylation. In *P. chrysogenum* DS68530, the deletion of the histone deacetylase gene *hdaA* resulted in a significant downregulation of expression of the *chy* genes and subsequent reduction of chrysogine biosynthesis (F. Guzmán-Chávez, O. Salo, and M. Samol, unpublished data).

Secondary metabolite pathways can provide a wide range of compounds from the initial scaffold molecule. Moreover, the same compounds can be produced through different paths. Branched secondary metabolite pathways have been described before

TABLE 3 Oligonucleotide primers used for amplifying the 5′ and 3′ flanking regions of the targeted genes and for qPCR

Primer name	Sequence (5′–3′)
chyA_5′_fw	GGGGACAACTTTGTATAGAAAAGTTGGGTACCGTTCGTACACACCATTCCGGCTG
chyA_5′_rv	GGGGACTGCTTTTTTGTACAAACTTGCATCGATCCTTGATGCCTACAGC
chyA_3′_fw	GGGGACAGCTTTCTTGTACAAAGTGGAAGAGATTGCGAGAGTTGGCTGG
chyA_3′_rv	GGGGACAACTTTGTATAATAAAGTTGGGTACCACTCGAAGGCTCCGTTCTCGGC
chyC_5′_fw	TTGAAGACAATGCCCCTGCAGGTGGGTCGGTATCACAACGACCG
chyC_5′_rv	TTGAAGACAATTGCGTCCCGTTCGCATGGTTACATAGCT
chyC_3′_fw	GGGGACAACTTTGTATAATAAAGTTGGGTACCACTCGAAGGCTCCGTTCTCGGC
chyC_3′_rv	TTGAAGACAAACTAGTTGAAGAAGTTGGTGTAGTTTGAGAATG
chyD_5′_fw	TTGAAGACAAGGAGCCTGCAGGGATCTCAAAGACTATTATCAAGGAAAGGA
chyD_5′_rv	TTGAAGACAAAGCGGGGTGTCGCATGATTATATCTATAGT
chyD_3′_fw	TTGAAGACAAGGAGTTTGAGATTGAGATGAAAGGATTTGGAAAG
chyD_3′_rv	TTGAAGACAAAGCGCCTGCAGGCGGGCATCTTCACGATCCAATAG
chyE_5′_fw	GGGGACAACTTTGTATAGAAAAGTTGCGTGCAGCAAAGACGACATTCG
chyE_5′_rv	GGGGACTGCTTTTTTGTACAAACTTGAGGTATTGGGAATAGACCGGCC
chyE_3′_fw	GGGGACAGCTTTCTTGTACAAAGTGGCAGTATATCTGACGAGGAAGTGGG
chyE_3′_rv	GGGGACAACTTTGTATAATAAAGTTGTCTCCTAGTATCCGACTTCTCCG
chyH_5′_fw	GGGGACAACTTTGTATAGAAAAGTTGGCATCGTAATATGCTCGATTTGG
chyH_5′_rv	GGGGACTGCTTTTTTGTACAAACTTGAGTCTATATAAGCGCTCGGAGGC
chyH_3′_fw	GGGGACAGCTTTCTTGTACAAAGTGGATGAGAGTGAAAGTGTTCAGTGCG
chyH_3′_rv	GGGGACAACTTTGTATAATAAAGTTGGAAGGACCCCTGAGACAGAACC
chyM_5′_fw	GGGGACAACTTTGTATAGAAAAGTTGAACTTCGAGTCGCAGTATGCGG
chyM_5′_rv	GGGGACTGCTTTTTTGTACAAACTTGGGTGTAATGGAACCCATTGCAAGG
chyM_3′_fw	GGGGACAACTTTGTATAGAAAAGTTGAACTTCGAGTCGCAGTATGCGG
chyM_3′_rv	GGGGACTGCTTTTTTGTACAAACTTGGGTGTAATGGAACCCATTGCAAGG
Pc21g12640_5′_fw	GGGGACAACTTTGTATAGAAAAGTTGCAAGAGATTGCCGATAACATTGTGG
Pc21g12640_5′_rv	GGGGACTGCTTTTTTGTACAAACTTGATGACTGGTCCGAGGTACTGG
Pc21g12640_3′_fw	GGGGACAGCTTTCTTGTACAAAGTGGATCATGCACGATGTGGTCATATGG
Pc21g12640_3′_rv	GGGGACAACTTTGTATAATAAAGTTGGCGGCCGCAGATTTCTCGACGTCCGATC
chyA_qPCR_fw	GCACAGGCCAAAGTAACACGTCC
chyA_qPCR_rv	CCGAGGGTTTGTGGTGGATGCC
chyC_qPCR_fw	GTAGACGCCGGTGAGACTTTGATCG
chyC_qPCR_rv	CAACCTAAGCGTCTAATTTTCATCGC
chyD_qPCR_fw	GGAATTCGCTGGCTAACTGGTCTCG
chyD_qPCR_rv	GGCATGTGGTAGACGAATTGGAGC
chyE_qPCR_fw	GGCAAGGGAAATGAATCCAGGTGGC
chyE_qPCR_rv	GATAGATGCCGCTTGTTCGGACC
chyH_qPCR_fw	GGTTGTGGAGCTCTACGAGGCTG
chyH_qPCR_rv	CTGGCAGGGCTCGTCGGTC
chyM_qPCR_fw	CCTGCATGCAGCTCCATACGAGC
chyM_qPCR_rv	CCAACAATAGGTGGAAACAGCTCAGAC
Pc21g12640_qPCR_fw	TGTCTCTCTGTGGGCTGTTCTCAG
Pc21g12640_qPCR_rv	CAAGAGTTCTTACGATGCGTGGCTG
actin_qPCR_fw	CGACTACCTGATGAAGATCCTCGC
actin_qPCR_rv	GTTGAAGGTGGTGACGTGGATACC

in *P. chrysogenum* (13). The chrysogine pathway is even more branched than the previously described roquefortine pathway, and in this case, chrysogine is the final product of one ramification. As a pigment, chrysogine could contribute to protecting the cell from UV light. No antimicrobial activity has been found for this metabolite (4) nor for *N*-acetylalanylanthranilamide (compound 2), which was also identified in a marine fungus (17). The functions of the other metabolites in the cell remain unknown. Nonetheless, the approaches used in this work and the established methods can provide a blueprint for the elucidation of novel secondary metabolite pathways that potentially specify unknown bioactive compounds. Moreover, the understanding of the biosynthetic mechanisms can help to develop new molecules by feeding with chemically modified intermediates.

MATERIALS AND METHODS

Fungal strains, media and culture conditions. *P. chrysogenum* DS68530 was kindly provided by DSM Sinochem Pharmaceuticals. Strain DS68530 lacks the penicillin gene cluster and the *hdfA* gene (15, 16). For RNA extraction and metabolite analysis, strains were pregrown in YGG medium (25) for 24 h. Next, 3 ml of culture inoculum was transferred into 22 ml of secondary metabolite production (SMP) medium (13), and growth was continued for the time indicated. The *Pc21g12630* (*chyA*) overexpression

TABLE 4 Oligonucleotide primers for amplification and checking of integration, gene absence, and deletion cassette amplification[a]

Primer name	Sequence (5′–3′)
P*pcbC*_fw	CAGTGGATCCACGCGTGTCTGTCAATGACCAATAATTGG
P*pcbC*_rv	CATGGTTTAAACGGCGCGCCGGTGTCTAGAAAAATAATGGTGAAAAC
*chyA*_cloning_fw	CATGGGCGCGCCATGGCTGCCCCATCCATATCGC
*chyA*_cloning_rv	CATGGTTTAAACTTACTCGAGATATTCGCAGACTGTCTCTTC
T*penDE*_fw	TCTGCGAATATCTCGAGTAAGTTTAAACCAATGCATCTTTTGTATGTAGCTTC
T*penDE*_rv	TCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCGGCCGCTGATATCCTGTCTTCAGTCTTAAGAC
*amdS*_hom_rec_fw	CTTATTAATTTGATGTAGGTAAGCCCGCCACAAATATATATTTTTACAAGATACCGTGGAAAAACTTCGTGCTA TCACAAAACAGTATACAAAAAATAAGTGGATCCCCCGGGCTGCAGG
*amdS*_hom_rec_fw	TCCCCTCGAGCTTGTCTGTGATTGCGTTTTTTCTAACACTTGTTGTTGCATCCGATCCGTCCCTACCAATTATT GGTCATTGACAGACACGCGTACCGCTCGTACCATGGGTTGAGTGGT
*amdS*_int_fw	ACAGCGGAAGACAAGCTTCTAATAAGTGTCAGATAGCAAT
*amdS*_int_rv	GTTGGCTCCCAGAGCAGCGGTGTCTTTCGTATTCAGGCAGCTAAAC
*chyA*_fw	CCATATCGCCGTTATTTGCC
*chyA*_rv	GACGGCAACATGTAGGAAAC
*chyC*_fw	ATGGCCCGCATCCTGATCAC
*chyC*_rv	TTAAGCTGGGAGCTTAATACCGGTGAT
*chyD*_fw	ATGTGTGGAATAAGTGCATTTCTGTGTC
*chyD*_rv	TCAGTTTGGCAGGGCACCAG
*chyE*_fw	ATGGACTCAGTGAGCAATCTAAAG
*chyE*_rv	CTATTCTGACAGCCACTGCAAA
*chyH*_fw	TCGCGATGCCGACTATAAAG
*chyH*_rv	GCCCATAGAAGCTGAACATC
*chyM*_fw	ATGGGTTCCATTACACCCTCGC
*chyM*_rv	TCACCAGAATGCTGCACACCG
*Pc21g12640*_fw	ATGTCTTCAGCCCCCGGTCT
*Pc21g12640*_rv	CTAGAATATGTCATCCTCGGATTGGAACC
*actin*_fw	ATGGAGGGTATGTTATTCCAGTTGTGG
*actin*_rv	TGCGGTGAACGATGGAAGGACC
phleo in *chyA* locus_fw	CAACGCCCACGAGCATCTGGT
phleo in *chyA* locus_rv	GCCAGAAACTCGACTCGTGGCTC
amdS in *chyC* locus_fw	TCACCAGAATGCTGCACACCG
amdS in *chyC* locus_rv	GATACCCCTTAGCCCGTCATCCAAA
phleo in *chyE* locus_fw	CCATGTCGGGTGTAGATCG
phleo in *chyE* locus_rv	GCCCATAGAAGCTGAACATC
amdS in *chyM* locus_fw	CTTGTCAAGTCTGCGACCAGCAC
amdS in *chyM* locus_rv	ACGAAGAGGCACTCGCGTCAC
amdS in *Pc21g12640* locus_fw	CAAACAGATGAAGACTGGGG
amdS in *Pc21g12640* locus_rv	GGCTCAAACTTGCGCTTAG
*phleo*_seq	ATGGCCAAGTTGACCAGTGCCGTT
*amds*_seq	TCCCCTAAGTAAGTACTTTGCTA

[a]Primers used for amplification of P*pcbC*, *chyA*, and T*penDE* for cloning into pDSM-JAK108; amplification of the *amdS* cassette for *in vivo* homologous recombination into pDSM108_AV1; checking the correct integration of the *amdS* cassette into pDSM108_AV1; and checking the absence of the genes in the knockout strains and amplification of the deletion cassettes into the genome. PCR products were sent for sequencing by using primers *phleo_seq* and *amdS_seq* in order to check the purity of the strains.

strain was grown in SMP medium lacking urea and CH_3COONH_4 and supplemented with 2 g/liter acetamide for plasmid maintenance. The Δ*chyA* strain was fed with 300 μM compound 14 or 15 after 48 h of growth. All cultivations were performed as 25-ml cultures in 100-ml Erlenmeyer flasks shaken at 200 rpm and 25°C.

Construction of deletion and overexpression plasmids. Plasmids for the deletion of the chrysogine genes were built by PCR amplification of 1 to 2 kbp of the 5′ and 3′ flanking regions of each gene, using genomic DNA (gDNA) from the DS68530 strain as the template. All primers used in this study are listed in Tables 3 and 4, and the constructed plasmids are shown in the supplemental material.

For the deletion of *Pc21g12630* (*chyA*), *Pc21g12570* (*chyE*), *Pc21g12590* (*chyH*), *Pc21g12610* (*chyM*), and *Pc21g12640* genes, the Multisite Gateway Three-Fragment Vector Construction kit (Invitrogen) was used. PCR products were inserted into the donor vectors pDONR4-R1 and pDONR2-R3 by the BP Clonase II reaction. The resulting plasmids were mixed with the vector carrying the selection marker (pDONR-amdS or pDONR-phleo), the destination vector pDESTR4-R3, and the LB Clonase II mixture to form the final constructs. The acetamidase gene *amdS* (25, 26) was employed as a marker for the deletion of *chyH*, *chyM*, and *Pc21g12640* genes, while the phleomycin resistance gene was used for selecting *chyA* and *chyE* deletion strains. The modular cloning (MoClo) system (27) was used for building *Pc21g12600* (*chyC*) and *Pc21g12620* (*chyD*) deletion vectors containing an *amdS* marker cassette.

Due to its strength, the *pcbC* promoter was chosen for overexpression of *chyA*, followed by the *penDE* terminator. All genetic elements were amplified from *P. chrysogenum* DS68530 gDNA, and the *chyA* expression cassette was built in subsequent steps of digestions and ligation, using pCM251 (Euroscarf) as the backbone vector. The promoter and terminator were digested with BamHI, PmeI, and NotI

enzymes for cloning into pCM251. *chyA* was inserted into the resulting pCM251 plasmid after digestion with AscI and PmeI. The expression cassette was digested with NotI for insertion into pDSM-JAK108 (28) to form pDSM108_AV1. pDSM-JAK108 contains the AMA1 (autonomous maintenance in *Aspergillus*) (29) sequence, the DsRed gene for visualization of the cells, and the essential gene *tif35*. In this study, the *tif35* gene on the plasmid was replaced with an *amdS* cassette by *in vivo* homologous recombination in *P. chrysogenum*. The *amdS* cassette containing 100-bp flanks homologous to pDSM108_AV1 was obtained by oligonucleotide extension-PCR, using pDONR-amdS as the template.

Transformation and purification procedures. The deletion plasmids (1.5 μg) were linearized and transformed into *P. chrysogenum* DS68530 protoplasts using a standard protocol (30). pDSM108_AV1 (1 μg) was linearized by digestion with the MluI enzyme and cotransformed with the *amdS* cassette (1 μg). The transformants were plated on respective selective media transformation agar [T agar] (25) and grown at 25°C for 5 days. For strain purification, the colonies were transferred to minimal selective solid media (selective agar [S agar]) and sporulation media (25). Rice batches were prepared for inoculation of conidia and long-term storage.

Analysis of the gene deletion strains. The absence of the deleted genes was verified by PCR with gDNA isolated from the knockout strains after 48 h of growth, using an adapted yeast gDNA extraction protocol (31). Primers binding outside the homologous flanking regions were used for amplification of the targeted fragment, after which the PCR products were further verified by sequencing (Macrogen, UK). To verify the correct integration of the *amdS* cassette into pDSM108-AV1, colony PCR was performed on red colonies (bearing the AMA1 plasmid, as seen by the DsRed marker on the plasmid).

RNA extraction, cDNA, amplification and qPCR analysis. Total RNA was isolated from the DS68530 and Δ*Pc21g12640* strains after 48 h of growth in SMP medium, by using the TRIzol (Invitrogen) extraction method with additional DNase treatment (Turbo DNA-free kit, Ambion). For the cDNA synthesis, 500 ng of RNA was used (iScript cDNA synthesis kit, Bio-Rad). The γ-actin gene was used for normalization. The expression levels were measured in technical duplicates with a MiniOpticon system (Bio-Rad) using the Bio-Rad CFX manager software, which automatically determines the threshold cycle (Ct) values by regression. The SensiMix SYBR Hi-ROX kit (Bioline) was used as the master mix for qPCR. The reactions were run as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s.

Metabolite profiling. All the strains used were grown in triplicates for metabolite analysis. Samples were collected after 48 h from the *chyA* overexpression strain and after 48 and 96 h from the deletion mutants and the parental strain. Samples were taken before the feeding of Δ*chyA*, immediately after the feeding, and then after 48 h. All the samples from the different experiments were centrifuged for 10 min, after which the supernatant was filtered with 0.2 μm polytetrafluoroethylene (PTFE) syringe filters and stored at −80°C. The analysis of secondary metabolites was performed with an Accella1250 HPLC system coupled with the ES-MS Orbitrap Exactive (Thermo Fisher Scientific, CA), following the method described by Salo et al. (32).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM .02246-17.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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