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Review

Penicillium roqueforti Secondary Metabolites: Biosynthetic Pathways, Gene Clusters, and Bioactivities

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Abstract: *Penicillium roqueforti* is a fungal starter culture used for the production of blue-veined cheeses, such as Roquefort, Gorgonzola, Stilton, Cabrales, and Danablue. During ripening, this species grows in the veins of the cheese, forming the emblematic blue-green color and establishing the characteristic flavor owin to its biochemical activities. *P. roqueforti* synthesizes a diverse array of secondary metabolites, including the well-known compounds roquefortine C, clavine alkaloids, such as isofumigaclavine A and B, mycophenolic acid, andrastin A, and PR-toxin. This review provides an in-depth exploration of *P. roqueforti*'s secondary metabolites, focusing on their biosynthetic pathways, the gene clusters responsible for their production, and their bioactivities. The presence of these compounds in blue cheeses is also reviewed. Furthermore, the silent clusters and the potential of *P. roqueforti* for producing secondary metabolites were discussed. The review highlights recently identified metabolites, including sesterterpenoids; tetrapeptides, D-Phe-L-Val-D-Val-L-Tyr, and D-Phe-L-Val-D-Val-L-Phe; *cis*-bis(methylthio)silvatin; and the 1,8-dihydroxynaphthalene (DHN)-melanin precursor, scytalone. Additionally, a gene cluster for DHN–melanin biosynthesis is presented. Finally, a revised cluster for roquefortine C biosynthesis comprising three rather than four genes is proposed.

Keywords: bioactivity; biosynthetic gene clusters; blue cheese; isofumigaclavine; melanin; natural products; *Penicillium roqueforti*; roquefortine C; secondary metabolites

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1. Introduction

Fungal secondary metabolites are chemically diverse natural products with low molecular weights [1]. Because of their biological activities, these compounds have a wide range of pharmacological applications. They are used as antibiotics, such as penicillin and cephalosporins; antifungals, like griseofulvin; immunosuppressants, including mycophenolic acid and cyclosporins; cholesterol-lowering drugs, such as lovastatin; and anti-migraine agents, e.g., ergotamine [1–4]. They are also important in the food and feed industry mostly because of their toxic properties [5]. Toxic fungal secondary metabolites, mycotoxins, were first described when large numbers of turkeys fed by the Brazilian groundnut meal died in 1960 [6,7]. The responsible contaminating fungus was identified as *Aspergillus flavus*, and the toxin it produces was named aflatoxin [7]. On the other hand, there are also beneficial secondary metabolites used in the food industry. For example, *Monascus* spp. pigments have been employed as food colorants in Southeast Asia for a long time [8].

Secondary metabolites serve a wide range of functions for the producing organism [9,10]. In nature, different organisms share the same environment and live in communities. These compounds have roles in the interaction of the producing fungus with the surrounding organisms [2]. The fungus might use these compounds to defend itself, compete with the other organisms, as communication signals, and even regulate symbiotic relationships [9,10]. These compounds might also serve as virulence factors for animal and plant pathogens [9]. Moreover, secondary metabolites have roles in fungal development; for example, proper formation of the fruiting bodies or spore germination depends on the produced metabolites [2].

Secondary metabolites are generally grouped into three major categories: acyl-CoA-derived polyketides, acyl-CoA-derived terpenes, and amino acid-derived peptides [2]. A combination of these is also commonly observed, such as alkaloids, meroterpenoids, and peptide–polyketide hybrids [11]. The genes involved in the biosynthesis of secondary metabolites are generally present as clusters in the genomes and are often co-regulated [2]. A secondary metabolite biosynthetic gene cluster primarily harbors a core gene encoding an enzyme synthesizing the backbone of the secondary metabolite, such as a polyketide synthase, a non-ribosomal peptide synthetase, or a terpene synthase or a terpene cyclase, in addition to genes, products of which, are used in further modification of the backbone [2]. The contiguous configuration of the biosynthetic genes allows for the regulation of the secondary metabolite biosynthesis epigenetically. Epigenetic regulation involves tightly or loosely packed chromatin by histone modifications that change the chromatin's availability to the transcriptional machinery [12]. Some clusters also harbor a transcription factor with binding sites located at the promoters of the cluster genes [2]. Environmental signals, including temperature, light, pH, solid/liquid medium, heat and oxidative stress, and nutritional factors, regulate the production of secondary metabolites [13].

Penicillium roqueforti is the filamentous fungal species responsible for the characteristic blue-green color of blue cheeses, produced under different names in different countries, such as Roquefort (France), Gorgonzola (Italy), Stilton (UK), Cabrales (Spain), and Danablue (Denmark) [14]. All of these cheeses were given Protected Designation of Origin/Protected Geographical Indication (PDO/PGI) status [15]. In the production of blue cheeses, *P. roqueforti* spores are either intentionally added to the milk or curd as a secondary starter or ripening culture or grow on the cheese spontaneously in the humid and cool atmosphere of the ripening cellars or caves [16]. After 2–3 weeks, the blue color appears in the cheese veins [17]. *P. roqueforti* not only gives the characteristic color of blue cheeses but also takes a significant role in aroma development with its biochemical activities [18]. While this species is a starter culture in blue cheeses, it is also a food contaminant in dairy products and rye bread and has been observed in other environments, such as silage and wood [19,20]. In addition, *P. roqueforti* has been proposed to have a cell factory potential for producing high-value-added molecules with its convenient fermentation properties, such as a wide pH tolerance and ability to metabolize both pentoses and hexoses [21].

P. roqueforti has been reported to produce a variety of secondary metabolites, e.g., roquefortine C, isofumigaclavines, mycophenolic acid, andrastin A, PR-toxin, citreoisocoumarin, and orsellinic acid [22–24]. The metabolites of cheese-associated fungi, including *P. roqueforti*, have been reviewed previously [18,25,26]. There are also reviews specifically on the secondary metabolites of *P. roqueforti* [27,28]. Garcia-Estrada and Martin [27] concentrated on biosynthesis and the corresponding gene clusters of roquefortine C, PR toxin, and mycophenolic acid. Chavez et al. [28] encompassed a broader spectrum of compounds and, notably, offered valuable insights into regulatory mechanisms.

The current review aims to provide the latest information on the biosynthesis, bioactivities, and toxicities of the secondary metabolites *P. roqueforti* produces. Additionally, the gene clusters responsible for synthesizing these compounds and their prevalence in blue cheeses were presented. Different from the previous reviews, this study includes the newly determined sesterterpenoids [29–31]; tetrapeptides, D-Phe-L-Val-D-Val-L-Tyr, and D-Phe-L-Val-D-Val-L-Phe; *cis*-bis(methylthio)silvatin; and the DHN–melanin intermediate, scytalone [32,33]. Furthermore, a putative gene cluster responsible for DHN–melanin biosynthesis in *P. roqueforti* has been introduced. Moreover, recent findings regarding the secondary metabolites were given, such as the subcellular compartmentalization in mycophenolic acid biosynthesis [34]. In addition, it is suggested that the *P. roqueforti* roquefortine C biosynthetic gene cluster, previously postulated to encompass four genes [35], should be reconsidered to comprise three genes, as per meticulous sequence comparisons.

2. Secondary Metabolites of *P. roqueforti*

2.1. Roquefortine C

Roquefortine C, first described to be isolated from *P. roqueforti* in 1975 [36,37], is produced by at least 30 *Penicillium* species associated with different environments [38]. This compound belongs to a group of secondary metabolites called indole alkaloids [39]. Roquefortine C has neurotoxic properties, as observed in mice and cockerels [37,40]. The compound was also detected in intoxication cases in dogs and cattle [41,42]. While an LD_{50} value (the dose causing the death of 50% of the tested animals) of 15–20 mg/kg was reported in mice in one study [37], another study reported 169–189 mg/kg in two different mice strains [43]. Cytotoxicity of roquefortine C on Caco-2 cells determined a 50% inhibition concentration (IC_{50}) of 48 µg/mL in one study [44] and >100 µM in another study [45]. The mechanism of toxicity is not precisely known, but the inhibition of rat and human cytochrome P450 enzymes by this compound was reported [46].

In addition to the neurotoxic effects, roquefortine C has antimicrobial properties against Gram-positive bacteria, such as *Staphylococcus aureus* and some *Bacillus* species [47]. The mechanism of the antibacterial effect could be the impairment of RNA synthesis [48]. On the other hand, the growth of lactic acid bacteria was only insignificantly impaired in the presence of roquefortine C [47]. During cheese ripening, the production of this metabolite might be useful for controlling unwanted bacterial growth while lactic acid bacteria remain unaffected.

Roquefortine C is produced by almost all of the studied *P. roqueforti* strains [49,50]. Production on solid substrates is much higher than in cheese [26]. The roquefortine C concentration in cheeses varies considerably; the average concentration was 858 ± 1670 µg/kg, determined using 83 blue cheese samples [51]. Based on this value and the average blue cheese consumption levels in France, a 21-day exposure representing chronic conditions was investigated on Caco-2 cells [45]. Chronic exposure did not change the intestinal barrier, suggesting that the amounts taken with blue cheese consumption do not cause significant toxicological effects [45].

Roquefortine C biosynthesis begins with the condensation of L-tryptophane and L-histidine to form a cyclodipeptide with a diketopiperazine ring by the action of a nonribosomal peptide synthetase (NRPS), named roquefortine dipeptide synthetase (RDS) (Figure 1A) [52]. After the formation of the cyclodipeptide, there is a grid in the biochemical pathway. The resulting dipeptide might first be prenylated to produce roquefortine D and dehydrogenated, or the pathway proceeds with dehydrogenation first and then prenylation to yield roquefortine C [38,53]. The relative amounts of the metabolites in the deletion strains in *P. chrysogenum* (renamed as *P. rubens*) indicate that the former path is the predominant one [53]. Some fungal species can convert roquefortine C to metabolites, such as meleagrin, oxaline, and neoxaline [38]. For example, *P. chrysogenum* produces meleagrin, and the formation of this compound requires three additional steps after roquefortine C is formed [38]. In parallel, the biosynthetic gene clusters responsible for the production of roquefortine C in *P. roqueforti* and meleagrin in *P. chrysogenum* are very similar (Figure 1B). However, while the cluster in *P. chrysogenum* harbors seven genes [53], the *P. roqueforti* cluster is a shorter version [35]. It has been suggested that the biosynthetic gene cluster for roquefortine C (*roq*) in *P. roqueforti* harbors four genes [35]. These genes include *gmt* (*roqN*, PROQFM164S01g003515), represented as a white arrow in Figure 1B, *rds (roqA)*, which encodes a nonribosomal peptide synthetase, *rdh (roqR)*, responsible for encoding a dehydrogenase, and *rpt (roqD)*, which codes for a roquefortine prenyltransferase. However, between *P. roqueforti* FM164 and *P. chrysogenum* Wisconsin 54-1255 genomes, three stretches of high identity, >85%, are observed; one corresponds to the *rpt (roqD)* gene, while the other long stretch harbors *rdh (roqR)* and *rds (roqA)* (Figure 1B). There is also a tiny stretch at the end of the analyzed regions. The *roq* clusters in the two *P. roqueforti* genomes, FM164 and CECT 2905, are 99.2% identical, as indicated by the bright red color of the Artemis Comparison Tool (ACT) in the entire region analyzed (Figure 1B). PROQFM164_S01g003515 (old name: Proq01g022760), shown with a white arrow in Figure 1B, was mentioned to be part of the

P. roqueforti cluster as a remnant homolog of the methyltransferase gene, *gmt (roqN)*, in *P. chrysogenum* [35]. However, the low sequence identity (22%) of this gene with *gmt* (*roqN*, Pc21g15440) and its higher sequence identity (79%) with another gene (Pc21g15540) located ~29 kb downstream in the *P. chrysogenum* genome (Figure S1), suggest that it is not part of the cluster. Therefore, it is likely that the *P. roqueforti* roquefortine C cluster comprises only three genes. Silencing of *rds* and *rpt* genes in *P. roqueforti* reduced roquefortine C production, indicating these genes' involvement in biosynthesis [35]. In *P. chrysogenum*, deletions of the genes in the cluster identified their functions in the pathway. The three genes that are absent in the *P. roqueforti* gene cluster, *nox* (*roqM*), *sro* (*roqO*), and *gmt* (*roqN*) in *P. chrysogenum* take part in the formation of meleagrin from roquefortine C [53]. An MFS transporter gene, *roqT*, in *P. chrysogenum* is also absent in *P. roqueforti*. *RoqT* deletion in *P. chrysogenum* did not inhibit the production and secretion of meleagrin; however, the concentrations of the intermediate compounds were altered [53]. RoqT in *P. chrysogenum* might facilitate the transport of the intermediates within the cell or the secretion of the final product, which would otherwise occur by passive transport or by an alternative transporter.

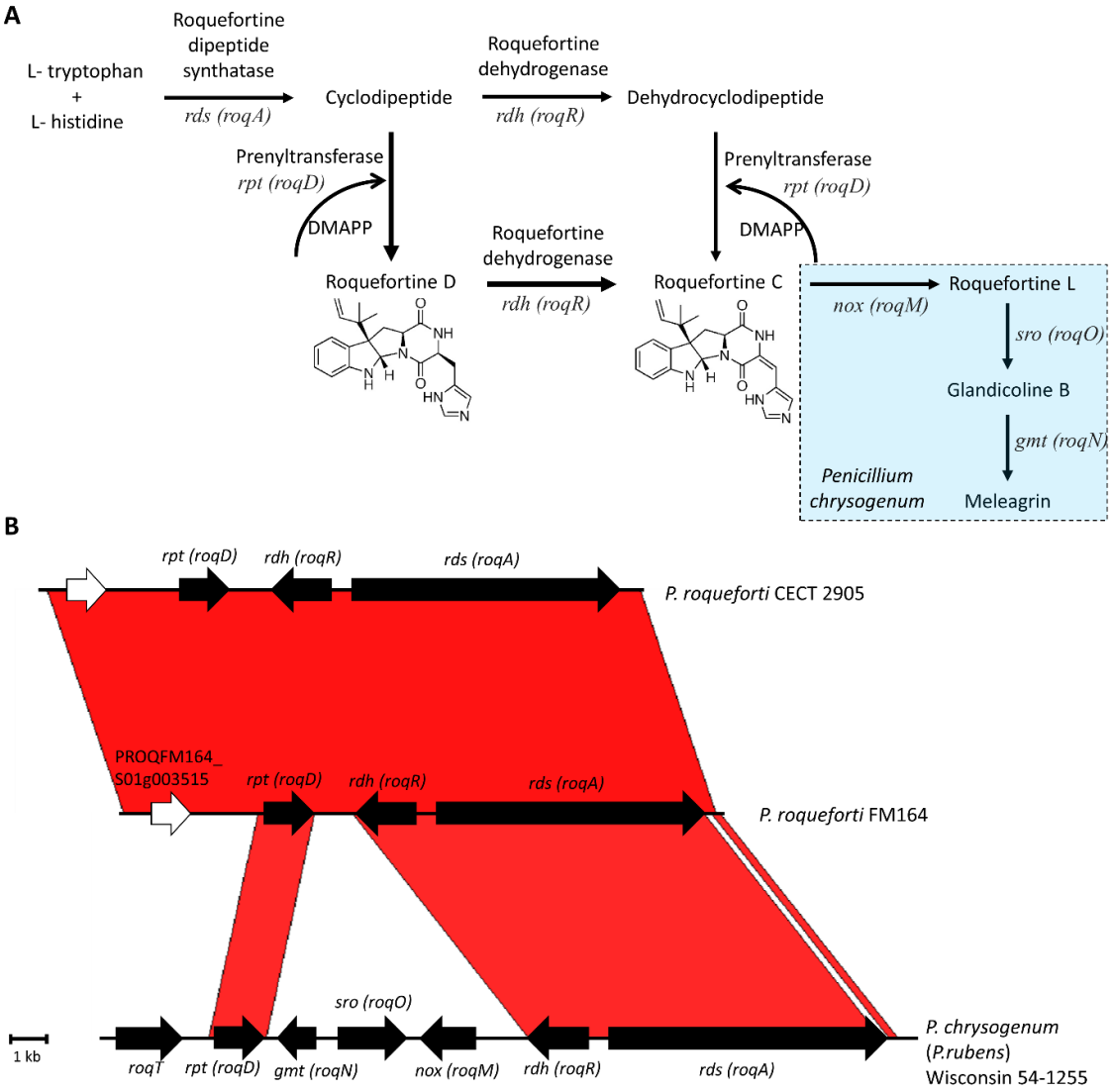


Figure 1. Roquefortine C biosynthesis and the associated gene clusters. (**A**) Roquefortine C biosynthetic pathway. The thicker arrows indicate the predominant path in the metabolic grid in *P. chrysogenum*. While the pathway stops at roquefortine C in *P. roqueforti*, the end product is meleagrin in *P. chrysogenum*, as indicated in the blue rectangle [35]. (**B**) Roquefortine C biosynthesis gene clusters in *P. roqueforti* CECT 2905 and FM164, and *P. chrysogenum* (*P. rubens*) Wisconsin 54-1255 [35]. The sequence

comparison file for the gene clusters was generated using BLAST and used in the Artemis Comparison Tool (ACT) Release 18.2.0 to obtain a visual image [54]. ACT indicates high sequence similarity in red and blue colors representing forward and reverse matches, respectively, with the color intensity proportional to the percent sequence identity. *P. roqueforti* CECT 2905 roquefortine cluster is in contig 377. *P. roqueforti* FM164 gene IDs are as follows: *rpt (roqD)*: PROQFM164_S01g003515, *rdh (roqR)*: PROQFM164_S01g003516, *rds (roqA)*: PROQFM164_S01g003517. *P. chrysogenum* Wisconsin 54-1255 gene IDs: *roqT*: Pc21g15420, *rpt (roqD)*: Pc21g15430, *gmt (roqN)*: Pc21g15440, *sro (roqO)*: Pc21g15450, *nox (roqM)*: Pc21g15460, *rdh (roqR)*: Pc21g15470, *rds (roqA)*: Pc21g15480. DMAPP: dimethylpyrophosphate.

2.2. Clavine Alkaloids

P. roqueforti was reported to produce clavine-type indole alkaloids, isofumigaclavine A [23,49,50,55–57], isofumigaclavine B [50,55], festuclavine [24,36], and agroclavine [24,58]. Because of the structural resemblance of clavine alkaloids to the neurotransmitters dopamine, serotonin, and adrenaline, they have diverse pharmacological properties [59]. Agroclavine was reported to enhance natural killer cell activity, an immunostimulatory effect [60]. In blue cheeses, limited amounts of isofumigaclavine A (<5 ppm) and only traces of isofumigaclavine B were reported [55]. The relatively low amounts might be attributed to the low oxygen conditions in cheese that do not allow the high number of oxidation steps in the biosynthetic pathway (Figure 2A) [18].

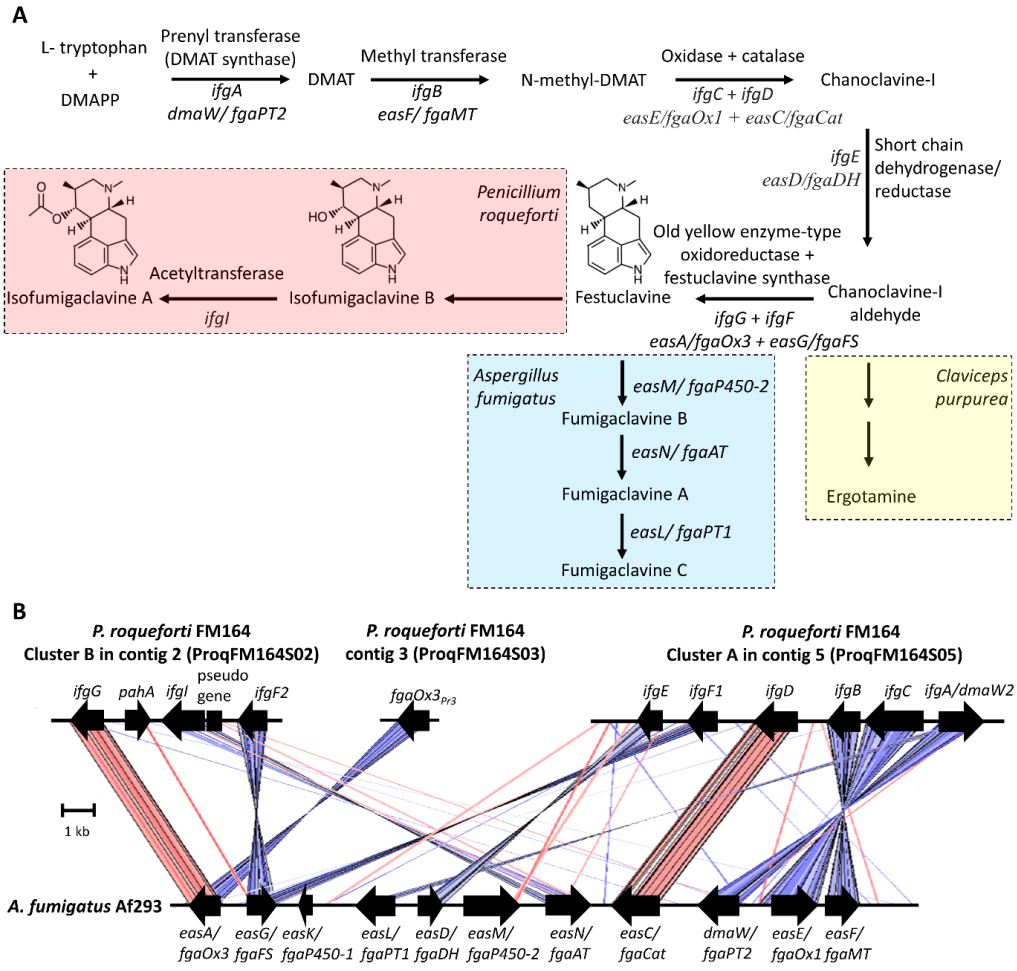


Figure 2. Biosynthesis of clavine alkaloids. (**A**) Biosynthetic pathway for the production of clavine alkaloids in *Penicillium roqueforti* and *Aspergillus fumigatus* and ergotamine in *Claviceps purpurea* [61,62]. (**B**) Gene clusters for the production of clavine alkaloids in *P. roqueforti* and *A. fumigatus*. The gene

IDs in the *P. roqueforti* FM164 genome are as follows; cluster A genes: *ifgA*/*dmaW2* (PROQFM164_S05g000511), *ifgC* (PROQFM164_S05g000510), *ifgB* (PROQFM164_S05g000509), *ifgD* (PROQFM164_S05g000508), *ifgF1* (PROQFM164_S05g000507), *ifgE* (PROQFM164_S05g000506); cluster B genes: *ifgG* (PROQFM164_S02g000300), *pahA* (PROQFM164_S02g000301), *ifgI* (PROQFM164_S02g000302), pseudogene (PROQFM164_S02g000303), *ifgF2* (PROQFM164_S02g000304). The old yellow enzyme gene homolog fgaOx3$_{Pr3}$ unlinked to cluster A or B: PROQFM164_S03g000127 (CDM33403). The *A. fumigatus* Af293 cluster was redrawn based on Bilovol et al. [63] and Wallwey et al. [64]. The color intensity created by ACT is proportional to the percent sequence identity. Red and blue colors represent forward and reverse matches, respectively. DMAPP—dimethylpyrophosphate, DMAT—dimethylallyltryptophan.

Clavine alkaloid biosynthesis has been largely elucidated in *Aspergillus fumigatus* (*Neosartorya fumigata*), which produces festuclavine and fumigaclavines [65]. The biosynthesis of clavine alkaloids starts with the condensation of tryptophan and dimethylpyrophosphate (DMAPP) by a prenyltransferase enzyme called dimethylallyltryptophan (DMAT) synthase (DmaW/FgaPT2) (Figure 2) [66]. The produced DMAT is methylated by a methyltransferase (EasF/FgaMT) [67], and by the action of at least two enzymes, an oxidase (EasE/fgaOx1), and a catalase (EasC/fgaCat), chanoclavine I, is produced [68,69]. After that, a short-chain dehydrogenase/reductase (EasD/FgaDH) converts chanoclavine I to chanoclavine I aldehyde [70]. From this point on, different fungi produce different metabolites using the intermediate chanoclavine I aldehyde. For example, while *Claviceps purpurea* produces the ergot alkaloid ergotamine, *A. fumigatus* produces fumigaclavines via festuclavine (Figure 2A) [61]. *P. roqueforti*, on the other hand, produces festuclavine similar to *A. fumigatus*; however, festuclavine is converted into isofumigaclavines. In the production of festuclavine from chanoclavine I aldehyde, two enzymes, one, a member of the old yellow enzyme family of oxidoreductases (EasA/FgaOx3), and the other, festuclavine synthase (EasG/FgaFS), are involved [61,64,71]. In *A. fumigatus*, festuclavine is hydroxylated by EasM/fgaP450-2) to fumigaclavine B [63], which is later acetylated by EasN/FgaAT to produce fumigaclavine A [72]. This compound is prenylated once more by easL/fgaPT1 and yields fumigaclavine C [73].

The discovery of the clavine alkaloids biosynthetic cluster in *P. roqueforti* started with the finding of a second prenyltransferase gene (*ifgA*/*dmaW2*, proq05g069320 [new ID: *PROQFM164*_S05g000511]), different from *rpt* involved in roquefortine biosynthesis [74]. The silencing of *ifgA* resulted in the suppression of isofumigaclavine A and three other intermediate products in *P. roqueforti* [62]. Interestingly, *ifgA* silencing leads to an increase in roquefortine C production [62], which might be the result of the unused precursors, tryptophan and DMAPP, directed to this pathway. *IfgA* was found in a cluster of six genes (cluster A) (Figure 2B). There is a methyltransferase gene in the cluster, *ifgB*, the product of which presumably methylates DMAT to produce N-methyl-DMAT [62]. The FAD-binding oxidoreductase (*ifg*C) and the catalase (*ifgD*) gene products are the putative enzymes that produce chanoclavine I [62]. The gene *ifgE* is a short-chain dehydrogenase/reductase that corresponds to the enzyme catalyzing the production of chanoclavine I aldehyde from chanoclavine [62]. The final gene in this cluster is a festuclavine synthase ortholog, named *efgF1*. In cluster A, only the function of the *ifgA*/*dmaW2* gene in clavine alkaloid biosynthesis has been demonstrated experimentally [62].

Because cluster A in contig 2 did not involve the gene for the old yellow enzyme family necessary for festuclavine production, the authors looked for this gene (*ifgG*) in the genome. They found one in a second cluster in contig 5 (Figure 2B) [62]. This cluster, cluster B, has three additional genes, another copy of the festuclavine synthase gene, *efgF2*, and an acetyltransferase gene, *ifgI*, which putatively catalyzes the acetylation of isofumigaclavine B to isofumigaclavine A, similar to the corresponding enzyme EasN/FgaAT in *A. fumigatus* that acetylates fumigaclavine B to fumigaclavine A [72]. There is also a phytanoyl-CoA hydroxylase gene, *pahA*, in cluster B [62,65]. While this gene is not present in the *A. fumigatus* cluster, it is found in some ergot alkaloid and clavine alkaloid gene clusters in different fungi [65]. For example, in *C. purpurea*, EasH, a phytanoyl-CoA hydroxylase gene

product, functions as a cyclolizing dioxygenase in ergopeptine synthesis [75]. Whether this gene in *P. roqueforti* has a function in clavine biosynthesis is unknown. In addition, the roles of the cluster B genes in the biosynthesis of clavine alkaloids in *P. roqueforti* have yet to be shown experimentally.

Meanwhile, Gerhards and Li [61] reported an additional old yellow enzyme gene homolog (CDM33403, named FgaOx3$_{PR3}$) in contig 3 of the *P. roqueforti* genome, unlinked to cluster A or B (Figure 2B). They cloned this gene, overexpressed the corresponding protein, and demonstrated the activity of converting chanoclavine I aldehyde to festuclavine in the presence of festuclavine synthase of *A. fumigatus*. The enzyme also increased the conversion yield of chanoclavine I to chanoclavine aldehyde, the previous step in the biosynthetic pathway, catalyzed by a short-chain dehydrogenase/reductase. The authors reported an amino acid-level sequence identity of 51% between FgaOx3$_{PR3}$ and IfgG, the old yellow enzyme in cluster B [61]. Meanwhile, *A. fumigatus* EasA/FgaOx3 amino acid sequence identity is 67.4% and 53.9% to IfgG and FgaOx3$_{PR3}$, respectively. The higher sequence identity between EasA/FgaOx3 and IfgG suggests that the enzyme responsible for festuclavine synthesis might be IfgG with a better activity towards chanoclavine-I aldehyde, which remains to be explored.

While in *A. fumigatus*, the genes (n = 11) responsible for the clavin biosynthesis pathway are grouped contiguously in a single cluster, the biosynthesis in *P. roqueforti*, is apparently associated with more than one gene cluster (Figure 2B) [65]. Generally, for ergot alkaloid biosynthesis in ascomycetes, this is not an uncommon configuration; a single cluster or two unlinked clusters can be involved in biosynthesis [76]. The conservation of the genes responsible for chanoclavine synthesis (*ifgA*/*dmaW2*, *ifgC*, *ifgB*, and *ifgD*) as a block between *P. roqueforti* and *A. fumigatus* except a single inversion is remarkable (Figure 2B). Eight of the eleven genes in the *A. fumigatus* cluster have homologs in *P. roqueforti* cluster A or B. One of the three missing genes is *easL*/*fgaPT1*, the product of which prenylates fumigaclavine A to yield fumigaclavine C (Figure 2B). Because in *P. roqueforti*, the pathway stops at isofumigaclavine A, this gene is not expected. The second missing gene is *easK*/*fgaP450*-1, whose function, if any, is unknown in *A. fumigatus*. The third gene is *easM*, the product of which is responsible for hydroxylating festuclavine. A homologous gene is not present in the *P. roqueforti* cluster A or B and might be elsewhere in the genome. When the *A. fumigatus* EasM amino acid sequence (Genbank ID: Q4WZ65.1) was used to search the genomes of *P. roqueforti* strains FM164 and LCP96 04111, different hits were obtained with a percent identity between 45–23%. How the hydroxylation reaction is catalyzed in *P. roqueforti* awaits further research.

2.3. Mycophenolic Acid

Mycophenolic acid is one of the main secondary metabolites *P. roqueforti* produces [22]. The compound was discovered by an Italian physician, Bartolomeo Gosio, in 1893 while he was studying spoiled corn to find an agent causing pellagra [77]. At those times, pellagra was believed to be associated with a toxic substance rather than a vitamin deficiency as we know it today. Gosio isolated a fungus from spoiled corn and purified a compound from the culture filtrate of this fungus. This compound had phenolic properties and stopped the growth of "anthrax bacillus" [78,79]. Later, in 1913, Alsberg and Black [80] isolated an acidic compound from a corn-spoilage *Penicillium* and named it mycophenolic acid. When Clutterbuck et al. conducted extensive studies on the metabolic products of *Penicillium brevicompactum* in 1932, they concluded that the compounds Gosio, Alsberg, and Black isolated was one and the same [81]. Because of Gosio's work, mycophenolic acid was prized later as the first fungal antibiotic crystallized [82].

After the first report on its activity against *Bacillus anthracis* by Gosio [77], mycophenolic acid was later shown to have antibacterial [82,83], antifungal [82,84], antiviral [85], antipsoriasis [86,87], and antitumor [88] properties. However, mycophenolic acid is used in medicine today primarily because of its immunosuppressive activity [79]. Two prodrugs of mycophenolic acid, mycophenolate mofetil (CellCept®, Roche Pharma, Basel,

Switzerland) and mycophenolic acid sodium salt (Myfortic®, Novartis Pharma, Basel, Switzerland), are used as immunosuppressants in transplantation to prevent organ rejection and to treat autoimmune diseases [89]. Mycophenolic acid acts as an inhibitor of the inosine 5′-monophosphate dehydrogenase (IMPDH), an enzyme taking the role of purine monophosphate formation during DNA synthesis [90]. The compound inhibits the proliferation of B- and T-lymphocytes, whose DNA synthesis is mainly dependent on this de novo synthesis pathway [90]. Other cells use both de novo synthesis and a salvage pathway that recycles the purines; therefore, the compound explicitly affects the B- and T-lymphocytes, a highly beneficial feature for an immunosuppressant [79,90].

A long-term toxicity study in rabbits given 80 and 320 mg/kg of mycophenolic acid orally for one year showed no apparent toxicity sign [91]. In rats and mice, relatively high concentrations were needed for toxicity. For example, LD_{50} for rats and mice are 2500 and 700 mg/kg (oral), and 500 and 450 mg/kg (intravenous), respectively [92]. However, using the compound in immunosuppressive therapy revealed some gastrointestinal side effects, such as diarrhea, abdominal pain, mucosal changes, and submucosal inflammation, which indicate gastrointestinal toxicity [93]. The IC_{50} value of mycophenolic acid on Caco-2 cells representing cytotoxicity was 780 µM, the solubility limit of the compound, indicating that even at this concentration, more than 50% of the cells were viable [45]. In blue cheeses, the average mycophenolic acid concentration was reported to be 841 ± 1271 µg/kg [51]. Chronic exposure (21-day experiment) on Caco-2 cells based on the value attained by ingesting the highest contaminated cheese led to decreased intestinal barrier function [45]. However, this decrease did not result in bacterial passage. Regarding mutagenicity, the Ames test conducted on *Salmonella* Typhimurium strains using mycophenolic acid up to 400 µg/plate did not result in mutagenic activity [94]. Nevertheless, a study on mouse mammary carcinoma cell line FM3A indicated mycophenolic acid-induced mutations at 0.032 and 0.1 µg/mL and chromosome aberrations at 0.1 µg/mL [95].

Mycophenolic acid is a meroterpenoid, a term first used in 1968 [96], with the prefix mero meaning part, partial, or fragment [97], to describe compounds that are of mixed biosynthetic origin and derived in part from terpenoids [98]. Mycophenolic acid is a polyketide–terpenoid hybrid compound (Figure 3A) [99]. Mycophenolic acid biosynthesis in *P. roqueforti* is governed by a seven-gene cluster spanning 24.4 kb (Figure 3B) [100], similar in structure to the corresponding cluster in *P. brevicompactum* [101,102]. The silencing of each of the seven genes in the mycophenolic acid biosynthetic gene cluster of *P. roqueforti* resulted in significant reductions in mycophenolic acid [100]. The mycophenolic acid biosynthetic pathway has been elucidated with studies involving isotope labeling, gene silencing, and heterologous expression, mainly conducted in *P. brevicompactum* [34,99,101–104]. The biosynthesis begins with the formation of the tetraketide 5-methylorsellinic acid (5-MOA) from one acetyl-CoA, three units of malonyl-CoA, and one S-adenosyl-L-methionine (SAM) with the action of a polyketide synthase, coded by *mpaC* (Figure 3A) [100,101]. 5-MOA is then hydroxylated to produce 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid (DHMB), and the ring is closed to form 5,7-dihydroxy-4-methylphthalide (DHMP). These two reactions are catalyzed by a bifunctional enzyme coded by the *mpaDE* gene having a P450 monooxygenase and a hydrolase domain [102]. After that, a *mpaA*-encoded prenyltransferase adds a farnesyl, a 15-carbon isoprenoid, group to DHMP to yield 6-farnesyl-5,7-dihydroxy-4-methylphthalide (FDHMP) [34,99]. Oxidative cleavage of the farnesyl side chain by an oxygenase, coded by *mpaB*, yields a three-fewer carbon atom containing FDHMP-3C, which is later methylated by an O-methyltranferase, encoded by *mpaG*, to produce MFDHMP-3C [34]. A recent fascinating discovery demonstrated the compartmentalized nature of mycophenolic acid biosynthesis [101]. While the steps until the production of MFDHMP-3C take place in the cytoplasm, MFDHMP-3C enters into the peroxisome and CoA-ligated, followed by β-oxidation-mediated chain shortening to yield mycophenolic acid-CoA [34]. An acyl-CoA hydrolase, coded by *mpaH*, hydrolyzes the mycophenolic acid-CoA to prevent further oxidation and yields mycophenolic acid [34,105]. There is also a gene, *mpaF*,

coding for a mycophenolic acid-insensitive IMPDH, which has been suggested to confer self-resistance [100,106].

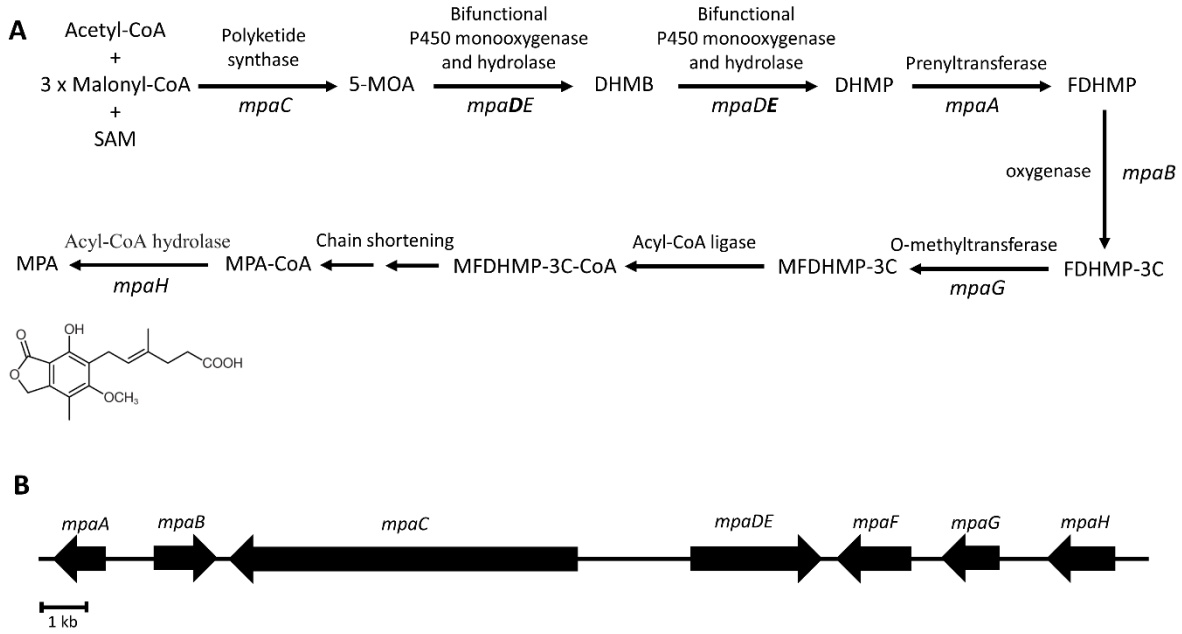


Figure 3. Mycophenolic acid biosynthetic pathway (**A**) Biosynthesis of mycophenolic acid (**B**) Mycophenolic acid biosynthetic gene cluster of *P. roqueforti* CECT 2905 and FM164 [100,107]. The gene IDs in the FM164 genome are as follows: *mpaA* (PROQFM164_S05g000561), *mpaB* (PROQFM164_S05g000560), *mpaC* (PROQFM164_S05g000559), *mpaDE* (PROQFM164_S05g000557), *mpaF* (PROQFM164_S05g000556), *mpaG* (PROQFM164_S05g000555), *mpaH* (PROQFM164_S05g000554). SAM—*S*-adenosyl-L-methionine, 5-MOA—5-methylorsellinic acid, DHMB—4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid, DHMP—5,7-dihydroxy-4-methylphthalide, FDHMP—6-farnesyl-5,7-dihydroxy-4-methylphthalide, FDHMP-3C—three-fewer carbon atom containing FDHMP, MFDHMP-3C—methylated FDHMP-3C, MPA—mycophenolic acid.

Mycophenolic acid production in *P. roqueforti* was reported to be strain-dependent [50,108]. In addition, some *P. roqueforti* strains were demonstrated to have a 174 bp deletion in the 3′-end of the *mpaC* gene, leading to a decrease in the protein length by 14 amino acids [107]. The strains with shorter *mpaC* produced low or no mycophenolic acid, suggesting a correlation between the deletion and the reduction or the absence of mycophenolic acid production [107]. However, even among the strains with no deletion on *mpaC*, significant differences in mycophenolic acid production were observed [107]; therefore, more factors, such as sequence variations in cluster genes or their regulatory regions or on a regulatory gene outside the cluster, are likely to be involved in the regulation of strain-dependent production. Although the mycophenolic acid cluster does not harbor a regulatory gene, several regulator genes for secondary metabolites were identified in *P. roqueforti* [109–111].

In addition to its variability, the mycophenolic acid production of *P. roqueforti* is lower than that of *P. brevicompactum* [112], known to be a good producer, as high as 6700 mg/L [113]. However, using a *P. roqueforti* mutant strain exposed to Gamma radiation and optimizing the fermentation media, El-Sayed and Zaki were able to increase the yield, which is of relevance in industrial production, up to 2933 mg/L [114].

2.4. Andrastin A

Similar to mycophenolic acid, andrastin A is also a meroterpenoid [115]. Andrastins (andrastin A, B, C) are farnesyl transferase inhibitors that might alter the activity and localization of Ras proteins; therefore, they have the potential to be used as anticancer

drugs [116,117]. In addition, andrastin A has been reported to enhance the cytotoxic effect of an anticancer drug, vincristine, in drug-resistant cancer cells [118].

Andrastin A is produced by *P. roqueforti* in solid and liquid media and has been consistently detected in different blue cheeses [119,120]. The concentration in cheeses depends on the strain used as the starter [119]. Positive or adverse effects of the presence of this metabolite in the human diet involving blue cheeses are unknown. The biological activity as a farnesyl transferase inhibitor might be beneficial for health; however, comprehensive toxicological data is needed to evaluate possible risks.

Isotope labeling studies in the andrastin-producing fungus *Penicillium* sp. FO-3929 showed that andrastins were synthesized from tetraketide 3,5-dimethylorsellinic acid (DMOA) and farnesyl diphosphate (Figure 4A) [116]. The andrastin biosynthetic pathway (Figure 4A) and the gene cluster with eleven genes, *adrA-adrK* (Figure 4B), in *P. chrysogenum*, were studied by Matsuda et al. [121]. The authors hypothesized that similar to austinol [122] and terretonin [123], andrastins were derived from epoxyfarnesyl-DMOA methyl ester based on the fact that the four genes coding for the enzymes catalyzing the formation of this intermediate in austinol and terretonin biosynthesis have high sequence homology to the *adrD*, *adrG*, *adrK*, and *adrH* in *P. chrysogenum* [121]. In analogy to austinol and terretonin biosynthesis, Matsuda et al. proposed that andrastin biosynthesis started with the formation of DMOA by an *adrD*-encoded polyketide synthase [121]. Then, a farnesyl group is added to DMOA by a prenyltransferase, coded by *adrG*. The resulting farnesyl-DMOA is methylated by an *adrK*-encoded methyltransferase, and the farnesyl unit is epoxidized by a flavin adenine dinucleotide (FAD)-dependent monooxygenase, AdrH, to produce epoxyfarnesyl-DMOA methyl ester [121], the common intermediate in the biosynthesis of related metabolites, austinol and terretonin. The authors then used a heterologous expression approach to show how the cyclization and the post-cyclization tailoring reactions take place [121]. According to these studies, epoxyfarnesyl-DMOA methyl ester is cyclized by the terpene cyclase, adrI, revealing the tetracyclic intermediate andrastin E. Then, the product of *adrF*, a short-chain dehydrogenase, oxidizes andrastin E to produce andrastin D, which is reduced by a ketoreductase encoded by *adrE* [121]. The resulting product, andrastin F, is acetylated by an acetyltransferase, AdrJ, to produce andrastin C [121]. The final steps in the pathway involve two consecutive oxidation reactions catalyzed by a cytochrome P450 monooxygenase, encoded by *adrA*, to produce andrastin B and then andrastin A [121].

The biosynthetic gene cluster for andrastin A, spanning 29.4 kb, has been defined in *P. roqueforti* and harbors ten genes (Figure 4B) [115]. The cluster is very similar to the andrastin biosynthetic cluster of *P. chrysogenum* [121], with the only exception that *adrB* is a pseudogene in *P. roqueforti* (Figure 4B) [115]. RNAi silencing of the ten genes in *P. roqueforti*, including the *adrC* gene, coding for a major facilitator superfamily (MFS) transporter, significantly reduced andrastin A production [115]. In secondary metabolite biosynthesis, MSF transporters are involved in the trafficking of the precursors, intermediary compounds, or final metabolites to specific locations within the cell [124]. For example, in *P. chrysogenum*, penicillin production involves at least three MSF transporters [124]. PenV, located in the vacuolar membrane, transports the vacuole-stored precursor L-α-aminoadipic acid into the cytosol; PaaT and PenM, which localize the peroxisomal membrane, translocate the intermediary compounds phenylacetic acid and isopenicillin N, respectively, from the cytosol to the peroxisome, where the final steps of penicillin production take place [125]. The *adrC*-silenced *P. roqueforti* strain did not have a difference in the andrastin A quantities in the mycelium and the surrounding agar, indicating that AdrC is not involved in the secretion of the final metabolite [115]. Whether this MSF transporter takes a role in the intracellular transport of intermediates or precursors is an open question that could be addressed in future studies.

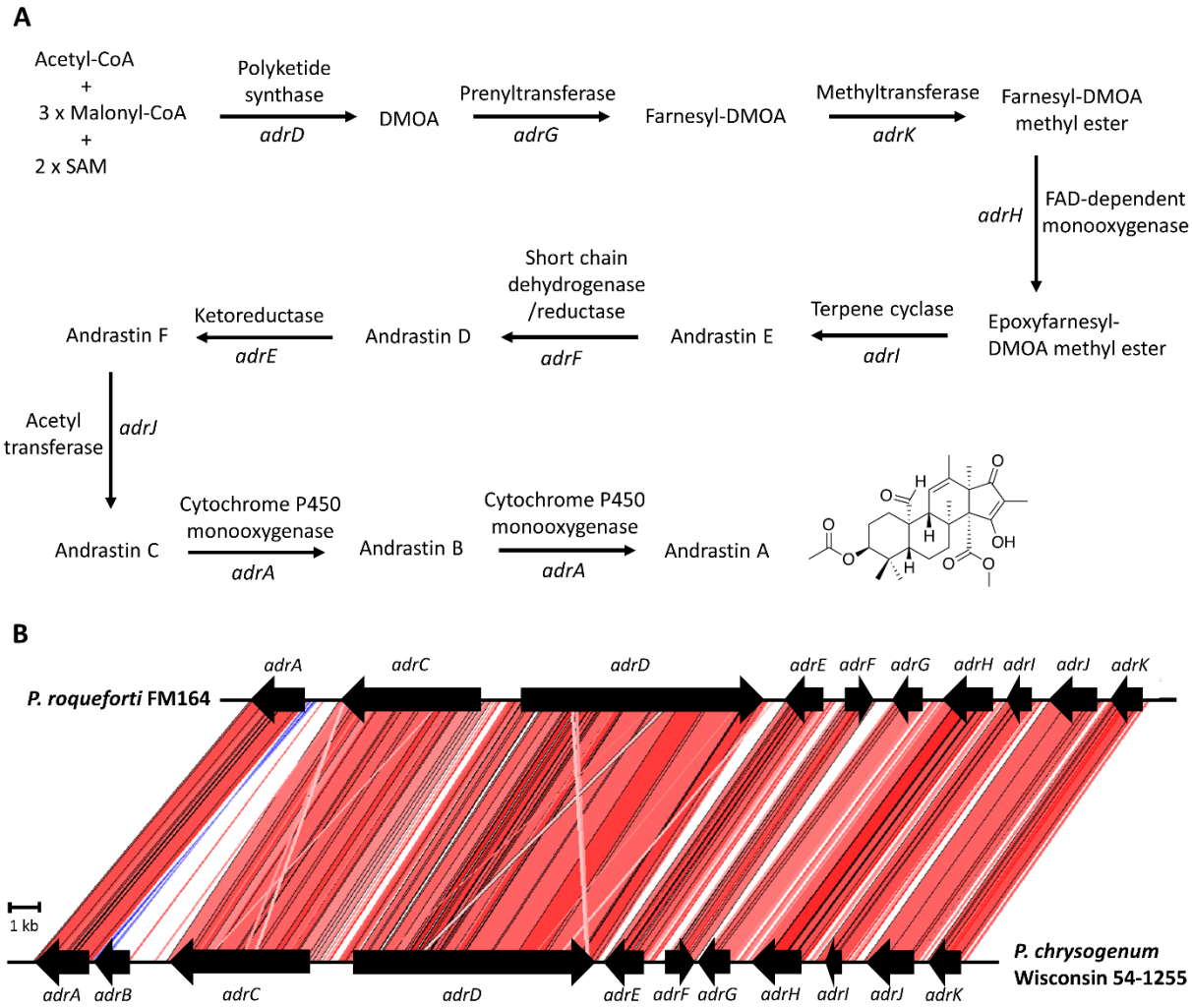


Figure 4. Andrastin A biosynthesis. (**A**) Proposed andrastin A biosynthetic pathway in *P. chrysogenum* [121]. (**B**) Biosynthetic gene clusters for andrastin A in *P. roqueforti* FM164 and *P. chrysogenum* Wisconsin 54-1255 [115,121]. *P. roqueforti* gene cluster is present in the genomic scaffold ProqFM164S01, and the gene IDs are as follows: *adrA* (PROQFM164_S01g001195), *adrC* (PROQFM164_S01g001194), *adrD* (PROQFM164_S01g001193), *adrE* (PROQFM164_S01g001192), *adrF* (PROQFM164_S01g001191), *adrG* (PROQFM164_S01g001190), *adrH* (PROQFM164_S01g001189), *adrI* (PROQFM164_S01g001188), *adrJ* (PROQFM164_S01g001187), *adrK* (PROQFM164_S01g001186). *P. chrysogenum* genes are in contig Pc00c22, *adrA-adrK* gene IDs are Pc22g22820- Pc22g22920. SAM—*S*-adenosyl-L-methionine, DMOA—3,5-dimethylorsellinic acid.

An interesting point in the andrastin A biosynthetic cluster is the pseudogenization of one of the genes of the *P. chrysogenum* cluster, *adrB*, in *P. roqueforti* [115]. In *P. roqueforti*, the region encoding the first ten amino acids is similar to *P. chrysogenum*; however, deletions after this point on the sequence introduced early stop codons, leaving *adrB* a residual pseudogene [115]. The role of this gene product in andrastin A biosynthesis was not proposed or analyzed in *P. chrysogenum* by Matsuda et al. [121]. *AdrB*-encoded ORF (Pc22g22830) was annotated in the *P. chrysogenum* genome (accession number: AM920437) as a hypothetical protein with no known functional domains. The mRNA transcript for Pc22g22830 was reported in two studies [126,127], indicating that the gene is active; however, the function, if any, in andrastin A production of *P. chrysogenum* remains to be explored.

2.5. PR-Toxin

The PR toxin, the most toxic metabolite of *P. roqueforti* [128], was first described in 1973 as a compound with toxic properties in rats [129]. The compound was extracted from a P. roqueforti strain isolated from contaminated animal feed. Later, the structure of PR toxin was elucidated as an eremophilane-type sesquiterpene [130]. The PR toxin has lethal effects on mice, rats, and cats, significantly damaging internal organs [131]. The toxin inhibits DNA synthesis [132], transcription [133], and protein synthesis [134], and has mutagenic [135] and carcinogenic [136] properties. In addition, it perturbs mitochondrial respiration and oxidative phosphorylation by disturbing the integrity of the mitochondrial membrane and inhibiting the succinate-cytochrome c reductase complex [137]. The cytotoxic effects were also demonstrated on human cell lines, Caco-2 and THP-1, inducing necrosis and inflammatory response in THP-1 cells [138].

Although stored cereals and maize silage are suitable substrates for PR-toxin production [139,140], this toxin was not found in blue cheeses [141]. Cheese production and maturation conditions, such as microaerophilic environment, low pH, and refrigeration temperatures, were determined to be unsuitable for PR-toxin production [142]. In addition, the PR toxin is not stable and is converted to other products, such as less toxic PR imine [43,143], in blue cheeses in the presence of amino compounds [144]. Not only in cheese but also in culture medium, PR toxin is converted to other products, PR imine, PR acid, and PR amide, in time [145,146]. Based on time-course studies, Chang et al. [147] proposed a degradation pathway for PR toxin, in which it is converted into either PR imine or sequentially to PR acid and later PR amide. The major metabolite among these compounds was determined as PR acid. The enzymes PR oxidase and PR amide synthetase, responsible for converting PR toxin to PR acid and PR acid to PR amide, respectively, were purified and characterized [148,149]. Although PR imine was detected in blue cheeses, the presence of PR acid or PR amide remains to be elucidated. The toxicities of PR acid or PR amide are unknown; however, they are not expected to be toxic because the aldehyde group responsible for the biological activity [143] is converted into an acid and an amide in these compounds.

PR toxin biosynthesis starts with the formation of the sesquiterpene aristolochene from farnesyl diphosphate by a terpene cyclase, aristolochene synthase (Figure 5A) [150]. In the biosynthetic pathway, the parent hydrocarbon aristolochene was suggested to be oxidized to 7-*epi*-neopetasone, a compound detected in culture extracts [151,152]. The presence of closely related other compounds, eremofortin A, eremofortin B, and eremofortin C, in culture fluids indicated that these compounds are intermediates in the biosynthetic pathway [153]. However, eremofortin A was also suggested to be a shunt product instead of an intermediate [151]. In a time course study, the production peaks were obtained in the sequential order: eremofortin B, eremofortin C, and later PR toxin, which suggested the biosynthesis pathway followed this sequence [153]. After the formation of 7-*epi*-neopetasone, a series of further oxidations were suggested to take place in the biosynthetic pathway producing eremofortin B, which could be both acetylated and further oxidized to yield eremofortin C [151]. The alcohol group at the C-12 position in eremofortin C is converted to an aldehyde group with toxic biological activity in PR-toxin [143] by eremofortin C oxidase [154,155]. This enzyme was suggested to be an oxidase rather than an alcohol dehydrogenase because it did not require NAD^+ or $NADP^+$ for activity [154].

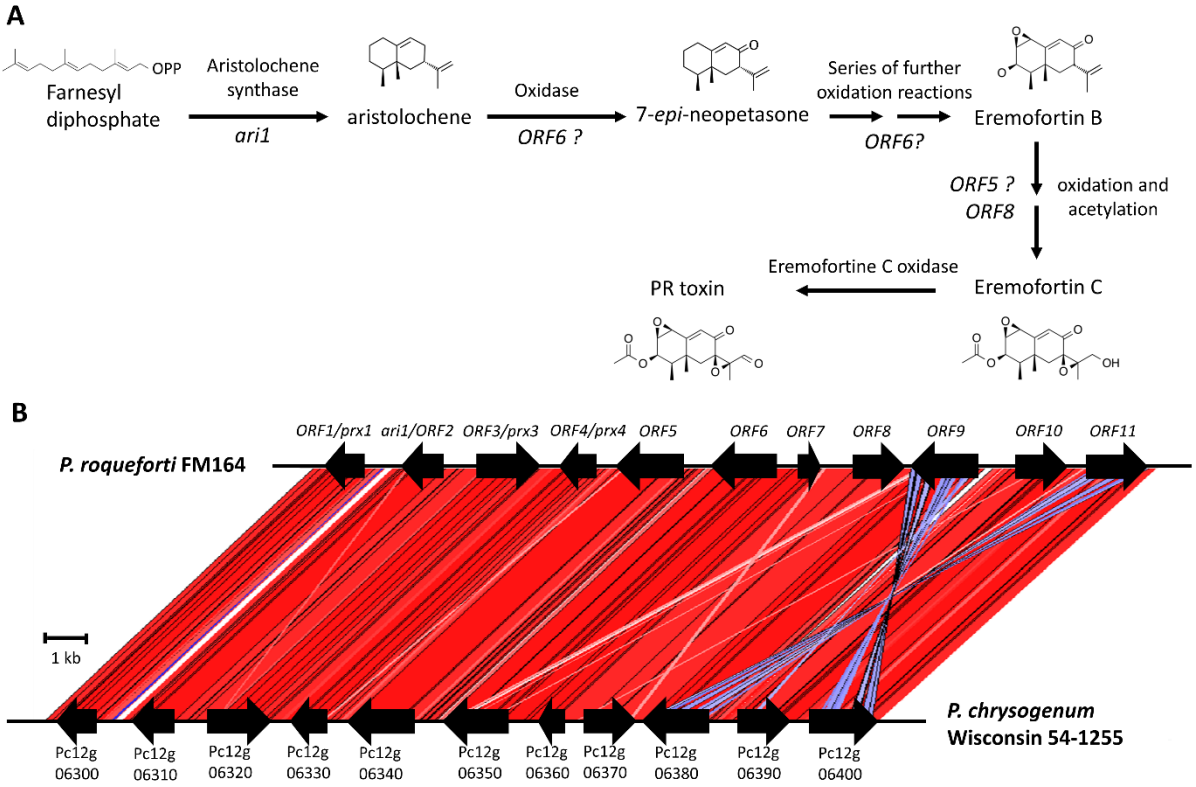


Figure 5. Biosynthesis of PR toxin. (**A**) Proposed biosynthetic pathway for PR-toxin [150–153] (**B**) Biosynthetic gene clusters for PR toxin biosynthesis in *P. roqueforti* and *P. chrysogenum* [156,157]. Sequence IDs for the *P. roqueforti* FM164 genes:. *ORF1/prx1* (PROQFM164_S02g001464), *ari1/ORF2* (PROQFM164_S02g001465),. *ORF3/prx3* (PROQFM164_S02g001466), *ORF4/prx4* (PROQFM164_S02g001467),. *ORF5* (PROQFM164_S02g001468), *ORF6* (PROQFM164_S02g001469), *ORF7* (PROQFM164_S02g001470), *ORF8* (PROQFM164_S02g001471), *ORF9* (PROQFM164_S02g001472), *ORF10* (PROQFM164_S02g001473), *ORF11* (PROQFM164_S02g001474). *P. chrysogenum* Wisconsin 54-1255 gene IDs are shown in the figure.

The gene coding for aristolochene synthase in *P. roqueforti* was determined to be *ari1*, revealed by overexpression in *E. coli* [158,159]. The search of this gene in a genomic library of the *P. roqueforti* type strain CECT 2905 revealed three additional genes in the vicinity of *ari1* (*prx2*, *prx* representing PR-toxin), named *prx1*, *prx3*, and *prx4*, coding for a short-chain oxidoreductase/dehydrogenase, an oxidase, and an alcohol dehydrogenase, respectively (Figure 5B) [156]. Silencing of each of these genes resulted in a reduction in PR toxin production, indicating the role of these genes in the biosynthetic pathway [156]. Later, when the *P. roqueforti* genome became available, more genes were revealed, resulting in a cluster spanning 22.4 kb and harboring 11 genes. In addition to *prx1-prx4*, which were named *ORF1-ORF4* in this later study, seven additional genes, *ORF5-ORF11*, were proposed to be part of the cluster (Figure 5B) [157]. While *ORF5*, *ORF6*, *ORF9*, and *ORF11* have cytochrome P450 domains, *ORF8* and *ORF10* have transferase and regulatory domains, respectively. There is also a gene (*ORF7*) with a domain of unknown function (DUF3237). Silencing *ORF5*, *ORF6*, and *ORF8* reduced PR toxin production levels, indicating that these genes are involved in PR toxin biosynthesis [157]. Silencing these genes also reduced eremofortin A, suggesting that the products of these genes take roles in the biosynthetic pathway before eremofortin A production. On the other hand, while *ORF6* silencing reduced the level of eremofortin B production, silencing *ORF5* and *ORF8* resulted in eremofortin B accumulation. This result indicates that *ORF6* has a function preceding eremofortin B production, whereas the roles of *ORF5* and *ORF8* are in later steps. While *ORF8* presumably functions in the transfer of the acetyl group, *ORF5* and *ORF6* have

cytochrome P450 domains responsible for oxidation reactions. *ORF10* encodes a regulatory protein, probably functioning in regulating the gene cluster.

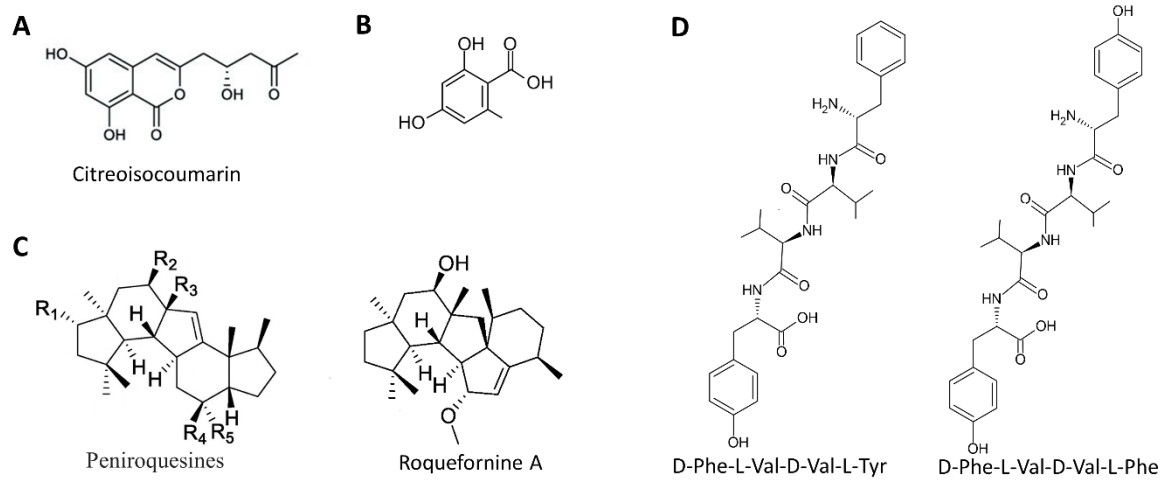
PR toxin gene clusters of *P. roqueforti* and *P. chrysogenum* are highly syntenic, including the intergenic regions (Figure 5B). The high similarity in gene content, gene directions, and intergenic regions indicates a recent acquisition of this cluster in these two species. The blue stretches between *ORF9* and Pc12g06400, and *ORF11* and Pc12g06380 represent inverse matches observed due to the similarity between *ORF9* and *ORF11*. They both encode cytochrome P450 monooxygenases, with 37.5% similarity at the amino acid level.

An interesting finding was the effect of the PR toxin on mycophenolic acid production. *Prx*-silenced strains produced significantly higher amounts of mycophenolic acid, indicating a common control mechanism [156].

2.6. Other Secondary Metabolites of P. roqueforti

2.6.1. Citreoisocoumarin

Citreoisocoumarin (Figure 6A) was produced by 42% of the *P. roqueforti* silage isolates (n = 79) [23]. This compound has been isolated more consistently from a closely related species, *Penicillium paneum*, such that all isolates (n = 78) were reported to produce citreoisocoumarin [23]. This metabolite has also been reported in other *Penicillium* species, such as *P. carneum* [24], *P. buchwaldii* [160], *P. citreoviride* [161], *P. nalgiovense* [162], and *P. vinaceum* [163], and other fungal genera, e.g., *Nectria* [164] and *Fusarium* [165]. Neither the biosynthetic gene cluster nor the biosynthetic pathway has been identified for *P. roqueforti*. However, in *Fusarium graminearum*, the pigment aurafusarin and citreoisocoumarin are synthesized by the same polyketide synthase, and the biosynthetic pathway is redirected from aurafusarin to citreoisocoumarin under nitrogen starvation conditions [165].



	1	2	3	4	5	6	7	A	B	C
R_1	H	H	OH	H	OH	OH	OH	H	H	H
R_2	OH	OH	H	H	H	H	OH	OH	OH	OH
R_3	CH_3	CH_3	CH_3	CH_3	CH_3	CHO	CH_3	CH_3	CH_3	CH_3
R_4	H	=O	H	OH	OH	OH	H	OH	H	H
R_5	OH	-	H	H	H	H	H	H	H	H
R_6	H	H	H	H	H	H	H	H	H	OH

Figure 6. Other secondary metabolites of *P. roqueforti*. (**A**) Citreoisocoumarin, (**B**) Orsellinic acid, (**C**) Sesterterpenes: Peniroquesines A–C and 1–7, and Roquefornine A, (**D**) Tetrapeptides, D-Phe-L-Val-D-Val-L-Phe and D-Phe-L-Val-D-Val-L-Tyr, and (**E**) *Cis*-bis(methylthio)silvatin.

2.6.2. Orsellinic Acid

Few strains of *P. roqueforti* were shown to produce orsellinic acid (Figure 6B) [24]. Methylated versions of orsellinic acid, 5-MOA, and DMOA are produced by the polyketide synthases in mycophenolic acid and andrastin A biosynthesis, respectively. Whether orsellinic acid is produced as a by-product in these pathways or is synthesized by a different biosynthetic pathway is unknown.

2.6.3. Sesterterpenoids: Peniroquesines A–C and 1–7, and Roquefornine A

P. roqueforti has recently been reported to produce sesterterpenes derived from geranyl farnesyl diphosphate (GFPP), a 25-carbon compound: peniroquesines (A–C, and 1–7), and roquefornine A with pentacyclic ring structures (Figure 6C) [29–31]. Some of these compounds demonstrated strong anti-inflammatory properties and/or cytotoxic activities toward human cell lines [29–31]. All these compounds were obtained from a plant-associated strain of *P. roqueforti*, YJ-14, isolated from *Aconitum vilmorinianum* using solid-state culture conditions. Whether these compounds are also produced by other *P. roqueforti* strains and under cheese production or maturation conditions are interesting questions to be addressed in future studies.

2.6.4. Tetrapeptides D-Phe-L-Val-D-Val-L-Phe and D-Phe-L-Val-D-Val-L-Tyr

Recently, the tetrapeptides, D-Phe-L-Val-D-Val-L-Phe and D-Phe-L-Val-D-Val-L-Tyr (Figure 6D) were detected in all four *P. roqueforti* strains tested and one *P. paneum* isolate [32,33]. These two metabolites were also detected in *Penicillium citrinum* [166], and one (D-Phe-L-Val-D-Val-L-Tyr) was determined in *Penicillium canescens* [167]; both of these studies reported antifungal activities for these tetrapeptides. However, biosynthetic routes and the corresponding genes have yet to be determined in *P. roqueforti*, or other *Penicillium* species reported to produce these metabolites.

2.6.5. *Cis*-bis(methylthio)silvatin

Cis-bis(methylthio)silvatin (Figure 6E) was detected from *P. roqueforti* ATCC10110 (CBS 221.30) in a recent study and demonstrated antibacterial activity for both Gram-positive and Gram-negative organisms [32]. This metabolite is a thiosilvatin, a sulfurous compound originating from the larger class diketopiperazines, cyclic peptides having two amide linkages [168]. Thiosilvatins generally contain two methylated sulfur atoms on the cyclopeptide structure [168]. *Cis*-bis(methylthio)silvatin, the most commonly observed member, has been detected in several *Penicillium* species, e.g., *P. brevicompactum*, *P. crustosum*, *P. commune*, and *P. bilaiae*, in addition to other fungi, such as *Fusarium*, *Trichoderma*, and *Cordyceps* [168]. This compound exerted cytotoxic effects on colon cancer cell lines HCT116 and Caco-2 but with no selectivity toward non-tumoral cell line HCEC [169]. In addition, it exhibited weak cytotoxicity against NS-1 mouse myeloma [170] and P388 murine lymphocytic leukemia cells [171]. The biosynthesis of thiosilvatins is unknown; however, in analogy to sulfide bridge-containing diketopiperazine compounds, such as gliotoxin and sirodesmin, the biosynthesis probably involves nonribosomal peptide synthetases to produce a cyclopeptide followed by sulfurization [172].

2.6.6. Scytalone and Melanin

Scytalone is a known intermediate in DHN–melanin biosynthesis [173] and has been detected in various *P. roqueforti* isolates [33]. The polyketide synthase gene (corresponding protein ID: Pro_LCP9604111_2 | g6432.t1) in *P. roqueforti* was disrupted by the CRISPR/Cas9 approach using the strain LCP9604111, resulting in white-colored mutants, suggesting the involvement of this gene in melanin production [174]. The study did not mention or analyze a gene cluster around the polyketide synthase gene. While in *Aspergillus* species, DHN melanin gene clusters are generally conserved in gene content and harbor six genes, *pksP/alb1*, *abr1*, *abr2*, *arp1*, *arp2*, and *ayg1*; these genes may not be found in a single cluster in *Penicillium* species [173,175]. The pathway is well-established in *A. fumigatus*

(Figure 7A) [176]. The polyketide synthase gene (*pksP/alb1*) is responsible for the production of the heptaketide naphtopyrone, which is converted to 1,3,6,8-tetrahydroxynaphthalene by the product of the heptaketide hydrolase (*ayg1*) [176]. After that, scytalone is produced by the action of the *arp2*-encoded reductase. Later in the biosynthetic pathway, scytalone is dehydrated by the *arp1*-coded scytalone dehydratase to 1,3,8-trihydroxynaphtalene, which is reduced by the reductase Arp2 again to vermelone. Vermelone is then transformed to DHN by the *abr1*-encoded multicopper oxidase, and DHN is polymerized into DHN–melanin by the action of the *abr2*-coded laccase [176].

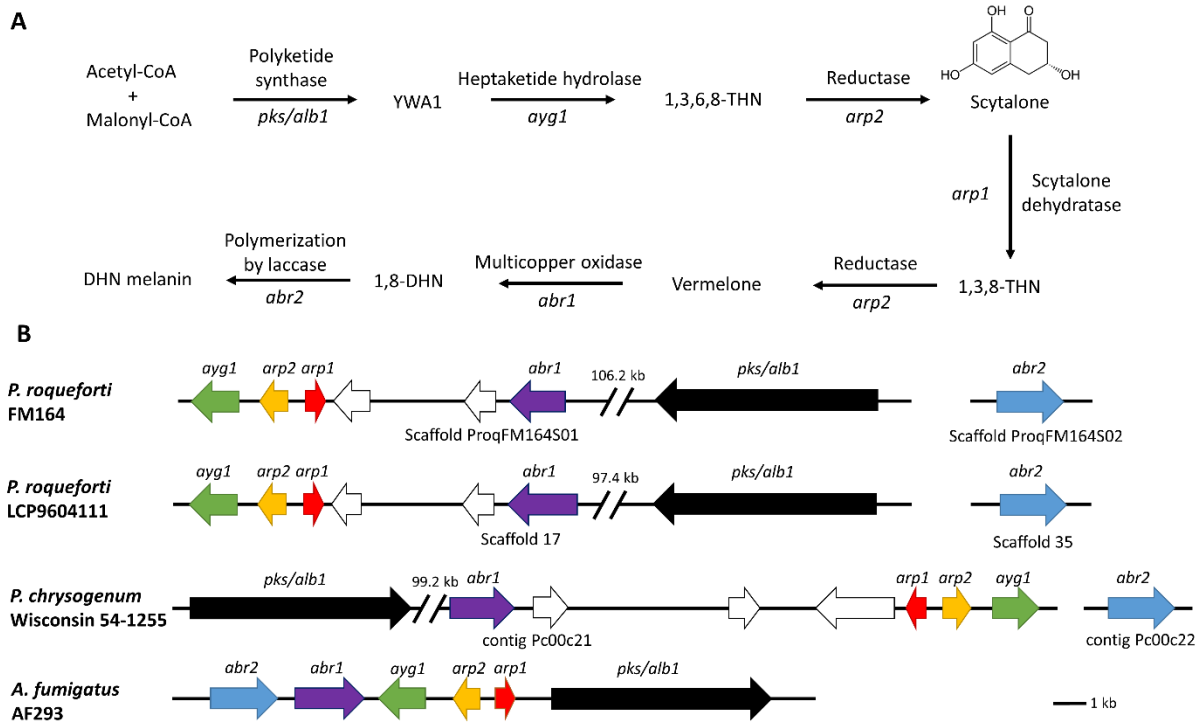


Figure 7. DHN melanin biosynthesis and associated gene clusters. (**A**) DHN melanin biosynthesis pathway [176]. (**B**) DHN melanin biosynthesis genes in *P. roqueforti* FM164 and LCP960411, *P. chrysogenum*, and *A. fumigatus*. Genes shown in white are not found in the *A. fumigatus* DHN melanin gene cluster. The gene IDs for DHN melanin biosynthesis genes for *P. roqueforti* FM164 and LCP960411 genomes are as follows: *ayg1* (PROQFM164_S01g003413, LCP9604111_6390), *arp2* (PROQFM164_S01g003414, LCP9604111_6391), *arp1* (PROQFM164_S01g003415, LCP9604111_6392), *abr1* (PROQFM164_S01g003418, CP9604111_6395), *pks/alb1* (PROQFM164_S01g003466, LCP9604111_6432), *abr2* (PROQFM164_S02g000511, LCP9604111_8601). *P. chrysogenum* Wisconsin 54-1255 gene IDs: *pksP/alb1*: Pc21g16000, *abr1*: Pc21g16380, *arp1*: Pc21g16420, *arp2:* Pc21g16430, *ayg1*: Pc21g16440, *abr2*: Pc22g08420. *A. fumigatus* Af293 gene IDs for *abr2*, *abr1*, *ayg1*, *arp2*, *arp1*, and *pksP/alb1* are AFUA_2G17530, AFUA_2G17540, AFUA_2G17550, AFUA_2G17560, AFUA_2G17580, and AFUA_2G17600, respectively. 1,3,6,8-THN—1,3,6,8-tetrahydroxynapthalene, 1,3,8-THN—1,3,8-trihydroxunaphthalene, 1,8-DHN—1,8-dihydroxynaphthalene.

When the genomes of *P. roqueforti* FM164 and LCP9604111 were searched for the *A. fumigatus* DHN–melanin biosynthetic pathway protein sequences using tblastn, five of the six genes were observed to be on the same contig (Figure 7B). The putative *abr2* gene (PROQFM164_S02g000511 and LCP9604111_02 | g8601.tr1) is located in a separate contig in both genomes. Additionally, the *pks/alb1* is ~100 kb away from the other four genes (*abr1*, *arp1*, *arp2*, and *ayg1*). This configuration is very similar to that in *P. chrysogenum* (Figure 7B). In addition, different genes (shown as white arrows in Figure 7B) have been recruited between *abr1* and *arp1* in *P. roqueforti* and *P. chrysogenum*.

3. Secondary Metabolite Potential of *P. roqueforti*: Yet Undiscovered Metabolites and/or Silent Gene Clusters

The genome sequencing of *P. roqueforti* CECT2905^T indicates the presence of 34 biosynthetic gene clusters [177]. Coton et al. [178] reported a similar but variable number of clusters, 34–37, that changed at the strain level. Few clusters (roquefortine C, clavine alkaloids, mycophenolic acid, andrastin A, and PR-toxin) related to the production of secondary metabolites have been uncovered until now, leaving many unstudied. There might be several alternatives for the remaining gene clusters. First, some of these clusters might be responsible for producing the metabolites whose biosynthetic gene cluster is unknown. For instance, *P. roqueforti* is known to produce citreoisocoumarin, but the associated cluster for this compound has yet to be identified. Second, the remaining clusters might be involved in the production of as-yet-undiscovered metabolites. For example, sesterterpenoids have only recently been discovered in a *P. roqueforti* strain [29–31], and the gene cluster involved in the biosynthesis of these compounds has not been elucidated yet. Third, some clusters identified in the published genomes might be non-functional because modifications, such as gene deletions or acquisition of early stop codons, might have happened; and these changes might prevent the production of the corresponding metabolite. Finally, the cluster might be silent due to an inactive pathway-specific transcription factor or epigenetic regulation [1]. In this case, overexpression of the pathway-specific transcription factor might result in the activation of the cluster. For example, in *Penicillium dangeardii*, the overexpression of the transcription factor *danS* resulted in the production of azaphilons that were not detected in the wild-type strain [179]. Another strategy to activate the silent clusters is by inactivating the epigenetic silencing machinery [12]. For example, the deletion of the histone H3 deacetylase of the endophytic fungus *Calcarisporium arbuscula* activated the transcription of more than 75% of the key metabolite genes, such as polyketide synthases, peptide synthetases, and terpene cyclases, which led to the recovery of new metabolites [180].

An example of the silent clusters in *P. roqueforti* is the recently discovered annullatin D cluster, harboring 11 genes (*anuA-anuK*) (Figure 8) [181]. The cluster harbors genes for a polyketide synthase (*anuA*), short-chain dehydrogenases/reductases (*anuB*, *anuD*, *anuF*, and *anuI*), an unknown protein (*anuC*), a cytochrome P450 oxygenase (*anuE*), a berberine-bridge enzyme (BBG)-like protein (*anuG*), a prenyltransferase (*anuH*), a dehydrogenase (*anuJ*), and a transcription factor (*anuK*) (Figure 8B). When the genomic region *anuA-anuK* of *P. roqueforti* FM164 was heterologously expressed in *Aspergillus nidulans* under the control of the strong constitutive *gpdA* promoter, four annullatin derivatives were detected in the *A. nidulans* culture extract [181]. The fact that annullatins were not observed in *P. roqueforti* FM164 and were not reported in *P. roqueforti* before indicates the silent nature of the cluster. As a result of the deletion of the heterologously expressed genes and feeding experiments with single expressed genes in *A. nidulans*, the authors proposed a biosynthetic pathway for annullatins (Figure 8A) [181]. According to this proposal, the annullatin backbone, 2-hydroxymethyl-3-pentylphenol, is first synthesized by the polyketide synthase anuA and some modification enzymes, which could be anuB and anuC. Then, this intermediate is hydroxylated by the cytochrome P450 AnuE to produce annullatin E, and added an isoprenyl group by the prenyltransferase anuH using a dimethylallyl diphosphate (DMAPP) to yield annullatin J. Afterward, two diastereomers are formed either by an enzymatic or a non-enzymatic way. One of the stereoisomers, (2*S*, 9*S*)-annullatin H, is converted to (2*S*, 9*S*)-annullatin D by the BBG-like enzyme anuG. Meanwhile, anuF dehydrogenates the other isomer to (2*R*)-annullatin F. The authors also detected a small amount of an acetylated form of (2*R*)-annullatin F, which might have been formed via an *A. nidulans* enzyme because the cluster does not have an acetyltransferase gene [181]. In addition, when they deleted *anuE*, they discovered a shunt pathway leading to the (*R*)-configurations of annullatin A and B, the metabolites produced by *Cordyceps annullata* [182]. These two compounds and annullatin D of *C. annullata* have potent agonistic or inverse agonistic activities towards cannabinoid receptors, which are promising therapeutic targets [182]. If the annullatins produced by the *P. roqueforti* cluster have bioactive properties is yet to be analyzed.

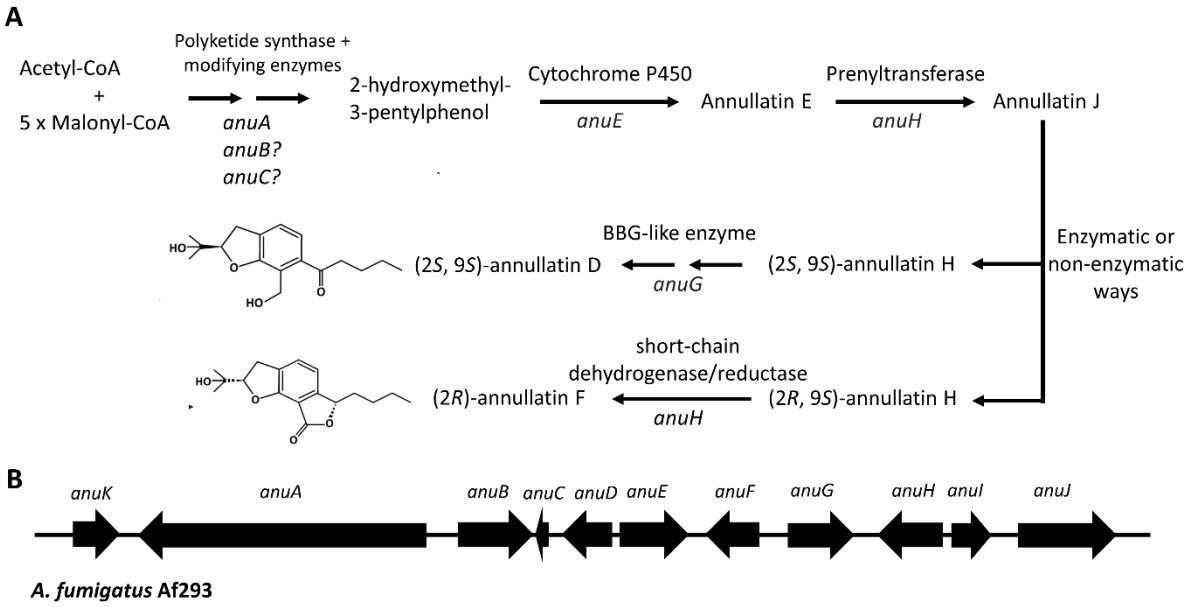


Figure 8. Annullatin biosynthesis and the corresponding gene cluster in *P. roqueforti* (**A**) Annullatin biosynthetic pathway [181] (**B**) Annullation biosynthesis gene cluster in *P. roqueforti* FM 164 [181]. The IDs of the genes in the FM164 genome: *anuK* (PROQFM164_S03g001173), *anuA* (PROQFM164_S03g001174), *anuB* (PROQFM164_S03g001175), *anuC* (PROQFM164_S03g001176), *anuD* (PROQFM164_S03g001177), *anuE* (PROQFM164_S03g001178), *anuF* (PROQFM164_S03g001179), *anuG* (PROQFM164_S03g001180), *anuH* (PROQFM164_S03g001181), *anuI* (PROQFM164_S03g001182), *anuJ* (PROQFM164_S03g001183). BBG—Berberine-bridge enzyme.

4. Conclusions and Future Directions

The blue cheese fungus *P. roqueforti* produces a variety of secondary metabolites. Some of these have long been known, such as mycophenolic acid, PR-toxin, andrastin A, roquefortine C, and clavine alkaloids. The biosynthetic pathways and the associated gene clusters for these metabolites have been mainly uncovered and presented in this review. However, the functions of most genes in clavine alkaloid and PR toxin biosynthetic pathways remain to be discovered. Other metabolites of *P. roqueforti* include citreoisocoumarin, orsellinic acid, and scytalone. While the citreoisocoumarin biosynthetic pathway is unknown, the methylated versions of the orsellinic acid are produced in mycophenolic acid and andrastin A biosynthesis. However, whether there is a separate pathway for orsellinic acid needs further research.

Scytalone is an intermediate compound produced during DHN–melanin biosynthesis. This study deduced the *P. roqueforti* genes putatively taking roles in DHN–melanin biosynthesis using their similarity to their homologs in *A. fumigatus*. Both *P. roqueforti* and *P. chrysogenum* putative DHN–melanin genes are found in two contigs, one harboring the laccase ortholog *abr2* and the other having the remaining five genes. However, these remaining genes are not contiguously organized like in *A. fumigatus*; the polyketide synthase gene, *pks/alb1*, is about 100 kb away from *abr1*, *arp1*, *arp2*, and *ayg1*. Moreover, additional genes have been acquired between *abr2* and *arp1,* both in *P. roqueforti* and *P. chrysogenum*.

There are also recently described metabolites of *P. roqueforti*, e.g., sesterterpenes, peniroquesines and roquefornine A; tetrapeptides, D-Phe-L-Val-D-Val-L-Tyr, and D-Phe-L-Val-D-Val-L-Phe; and *cis*-bis(methylthio)silvatin. The biosynthetic pathways for these compounds remain to be explored.

The secondary metabolites of *P. roqueforti* hold significance for two distinct industries. The first is the food industry, where *P. roqueforti* is a fungal starter culture for blue cheeses. Furthermore, this species is a common contaminant in other cheeses and certain food products. The prevalence of secondary metabolites in foods is of significant concern due to their potential impact on toxicological attributes. Fortunately, the most toxic metabolite

of *P. roqueforti*, known as PR toxin, is unstable under cheese production and maturation conditions and is converted into less toxic other products. Moreover, certain metabolites, such as roquefortine C and clavine alkaloids, are in low quantities in cheeses and are not expected to have significant effects. On the other hand, the quantity of mycophenolic acid in cheeses varies considerably depending on the specific strain used as the starter culture. Despite its pharmacological application as an immunosuppressive agent for organ transplant recipients, this compound has been observed to cause gastrointestinal side effects. Consuming an amount equivalent to the most contaminated cheese might affect the gastrointestinal system. Another metabolite, andrastin A, was consistently detected within cheese matrices, and necessitates further investigation to ascertain its potential positive or adverse effects, if any.

The other sector wherein secondary metabolites assume significance is the pharmaceutical industry. The increasing number of identified genomes of *P. roqueforti* strains underscores a substantial genomic reservoir for the biosynthesis of secondary metabolites. The functional elucidation of one such silent gene cluster, the annullatin biosynthetic gene cluster, was accomplished via heterologous expression in *A. nidulans*. Overexpression of the pathway-specific or global transcription factors and modulation of epigenetic regulatory mechanisms hold promise to activate additional silent clusters of *P. roqueforti*. The comprehensive exploration of the latent potential inherent in the secondary metabolism of *P. roqueforti* carries the prospect of yielding novel pharmaceutical agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090836/s1, Figure S1. Comparison of the roquefortine C gene cluster in *P. roqueforti* FM164 and larger section harboring the meleagrin cluster in *P. chrysogenum* Wisconsin 54-1255.

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Data Availability Statement: No new dataset has been created in this study. The sequences available in the NCBI database were used. The accession numbers used in the analyses are given in the text and the Figure legends.

Conflicts of Interest: The author declares no conflict of interest.

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