Secondary Metabolites in Cheese Fungi

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Contents

Abstract

Several filamentous fungi grow on the surface or inside different types of cheese, produce secondary metabolites, and contribute to the organoleptic characteristics of mature cheese. Particularly relevant is the contribution of *Penicillium roqueforti* to the maturation of blue-veined cheeses (Roquefort, Danablu, Cabrales, etc.). *P. roqueforti* is inoculated into these cheeses as a secondary starter. This fungus is closely related taxonomically to *Penicillium carneum* and *Penicillium paneum*, but these two species are not used as starters because they

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produce the potent toxin patulin. *P. roqueforti* Thom has the capability to produce about 20 secondary metabolites of at least seven different families, but it seems that only some of them are produced in microaerobic conditions and accumulate inside the cheese (e.g., andrastins). This article focuses on the biosynthetic pathways, gene clusters, and relevance of the known metabolites of *P. roqueforti* including roquefortines, PR-toxin and eremofortins, andrastins, mycophenolic acid, clavines (agroclavine and festuclavine), citreoisocoumarin, and orsellinic acid. In addition the biosynthesis of patulin (a *P. paneum* and *P. carneum* product) is discussed. *Penicillium camemberti* grows on the surface of Camembert, Brie, and related white rind cheeses, and the penetration of secondary metabolites inside the cheese is relevant. One of the *P. camemberti* metabolites, cyclopiazonic acid, is important because of its neurotoxicity and its biosynthesis is reviewed. The removal of toxic metabolites gene clusters by precise gene excision while preserving all other characteristics of the improved starter strains, including enzymes involved in cheese ripening and aroma formation, is now open. A possible strain improvement application to the cheese industry is of great interest.

Keywords

Cheese fungi • Blue-veined cheeses • *Penicillium roqueforti* • *Penicillium camemberti* • Secondary metabolites biosynthesis • Roquefortines • PR-toxin • Eremofortins • Andrastins • Mycophenolic acid • Clavine alkaloids • Cyclopiazonic acid

List of Abbreviations

ACP	Acyl-carrier protein
AT	Acyltransferase
ATCC	American Type Culture Collection
DMA-PP	Dimethylallyl diphosphate
DMAT	Dimethylallyltryptophan
DMOA	3,5-Dimethylorsellinic acid
FPP	Farnesyl diphosphate
KS	Ketosynthase
MFS	Major facilitator superfamily
MPA	Mycophenolic acid
6-MSAS	6-Methyl salicylic acid synthase
MT	Methyltransferase
nr-PKS	Non-reductive polyketide synthase
NOX	N1 hydroxylase
RAPD	Random amplified polymorphic DNA
RDH	Roquefortine D dehydrogenase
RPT	Roquefortine prenyltransferase
SAR	Starter unit acyltransferase

1 Introduction

Many *Penicillium roqueforti* strains are used in different countries in the world as secondary starters for the production of blue-veined cheese. More than one hundred of these strains have been characterized morphologically [1], and all of them are closely related to the original type strain described by Charles Thom [2] as *P. roqueforti* Thom ATCC10110. Taxonomically, this strain is referred as *P. roqueforti* subspecies *roqueforti*. Some strains of the *P. roqueforti* cluster (initially identified as *P. roqueforti*) differ in the metabolites that are separated by thin layer chromatography [3] and in the pigmentation of the reverse of the colonies [4]. These authors found a group *of P. roqueforti* strains that are less pigmented and produce patulin instead of PR-toxin and named this group *P. roqueforti* subspecies *carneum* because they are found associated with spoiled meat products.

Later Boysen et al. [5] using rRNA sequences and RAPD (random amplified polymorphic DNA) techniques divided the "*P. roqueforti*" strains into three species, namely, *P. roqueforti* sensu stricto, *Penicillium carneum*, and *Penicillium paneum*. The last one was associated with molded bread, flour, and cereal grains.

Recently Houbraken et al. [6] discovered a new member of the *P. roqueforti* series in cold-preserved apples. This strain, which grows and forms sexual cleistothecia at low temperature, has been classified as *Penicillium psychrosexualis*. It produces patulin as *P. carneum* and *P. paneum* at difference of *P. roqueforti* (Table 1). So far, *P. psychrosexualis* has not been found in cheeses and appears to be mainly associated with fruits such as apples and pears, in which it may produce pigmented spots. Therefore, the relevance of the secondary metabolites of this species in cheese is lower than that of *P. roqueforti*, *P. carneum*, and *P. paneum*.

1.1 Fungal Secondary Metabolites

Frequently filamentous fungi produce a few dozens of molecules belonging to different classes of secondary metabolites (polyketides, terpenes, nonribosomal peptides, aromatic compounds, heterocyclic metabolites, etc.) [7]. Usually they are produced as mixtures of chemically related molecules (e.g., roquefortines C, D, L, M or andrastins A to D). Each family of these compounds derives from a set of enzymes encoded by a gene cluster. Genetic information, in the form of gene clusters, for about 15 to 30 secondary metabolites have been found in the sequenced genome of ascomycetes [8, 9]. In some fungi, several gene clusters have been characterized by genetic and biochemical analysis (e.g., *A. nidulans*, *A. fumigatus*), whereas in others only a small number of secondary metabolite gene clusters has been identified so far (e.g., *P. chrysogenum* or *P. roqueforti*) and many other gene clusters remain cryptic, i.e., encoding unknown products [7, 10]. In addition, a number of gene clusters remain fully silent or nearly silent, although in some cases their expression may be activated by specific methods [10, 11]. In this article, we focus on the study of the

Table 1 Secondary metabolites produced by *P. roqueforti* and the closely related *P. carneum*, *P. paneum*, and *P. psychrosexualis*

Metabolites in *P. roqueforti*	Metabolites of *P. carneum*	Metabolites of *P. paneum*	Metabolites of *P. psychrosexualis*
Agroclavine	Agroclavine	Agroclavine	
Andrastins A, B	Andrastins A, B	Andrastins A, B	Andrastin A
Citreoisocoumarin	Citreoisocoumarin	Citreoisocoumarin	
Eremofortins A, B			
Festuclavine	Festuclavine	Festuclavine	
16-Hydroxyroquefortine	16-Hydroxyroquefortine	16-Hydroxyroquefortine	
		Marcfortins A, B, C	
Mycophenolic acid	Mycophenolic acid	Mycophenolic acid	Mycophenolic acid
Orsellinic acid	Orsellinic acid	Orsellinic acid	
PR-toxin			
Roquefortines C, D, L	Roquefortines C, D, L	Roquefortines C, D, L	Roquefortine C
	Patulin	Patulin	Patulin
	Penitrem A	Penitrem A	
		VM55599	
			"Fumu", uncharacterized

(1) *P. carneum* and *P. paneum* may produce variable amounts of all other *P. roqueforti* secondary metabolites with the exception of eremofortins and PR-toxin (see text). An early description of penicillic acid production in a *P. roqueforti* strain is now explained due to a misclassification of the producer strain

secondary metabolites produced by the cheese fungus *P. roqueforti* (Table 1). The metabolites produced by the related fungi *P. paneum* and *P. carneum*, which are only rarely found in blue cheeses, and *P. psychrosexualis* are listed in Table 1, but they are reviewed succinctly at the end of the chapter (e.g., patulin produced by *P. paneum* and *P. carneum*) [12]. Also the biosynthesis of the neurotoxin cyclopiazonic acid by *Penicillium camemberti* is included in this article.

2   Secondary Metabolites Produced by *P. roqueforti*

In the last decades, increasing evidence has been reported on the ability of *P. roqueforti* to produce secondary metabolites in different culture media and inside the blue cheeses [13, 14]. The biosynthetic pathway of some of these metabolites and the gene clusters encoding their pathways have been located in the genome of the producer fungi [15–18], although the pathways for some of the rare secondary metabolites remain unknown. The full genome sequence of *P. roqueforti* FM164 has been made available [19], and this information will contribute to a better understanding of the ability of this fungus to express the genes encoding secondary metabolites under different growth conditions. So far the information available about

the expression of the genes encoding enzymes for secondary metabolites biosynthesis inside the blue-veined cheeses is very scarce.

2.1   Roquefortines

Roquefortines were discovered many decades ago [20, 21] and are among the best known *P. roqueforti* secondary metabolites [22, 23]. The roquefortine family includes roquefortine C and the related roquefortines D (3, 12-dihydroroquefortine C), 16-hydroxyroquefortine C, roquefortine L, and some other minoritary roquefortines [24]. These compounds are members of the prenylated indole alkaloid class of compounds (Fig. 1) (reviewed in reference [25]). Roquefortine C is produced by *P. roqueforti* growing in a variety of solid substrates, but its formation in blue cheeses does not occur in significant amounts, and there is a consensus that roquefortines in cheese do not pose a health problem for humans [12, 14, 22, 26].

   The compounds of the roquefortine family derive from L-tryptophan, L-histidine, and mevalonate [25]. Roquefortines are produced by several *Penicillium* species including *P. roqueforti*, *P. chrysogenum* [15, 16], and other plant-associated or saprophytic fungi [27, 28]. However, it was unknown if the biosynthetic pathway of roquefortine alkaloids is identical in all these fungi. Recent evidence [18] demonstrated that the roquefortine pathway in *P. roqueforti* is shorter than that for roquefortine/meleagrin in *P. chrysogenum* (see below).

2.1.1   Biosynthesis of Roquefortine and Meleagrin

One of the common early intermediates of prenylated indole alkaloids is a molecule of dimethylallyl-tryptophan (DMAT) that is formed by a prenyltransferase that uses L-tryptophan (or a L-tryptophan-containing cyclopiperazine dipeptide) and dimethylallyl diphosphate (DMA-PP) as substrates [15, 16, 25]. In roquefortine alkaloids, precursor condensation of these substrates occurs at C-3 of L-tryptophan with the 3′carbon atom of DMA-PP (named "reverse condensation") [29].
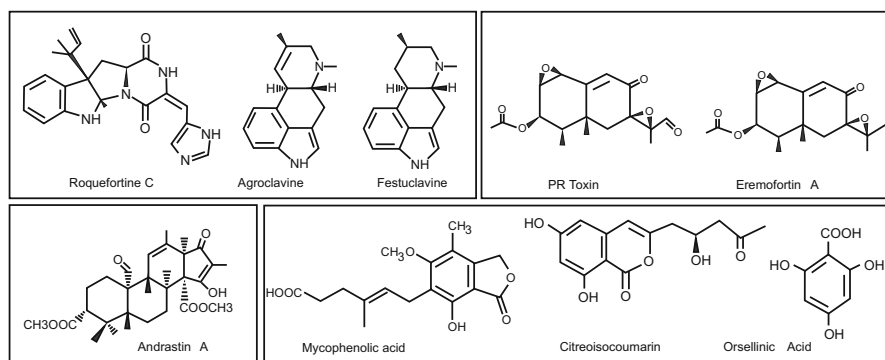
Fig. 1 Structure of the major secondary metabolites produced by *P. roqueforti*. The metabolites produced by *P. carneum*, *P. paneum*, and *P. camemberti* are not included in the figure

Following initial precursor studies in *P. roqueforti* [30, 31], it was established that the tryptophan-histidine cyclopiperazine nucleus of roquefortine C and the related compounds glandicolines A and B, meleagrin, and neoxaline derive from the precursor compounds L-tryptophan and L-histidine. These two amino acids are condensed by a dimodular nonribosomal peptide synthetase, named RDS (roquefortine dipeptide synthetase) consisting of two similar modules with the domain sequence ATCATC, where A indicates adenylation domain (amino acid activation), T thiolation (peptidyl carrier) domain, and C condensation domain. The amino acid specificity of each domain has been elucidated [15, 25]. The cyclodipeptide (*cyclo*-trp-his) is then prenylated by the roquefortine prenyltransferase (RPT) that introduces an isopentenyl group at C-3 of tryptophan (Fig. 2b). The resulting prenylated compound is roquefortine D (3, 12-dihydroroquefortine C). In the last step of the roquefortine pathway, roquefortine D is oxidized by the roquefortine D dehydrogenase (RDH), losing two H atoms with the formation of a double bond between carbons 3 and 12 resulting in roquefortine C (Fig. 2b). The order of the second and third biosynthetic reactions
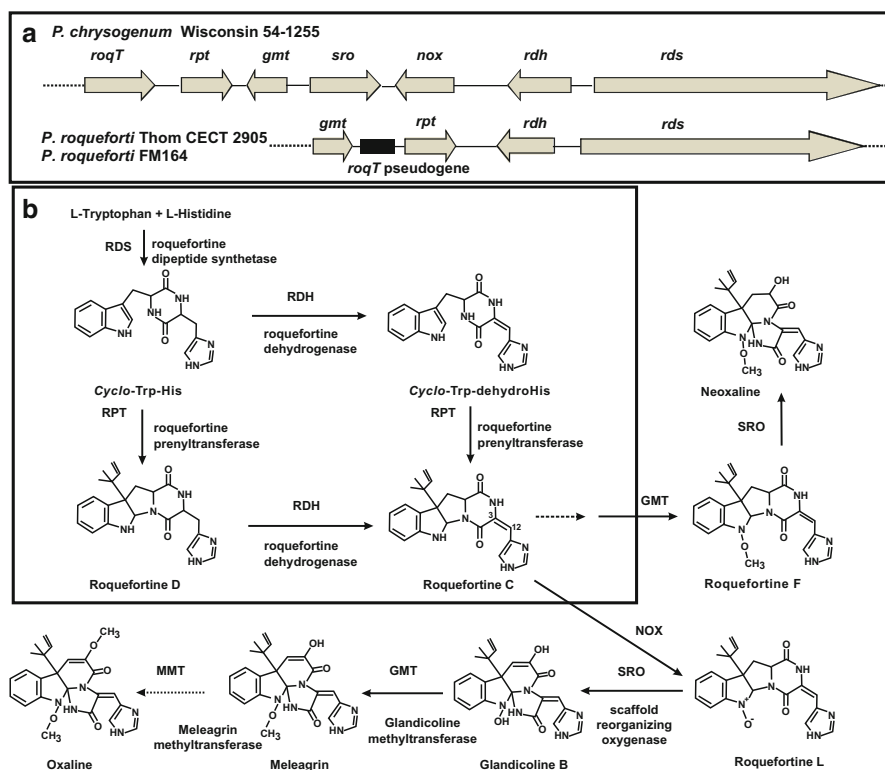
Fig. 2 (**a**) Gene cluster for roquefortine/meleagrin in *P. chrysogenum* compared to the roquefortine cluster in *P. roqueforti*. (**b**) Biosynthetic pathway of roquefortine C (*boxed area*), meleagrin, neoxaline, and oxaline in *P. chrysogenum* (Modified from Ref. [28])

(prenylation and roquefortine dehydrogenation) is indifferent [16], and therefore, a metabolic grid occurs in these early steps of the pathway [25].

In some fungi (e.g., *P. chrysogenum* and *P. oxalicum*), roquefortine C is later converted to roquefortine L (or in other fungi to glandicoline A) and then to glandicoline B and meleagrin (see structure in Fig. 1) [31]. The conversion of roquefortine C (or roquefortine L in *P. chrysogenum*) to the late pathway products involves (i) a carbon scaffold reorganizing oxygenase (SRO), similar to the FtmG oxygenase of *Aspergillus fumigatus* involved in fumitremorgin biosynthesis, and (ii) a N1 hydroxylase (NOX) and a N-OH methyltransferase (Fig. 2b). The role of each of these enzymes in the roquefortine and meleagrin pathways has been reviewed elsewhere [25, 28].

2.1.2 The Roquefortine/Meleagrin Gene Cluster

Molecular genetic studies on the roquefortine/meleagrin gene cluster (*roq/mel*) were performed first in *P. chrysogenum* Wis54-1255 leading to the characterization of the gene cluster and the proposal of a roquefortine/meleagrin biosynthetic pathway [15]. The roquefortine/meleagrin gene cluster was later confirmed by Ali et al. [16] and Ries et al. [24], who reported the formation in *P. chrysogenum* of roquefortine L (instead of glandicoline A) as an intermediate in the pathway, in addition to other minoritary roquefortines derived from late branches of the pathway. The entire pathway in *P. chrysogenum* is encoded by a seven-gene cluster (Pc21g15420 to Pc21g15480) (Fig. 2a).

An important question is if the many natural isolates (strains) of *P. roqueforti* obtained from different geographical areas [1] have genetic differences in their capability to synthesize roquefortine and the related indole alkaloids [14, 26] and whether these differences are due to changes in the roquefortine gene cluster.

The roquefortine gene cluster of *P. roqueforti* has recently been investigated [18]. The initial steps of roquefortine biosynthesis in *P. roqueforti* are identical to those of *P. chrysogenum*, but we found that *P. roqueforti* lacks the genes that encode the enzymes for the "late" conversion of roquefortine C to roquefortine L, glandicoline B, and meleagrin [18]. A natural short pathway was found in *P. roqueforti* that is dedicated to the production of roquefortine C but is unable to form derivatives containing the meleagrin scaffold [18, 28].

A comparative analysis of the *roq* cluster of *P. roqueforti* and the *roq/mel* cluster of *P. chrysogenum* revealed that two key genes located in the central region of the *roq/mel* cluster in *P. chrysogenum* (*sro* and *nox*) have been lost in *P. roqueforti* during evolution and the order of two of the conserved genes has changed during gene reorganization. Furthermore, the *roqT* gene, encoding a transmembrane transport protein in *P. chrysogenum*, has been rearranged into a pseudogene (Fig. 2a) that encodes only residual peptides [18, 28]. As a result of the *roq/mel* cluster reorganization, *P. roqueforti* is unable to convert the roquefortine-type carbon skeleton into a meleagrin-type scaffold and is incapable to produce glandicolines. The cluster reorganization is not a recent event derived from "industrial" strain selection. Rather, it seems to be an ancient phenomenon that occurred probably millions of years ago during adaptation of a progenitor *Penicillium* to cheese environments [28].

PR-Toxin and Eremofortins PR-toxin and eremofortins are isoprenoid secondary metabolites. PR-toxin is probably the most potent mycotoxin produced by *P. roqueforti* [12, 23]. This isoprenoid mycotoxin is clearly toxic for mice, rats, hamsters, and some domestic animals in vivo. Furthermore, PR-toxin has mutagenic action in vitro, as shown in studies using the Ames test. Actually, PR-toxin is considered to be the causative agent of cow toxicosis produced by poorly conserved moldy silages [12]. Fortunately, PR-toxin is modified to less toxic derivatives by *P. roqueforti* cells, and its toxic form does not seem to be accumulated in large amounts in blue cheese [22].

Recently, we have studied the biosynthesis of PR-toxin and its intermediates the eremofortins [17]. PR-toxin derives from the sesquiterpene (15 carbon atoms) aristolochene; this first intermediate is formed by aristolochene synthase (encoded by the gene *ari*1). Hidalgo et al. [17] cloned and sequenced a partial PR-toxin cluster containing four genes that include the *ari*1 (*prx*2) gene reported previously in *P. roqueforti* (Fig. 3a). Gene silencing of each of the four genes, named *prx*1 to *prx*4 (*prx,* abbreviation for PR-toxin), caused a reduction of 65–75 % in the production of PR-toxin indicating that these four genes encode enzymes involved in PR-toxin biosynthesis. An eleven gene cluster (Pc12g06260 to Pc12g06370) that includes the above-mentioned four *prx* genes and a 14-TMS (transmembrane spanner domain) drug/H^+ antiporter of the MFS family was found in the genome of *P. chrysogenum* (Fig. 3a). A detailed analysis of the published genome sequence of *P. roqueforti* FM164 [19] revealed that this strain contains in two subclusters 10 of
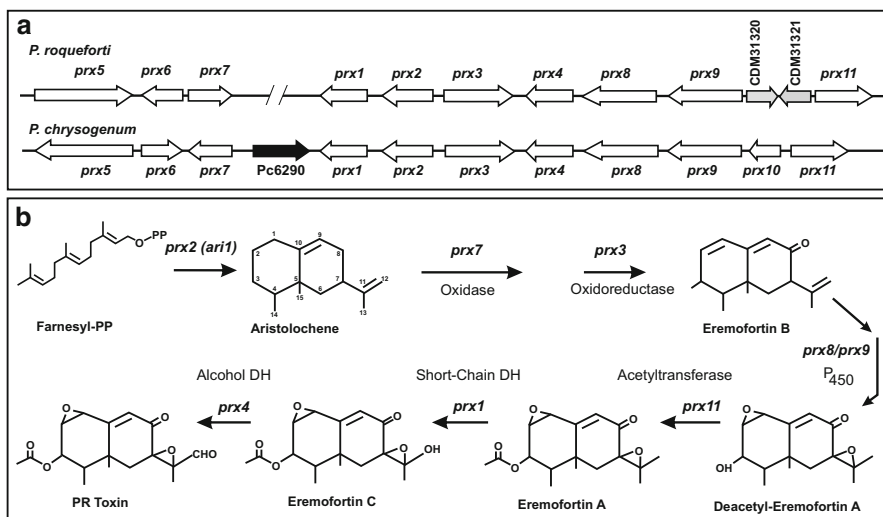
Fig. 3 (**a**) Gene clusters of PR-toxin in *P. chrysogenum* Wis 54–1255 and *P. roqueforti* FM164. The location of a pseudogene in the *P. chrysogenum* cluster (*shadowed in black*) is shown. In *P. roqueforti*, three of the *prx* genes (*prx*5 to 7, proteins CDM35712 to 35710) are located elsewhere (Contig Proq06g) in the genome in relation to the *prx*1 to *prx*11 gene cluster (contig Proq02g, proteins CDM31314 to 31322). (**b**) Proposed PR-toxin biosynthetic pathway (see text for details)

the 11 *prx* genes described in *P. chrysogenum*. The exception is *prx*10 that was reported as encoding a protein of unknown function [17]. As shown in Fig. 3a, seven of the 10 *prx* genes (*prx* 1 to 4, *prx* 8, 9, and 11) are clustered together in contig Proq02g of *P. roqueforti*, whereas the three remaining genes (namely, *prx*5, 6, and 7) are located elsewhere (contig Proq06g) in the genome.

PR-toxin biosynthesis pathway from farnesyl diphosphate was proposed based on all available evidence [17]. It proceeds to PR-toxin through aristolochene and the eremofortins (Fig. 3b). The PR-toxin pathway is divided in two parts. The first part corresponds to the conversion by oxidative enzymes of the 15-carbon atom aristolochene to 3-hydroxy, 8-oxo, 12-dehydroaristolochene, eremofortin B, and deacetyl-eremofortin A (DAC-EreA), all containing 15 carbon atoms. In the second half, DAC-eremofortin A is acetylated to the 17-carbon eremofortin A by an acetyltransferase encoded by *prx11*, and then eremofortin A is converted to eremofortin C and finally to PR-toxin.

Both eremofortins and PR-toxin are probably secreted by the MFS transporter encoded by the *prx5* gene in the *prx* cluster, as proposed for several antibiotics and other secondary metabolites [32].

The PR-toxin is converted in vitro and probably also in vivo to PR-amide and PR-imine by reaction of the PR-toxin carboxylic group with ammonium ions or primary amines in the culture medium or in the cells [33, 34], and these derivatives appear to be less toxic than the PR-toxin itself.

2.2 Andrastins

Another important family of *P. roqueforti* secondary metabolites is the andrastins that belong to the polyketide-isoprenoid class. They are inhibitors of the farnesyl-transferase of the *ras*-encoded oncogenic protein [35, 36]. Prenylation (farnesylation) of the human Ras protein is essential for its biological activity that may cause tumor formation. Therefore, inhibitors of the Ras prenyltransferase activity are interesting for their use as potential antitumor agents [37].

The andrastins belong to the meroterpenoid class of secondary metabolites that include compounds with interesting pharmacological activities [38]. Andrastins A, B, C, and D were discovered by S. Omura (Nobel Prize 2015) and coworkers at the Kitasato Institute in Japan in a screening of antitumoral agents. These compounds were first identified in the culture broth of *Penicillium* sp. FO4259 [36, 39, 40], and they are produced by several other *Penicillium* species [41].

Nielsen et al. [13] and Fernández-Bodega et al. [14] found that *P. roqueforti* produces andrastins and that andrastin A (the final product of the biosynthetic pathway) is accumulated inside blue cheeses inoculated with *P. roqueforti* as a secondary starter. Andrastin A concentrations in different blue cheeses such as Roquefort, Danablu, Cabrales, Bejes-Tresviso, and Valdeón vary depending on the particular *P. roqueforti* strain used as starter and ripening conditions [14]. Andrastins are considered to be beneficial for human health because of their *ras*

prenyltransferase inhibitory activity, but there are no studies that support its lack of toxicity when accumulated in high concentrations in cheese.

2.2.1   Biosynthesis of Andrastins

Initial precursor incorporation studies showed that the andrastins derive from 3, 5-dimethylorsellinic acid (DMOA) and the terpene precursor farnesyl diphosphate (FPP) [39]. In fungi, orsellinic acid is formed by the condensation of one unit of acetyl-CoA (starter unit) and three units of malonyl-CoA (elongation unit) followed by cyclization of the tetraketide to form the aromatic ring of orsellinic acid. These reactions are catalyzed by a specific nonreducing polyketide synthase (nr-PKS). The precursor incorporation studies suggested that the two methyl groups of DMOA derive from methionine [39] although it is not entirely clear if the incorporation of the methyl groups occurs during polyketide elongation or after orsellinic acid is formed. Based on the information available on the molecular genetics of the biosynthesis of other farnesylated-DMOA-derived fungal metabolites (e.g., austinol or terretonin), Matsuda et al. [42] identified a gene cluster encoding enzymes for andrastin biosynthesis in *P. chrysogenum*. The biosynthetic pathway of farnesyl-DMOA containing meroterpenoids [38] indicates that DMOA is converted to farnesyl-DMOA by a specific farnesyltransferase and then the farnesyl-DMOA is converted into farnesyl-DMOA methyl ester by the action of methyltransferase. A FAD-dependent monooxygenase converts the terminal double bond of farnesyl-DMOA methyl ester into its epoxy derivative (Fig. 4b).

The epoxy farnesyl-DMOA methylester is later cyclized to a polycyclic meroterpenoid by a characteristic terpene cyclase. The cyclases of each meroterpenoid gene cluster may yield a (slightly) different cyclic structure [43]
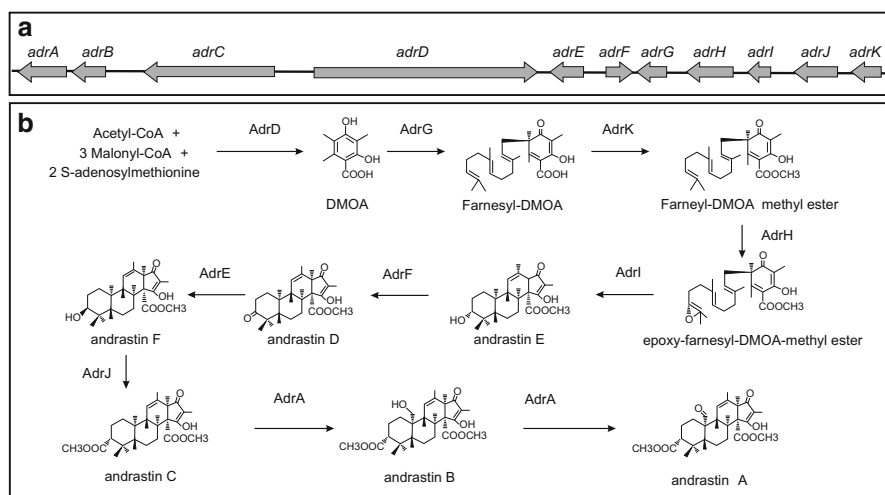
Fig. 4 (**a**) Gene cluster for andrastin in *P. chrysogenum* (see text for details). (**b**) Biosynthetic pathway of andrastins (Modified from Ref. [42])

that are converted to different final meroterpenoid molecules by "late" modification enzymes (so-called tailoring enzymes) (Fig. 4b).

2.2.2   Andrastins Gene Cluster

The andrastin A gene cluster of *P. chrysogenum* (Fig. 4b) comprises eleven genes (Pc22g22820 to Pc22g22920) of which nine correspond to enzymes that are directly involved in andrastin A (the most modified final product) biosynthesis. These nine enzymes include (i) an iterative type I, nonreductive polyketide synthase that forms DMOA (named AdrD); (ii) a prenyltransferase that attaches the farnesyl group of FPP to the DMOA moiety (AdrG); (iii) a methyltransferase that methylates the carboxyl group of farnesyl-DMOA forming farnesyl-DMOA methyl ester (AdrK); (iv) a FAD-dependent monooxygenase that converts farnesyl-DMOA methyl ester to epoxy-farnesyl-DMOA methyl ester (AdrH); (v) a terpene cyclase (AdrI) that cyclizes the epoxyfarnesyl-DMOA methyl ester intermediate to form andrastin E, the first member of the andrastin family; and (vi) four additional tailoring enzymes that convert andrastin E to andrastins D, F, C, B, and A (final product) (Fig. 4b). These tailoring enzymes include a short-chain dehydrogenase (AdrF), a ketoreductase (AdrE), an acetyltransferase (AdrJ) forming andrastin C, and finally a P450 monooxygenase (AdrA) involved in the consecutive oxidations of the C-23 methyl group of andrastin C to form andrastin B and then andrastin A that contain an alcohol and an aldehyde group at the C-23 position, respectively (Fig. 4b). The involvement of these 9 genes in andrastin A biosynthesis was confirmed by heterologous expression of a reconstructed gene cluster in *Aspergillus oryzae* that resulted in the production of andrastin A [42]. The andrastin gene cluster of *P. roqueforti* has not been characterized so far, although is likely to be similar to that of *P. chrysogenum*.

2.3   Mycophenolic Acid

Another important secondary metabolite of *P. roqueforti* is mycophenolic acid (MPA). This compound was already known at the beginning of the twentieth century, before the discovery of penicillin, as an antibiotic active against *Bacillus anthracis*, produced by a *Penicillium* sp. strain. Production of MPA in liquid cultures has been studied in *Penicillium brevicompactum* [44], the fungus which is used for MPA industrial production and in *P. roqueforti* [17]. Mycophenolic acid, discovered initially as antibacterial agent, was later found to have other important biological activities [45]. Particularly relevant is its activity as immunosuppressant used successfully to prevent organ rejection in transplants [46]. In addition, MPA has antitumor, antiviral, and antifungal activities and is used in the treatment of psoriasis [47–51].

2.3.1   Mycophenolic Acid Biosynthesis and Resistance Genes

Initial precursor incorporation studies [52] suggested that MPA is a compound synthesized through the hybrid polyketide-terpene pathway. Recently, the *mpa*

gene cluster was cloned from a *P. brevicompactum* strain [53] and later confirmed in the sequenced genome of a different *P. brevicompactum* strain [54]. In both strains, the *mpa* cluster comprises seven genes (Fig. 5), namely, *mpaA* (encoding a prenyltransferase), *mpaB* (encoding a protein of unknown function), *mpaC* (encoding a polyketide synthase), *mpaDE* (encoding a bifunctional fused protein with two domains corresponding to a P450 monooxygenase and a hydrolase), *mpaF* (encoding an inosine-5′-phosphate dehydrogenase), *mpaG* (encoding an O-methyltransferase), and *mpaH* (encoding an oxidative cleavage enzyme).

A key enzyme in MPA biosynthesis is the non-reductive iterative PKS encoded by *mpaC*. This protein contains the following domains: a starter unit acyltransferase (SAT), a ketosynthase (KS), an acyl-carrier protein (ACP), a methyltransferase (MT), and a standard acyltransferase (AT). These activities are required for the synthesis of the MPA intermediate 5-methylorsellinic acid from one starter acetyl-CoA, three malonyl-CoA extender units, and a methyl group.

Involvement of the *mpaC* gene encoding the non-reductive PKS and *mpaDE* encoding the bifunctional P450 monooxygenase-hydrolase in MPA biosynthesis has been confirmed by disruption of these genes in *P. brevicompactum* and by their expression in the heterologous host *Aspergillus nidulans*, a nonproducer of MPA that lacks the orthologous genes [53, 55]. More recently Zhang et al. [54] proved that *mpaG* encodes a S-adenosylmethionine (SAM)-dependent O-methyltransferase that converts in vitro demethylmycophenolic acid to MPA, the last step in the pathway (Fig. 5). This methyltransferase was purified after expression of the *mpaG* gene in *E. coli.* The enzyme showed similar substrate kinetics to O-methyltransferase
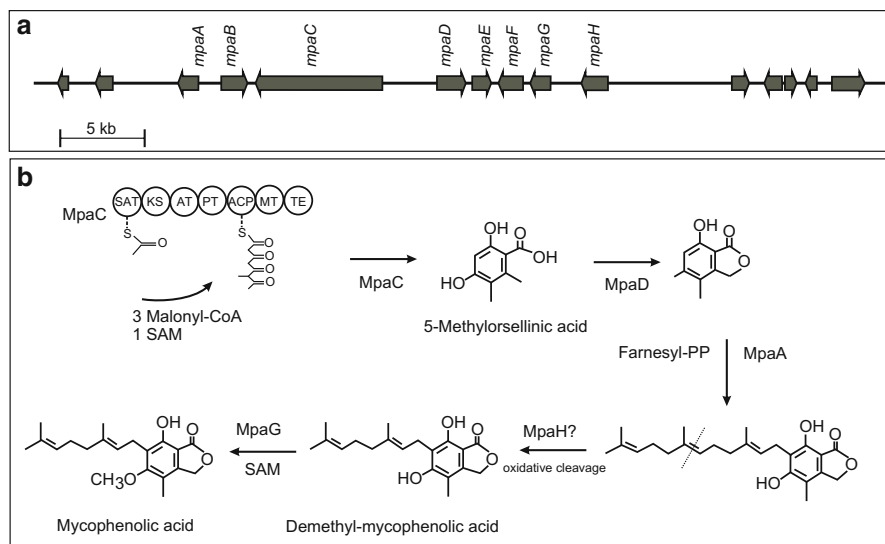
Fig. 5 (**a**) Gene cluster of mycophenolic acid in *P. brevicompactum.* (**b**) Proposed biosynthetic pathway for mycophenolic acid (Modified from Ref. [53])

obtained from *P. stoloniferum* (gene not yet cloned), another mycophenolic acid-producing fungus.

The MPA-producing fungi have to protect themselves against the antifungal activity of MPA. This resistance to MPA appears to be exerted by an IMP-dehydrogenase encoded by *mpaF* [56], although other mechanisms such as active MPA secretion and lack of uptake of the secreted extracellular MPA may also contribute to the resistance as occurs with other secreted metabolites [32].

Mycophenolic acid is active against fungi and human lymphocytes (involved in immune response) because it exerts a strong inhibition of the inosine-5′-phosphate (IMP) dehydrogenase, a key enzyme in *de novo* purine biosynthesis in those cells that lack the purine recycling pathway (as it is the case in lymphocytes). Overexpression of the *mpaF* gene in *A. nidulans* drastically increases the resistance to MPA in this fungus [55, 56]. Indeed, these authors reported that six different fungi, including those that produce MPA and also some putative nonproducers, contain two IMP dehydrogenase genes, one of them presumably located within the *mpa* gene cluster [56].

While this manuscript was in the proof stage, a recent report described the mycophenolic acid gene cluster in *P. roqueforti* (see note added in proof).

2.4 Agroclavine and Festuclavine

There are two subgroups of alkaloids produced by fungi: (i) the clavine alkaloids represented by fumigaclavine, synthesized by *A. fumigatus* [57], and agroclavine and festuclavine produced by *P. roqueforti*, and (ii) the lysergic acid-containing ergot peptide alkaloids produced by species of *Claviceps* [58]. Several *P. roqueforti* strains of different origins produce the clavine-type alkaloids agroclavine and festuclavine (Fig. 1). Festuclavine is also produced by *P. carneum* [12].

The clavine alkaloids have a tricyclic or tetracyclic structure with small structural differences between them. There are no detailed studies on the biosynthesis of agroclavine and festuclavine in *P. roqueforti*, but the biosynthesis of fumigaclavines in *A. fumigatus* [57] and ergot alkaloids in *Claviceps purpurea* [58] has been extensively studied.

2.4.1 Biosynthesis of Agroclavine and Festuclavine

All these compounds derive from the precursors L-tryptophan, dimethylallyl diphosphate, and the methyl group of methionine. The first step in the biosynthesis of these clavine alkaloids is prenylation of L-tryptophan at C-4 to form 4-dimethylallyltryptophan (DMAT) by the enzyme DMAT synthase, a prenyltranferase encoded by the gene named *fga*PT2 in *A. fumigatus* (or *dmaW* in *Claviceps*). In the second step, the primary amino group of DMAT is methylated using S-adenosylmethionine as methyl donor. The N-methyltransferase is encoded by the *fga*MT gene located in an 11-gene cluster that comprises all genes involved in the pathway [59, 60]. The N-methyl-DMAT is then cyclized and oxidized to chanoclavin-1 by a FAD-containing oxidoreductase named chanoclavine synthase,

encoded by the *casA* gene (also named *case* by other authors) [61]. This conversion also requires the product of a cluster-located gene, *casC*, encoding a catalase-like protein that is involved in oxidation of the 3′-methyl group of the dimethylallyl moiety (derived from DMA-PP) to a CH_2OH [62]. Disruption of this catalase-like gene results in the interruption of the clavine or ergot alkaloids biosynthetic pathways in the producer organisms with accumulation of the N-methyl-DMAT intermediate. The exocyclic alcohol group of chanoclavine-I is then oxidized to form chanoclavine-1-aldehyde by the enzyme chanoclavine-1 dehydrogenase encoded by the *fgaDH* gen (also named *casD*) [58, 63]. The chanoclavine-1-aldehyde is the branching point intermediate in the biosynthesis of the different clavines and lysergic acid-containing alkaloids in different fungi. *P. roqueforti* produces the tetracyclic compound festuclavine that in *A. fumigatus* (but apparently not in most *P. roqueforti* strains) is later converted to fumigaclavine. The conversion of the tricyclic intermediate chanoclavine-1-aldehyde to festuclavine requires two enzymes encoded by the genes *fgaFS* and *fgaOx3* [64]. The exact mechanism by which these two enzymes convert chanoclavine-1-aldehyde to festuclavine is still a matter of debate [64, 65]

The second tetracyclic clavine alkaloid produced by *P. roqueforti* is agroclavine. Agroclavine differs from festuclavine in that the former has a double bond between carbons 8 and 9 that is already present in the previous intermediate chanoclavine-1-aldehyde but is saturated (reduced) in festuclavine (Fig. 1), suggesting that there is an enzyme activity involved in the reduction of the double bond. At difference of *P. roqueforti*, *A. fumigatus* does not seem to accumulate agroclavine, probably because the pathway continues to fumigaclavine.

Formation of agroclavine from chanoclavine-1-aldehyde (the branching point intermediate) has been reported in *Claviceps* [66]. The agroclavine synthase EasG of *Claviceps* is a homologous enzyme to festuclavine synthase of *A. fumigatus*. Indeed, Cheng et al. [65] reported that the festuclavine synthase of *A. fumigatus* (about 65 % similarity to the agroclavine synthase of *Claviceps*) is able to produce agroclavine when incubated with the substrate chanoclavine-1-aldehyde in the presence of a FgaOx3 enzyme from *Neotyphodium lolii*. The difference between both homologous enzymes may explain the lack of the hydrogenase (reductase) activity characteristic of *A. fumigatus* (a festuclavine producer) in the agroclavine producers, such as *Claviceps purpurea*. Interestingly, *P. roqueforti* produces both agroclavine and festuclavine [12]. In summary, it seems likely that *P. roqueforti* synthesizes agroclavine by the action of a FgaFS-homologous enzyme as a less reduced product of the pathway.

Other *P. roqueforti* Metabolites   Several strains of *P. roqueforti* are also known to produce citreoisocoumarin and small amounts of orsellinic acid. Very little is known about the biosynthesis of these compounds.

Orsellinic Acid. The biosynthesis of orsellinic acid is related to that of methylorsellinic acid and dimethylorsellinic acid described above. In the absence of experimental information in *P. roqueforti*, it is unclear if there is a separate polyketide synthase without the methylation domain, specific for orsellinic acid biosynthesis or

whether this compound is formed by the 5-methylorsellinic acid PKS of the mycophenolic acid pathway (encoded by *mpaC*) when the methyltransferase domain is bypassed by the "domain skipping" mechanism occurring in some of these synthases. In support of this last possibility is the fact that orsellinic acid is produced in very small amounts in the tested strains of *P. roqueforti* [12]. A similar nr-PKS is the DMOA synthase involved in andrastins biosynthesis (see above).

Citreoisocoumarin. Another metabolite produced by *R. roqueforti* is citreoisocoumarin. There is no information on the biosynthesis of this compound in *P. roqueforti*, but in *Fusarium* species, citreoisocoumarin is known to be a byproduct of the biosynthesis of aurofusarin. Both compounds derive from a precursor polyketide that may be cyclized by (i) a carbon-to-carbon (C-C) Claysen-type condensation giving aurofusarin or (ii) by formation of an internal lactone resulting in citreoisocoumarin [67]. This last type of cyclization (lactone formation) appears to predominate in *P. roqueforti*, but the enzyme(s) and molecular basis underlying citreoisocoumarin biosynthesis are still unknown.

3   Metabolites of *Penicillium carneum* and *Penicillium paneum*

P. carneum and *P. paneum* are closely related to *P. roqueforti* Thom, although as indicated in the Introduction section, they are classified as separate species [5, 12]. Indeed, *P. carneum* and *P. paneum* differ from *P. roqueforti* and among themselves in their ability to produce some secondary metabolites (Table 1). *P. carneum* predominates in some spoiled meat products, whereas *P. paneum* is associated with molded bread or grains and grass silages. Both fungi may occur in the surface of some cheeses, but they are not used as secondary starters in blue-veined cheese [1, 23, 26], although they may be present in homemade artisanal blue cheeses, particularly in those produced in some developing countries. *P. carneum* and *P. paneum* may be included in the group of cheese contaminant fungi [22], and their absence in most blue cheeses makes their secondary metabolites less relevant for human health.

The main mycotoxins produced by these fungi are patulin, marcfortins, penitrem, and botryodiplodin (Table 1). *P. paneum* is more different from *P. roqueforti*; it lacks the ability to produce PR-toxin but synthesizes patulin that is not found in *P. roqueforti*. *P. carneum* produces most of the described metabolites of *P. roqueforti* and also patulin. Penitrem A is produced by *P. carneum* but not by *P. roqueforti* or *P. paneum*. The marcfortins A, B, C are produced only by *P. paneum*. On the other hand, PR-toxin and the intermediates eremofortins A, B, and C are produced exclusively by *P. roqueforti* [12]. In summary, *P. paneum* is different from the other two related species in its set of secondary metabolites, whereas *P. carneum* is more similar to *P. roqueforti*. The biosynthesis of these metabolites is poorly known with exception of that of patulin.

3.1   Patulin

Patulin is a potent mycotoxin that causes neurological and immunological disorders and gastrointestinal alterations in humans [68]. Patulin is a common mycotoxin produced by many species of *Penicillium* (including *P. paneum* and *P. carneum*), *Aspergillus*, *Paecilomyces*, and *Byssochlamys nivea* [69, 70]. Patulin is frequent in fungi-spoiled apples, and the levels of patulin allowed in apple-derived products, such as cider, apple jellies, or apple-derived infant foods, are strictly limited by the food safety agencies of many western countries. Among the producer fungi, *Penicillium expansum* is known to cause the soft rot of apples and pears and appears to be the major producer of patulin in fruits [71].

Fragmented evidence reported over the last three decades has shown that the first intermediate, 6-methylsalicylic acid (6-MSA), is converted to patulin through the intermediates m-cresol, m-hydroxybenzyl alcohol, and isoepoxydon. Biochemical and genetic studies in different producer fungi, including *P. paneum*, identified two enzymes, namely, 6-methyl salicylic acid synthase (6-MSAS) and isoepoxydon dehydrogenase (IDH) involved in patulin biosynthesis [72–74]. More recently two P450 monooxygenases have been found to be involved in the conversion of 6-MSA to m-cresol and m-hydroxybenzyl alcohol [75]. When the genome of the patulin producer *Aspergillus clavatus* was sequenced (TIGR http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/index.php ∼ main), a 15-gene cluster putatively encoding the entire patulin pathway was identified. This cluster includes the *msas* gene (encoding 6-MSA synthase), the *idh* (encoding the IDH), and the two P450 monooxygenase encoding genes. The patulin gene cluster of *P. paneum* or *P. carneum* has not been reported yet but is likely to be similar to that of *A. clavatus.*

4   *Penicillium camemberti:* Cyclopiazonic Acid

Several filamentous fungi may grow on the surface of cheeses during the ripening process. Most of them are strictly external, but some of them, e.g., *P. camemberti* and *P. nalgiovense*, may contribute to the organoleptic characteristics of mature cheeses.

P. camemberti is associated with ripening of white rind soft cheeses such as Camembert and Brie cheeses [76]. Selected strains are routinely used in production of soft cheeses. Although many of the *P. camemberti* secondary metabolites remain unexplored, one of them, cyclopiazonic acid, acquired relevance because of its well-known neurotoxicity. Cyclopiazonic acid (CPA) is a highly active inhibitor of Ca^{2+}-dependent ATPases of animal cells and is a potent neurotoxin for humans and other mammals [77].

Cyclopiazonic acid is a prenylated indole alkaloid containing a tetramic acid ring produced by several *Aspergillus* and *Penicillium* species, including *P. camemberti.* The biosynthesis of CPA has been studied in *Aspergillus flavus* and *A. oryzae* but not

in detail in *P. camemberti*, although the biosynthetic pathway is likely to be conserved in all fungi.

CPA derives from L-tryptophan, a four-carbon unit, and a dimethylallyl diphosphate units [77]. The four-carbon unit is formed by condensation of an acetyl-CoA and a malonyl-CoA. The first step in the CPA biosynthetic pathway is catalyzed by a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) that activates L-tryptophan and condenses this amino acid with acetyl-CoA and malonyl-CoA units forming the acetoacetyl-L-tryptophan (AA-L-trp) intermediate. This key enzyme is encoded by the *cpaA* (also named *cpaS*) gene. The NRPS component of the hybrid PKS-NRPS has four domains C-A-T-R* with activities for condensation, L-tryptophan activation and thiolation (peptidyl/acyl carrier), and peptide product release. The R* domain is a reductase that lacks the catalytic triad of NADH-dependent reductases and is proposed to release the N-acetoacetyl-tryptophan intermediate by cyclization via a Dieckmann condensation to form the cyclo-acetoacetyl-L-tryptophan intermediate that is released from the phosphopantetheinyl arm of the T domain of the enzyme [78].

In the second step of the CPA biosynthetic pathway, the cyclo-acetoacetyl-L-tryptophan intermediate is prenylated at C-4 of the indole nucleus by the enzyme cyclo-AA-trp prenyltransferase that introduces an isopentenyl group from the DMA-PP donor. The enzyme encoded by the *cpaD* gene is a member of the well-known family of prenyltransferases [79]. In *A. clavatus* and *A. oryzae*, there is a third step of the pathway catalyzed by a FADH-dependent oxidoreductase *cpaO* that converts β-cyclo-acetoacetyl-tryptophan into the final product, CPA [77, 80, 81]. The *cpa* gene cluster of *A. flavus* includes in addition to *cpaA-epaD-cpaO* structural genes two additional genes, *epaM* that encodes a transmembrane protein of the MFS family and *cpaR* that encodes a regulatory protein of the C6 Zn^{2+} finger type [82]. The CpaM transporter presumably is involved in CPA secretion [32].

CPA is the final product of the biosynthetic pathway in *A. flavus* and probably also in *P. camemberti* since CPA is secreted and accumulated in these two fungi. However, *A. oryzae* that is considered to be a "domesticated" variant of *A. flavus* widely used in Japanese food industries contains an additional gene in the cluster, *cpaH*, that encodes a P450 monooxygenase which converts CPA to 2-oxo-CPA. This compound is much less toxic than CPA [83], and the authors proposed that this P450 monooxygenase is a toxicity-reducing "safeguard" enzyme evolved in *A. oryzae* during its adaptation to grow in fermented foods [83]. The mechanism of adaptive safeguard appears to be more complex since some *A. oryzae* strains have lost completely the ability to produce CPA and 2-oxo-CPA due to a mutation in the N-terminal region of the CpaA hybrid PKS-NRPS. It will be interesting to confirm that *P. camemberti* lacks the "safeguard" *cpaH* gene and therefore maintains the high toxicity characteristic of CPA.

Other secondary metabolites have been reported to be produced by *P. camemberti*, e.g., asperenone, asperrubrol-like compounds, methyl-isoborneol, and hadacidin [27], but it is unknown if these compounds pose a health problem for humans.

5   Conclusions and Future Perspectives

Blue-veined cheese is a gourmet food consumed all over the world. These cheeses are maturated in different countries with *P. roqueforti* as a secondary starter that grows inside the cheese in microaerobic conditions. As described in this article, *P. roqueforti* is able to produce about 20 different secondary metabolites belonging to at least seven different families. Some of these compounds are highly toxic, e.g., PR-toxin, whereas others, e.g., andrastins, are considered to be beneficial for human health. Selection of fungal strains over centuries has favored the use of *P. roqueforti* Thom that lacks production of patulin, over the closely related *P. carneum* and *P. paneum* that produce this mycotoxin. Most *P. roqueforti* secondary metabolites are produced in rich solid (agar plates) and liquid cultures, but there is very limited information on the production and accumulation of secondary metabolites inside the cheese, under the microaerobic conditions. Andrastin A is known to be formed and accumulated in the blue-veined cheeses [13, 14]. Humans and other animals consume a variety of plants and fungal secondary metabolites; many of them are probably nontoxic, but in other cases, animals reject certain plants or mushrooms because they contain toxic secondary metabolites known as feeding deterrents. It is possible now to construct strains lacking certain secondary metabolite gene clusters. The possible removal of toxic metabolites gene clusters by precise gene excision, while preserving all other characteristics, including enzymes involved in cheese ripening and aroma formation, is now a possible application of the molecular genetics of *P. roqueforti* to the cheese industry. This "domestication" process would be equivalent to the "natural domestication" process that has evolved in *A. oryzae* for removal of CPA.

6   Authors' Note

When this article was in press Del Cid and coworkers reported the mycophenolic acid gene cluster of *P. roqueforti* (Del-Cid A, Gil-Durán C, Vaca I, Rojas-Aedo JF, García-Rico RO, Levicán G, Chávez R. (2016) Identification and Functional Analysis of the Mycophenolic Acid Gene Cluster of *Penicillium roqueforti*. PLoS One. 1(1):e0147047.

The *P. roqueforti mpa* cluster is almost identical to that known for *P. brevicompactum* (Fig. 5) and the conclusion obtained by these authors are similar for both fungi.

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