Reconciliation and evolution of *Penicillium rubens* Genome-Scale Metabolic Networks – What about specialised metabolism?

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Abstract

Mining filamentous fungal genomes have revealed a high proportion of specialised metabolites in the past few years. However, many of these metabolites, produced by biosynthetic gene clusters, remain silent in the laboratory, and one hypothesis for soliciting their production lies in modifying their growth conditions. Though, it remains a time-consuming and costly process. Therefore, the preliminary use of *in silico* modelling, such Genome-Scale Metabolic Network (GSMN), might be a clever alternative to explore and understand the production potential of a given organism. Consequently, *Penicillium rubens* Wisconsin 54-1255 strain was selected as the model organism. Following current convention standards and quality criteria, the proposed reconstruction results from the following four elements. In parallel, (1) an updated functional annotation of the *P. rubens* genome was carried out and supplemented by (2) an orthology search with different GSMNs templates. This first draft was enriched (3) by integrating data from *P. rubens* previous GSMN reconstructions and complemented (4) by manual curation steps targeting basal and specialised metabolites. The proposed high-quality GSMN, *i*Prub22, has a MEMOTE score of 68% and a metabolic coverage of 45%. The reconstruction is composed of 5,192 metabolites interconnected by 5,897 reactions, of which 5,033 are at least supported by a genomic sequence. *In fine*, the presence within *i*Prub22 of the specialised metabolisms highlights that many bottlenecks still exist. Solving such problems is the mandatory step to get access to specialised metabolic dedicated GSMN suitable for the exploration of metabolic regulation responsible for natural product synthesis.

Introduction

Comparable to Alexander Fleming's pioneering discovery of penicillin in 1929, the elucidation of natural products (NPs) has frequently been serendipitously achieved through the random screening of plant, fungal, and bacterial extracts. Natural molecules, synthesised by living organisms, can be categorised into two types based on their function within them: constitutive molecules, which are essential for the organism's growth, and specialised molecules, which provide competitive advantages towards the organism's environment, historically referred to as secondary metabolites. The latter is of great interest in pharmacology, both in the field of health and agriculture, since many drugs are originated from these biologically active natural molecules [1]. However, since the early 2000s, the discovery rate of new biologically active NPs has slowed down or even become scarce. Moreover, the time-consuming, inefficient and costly traditional methods used for bioprospecting new NPs have been slowly abandoned [2]. Given the increasing antibiotic resistance [3], it is essential to relaunch such NP research using more rational and predictive approaches. The confluence of genomics and metabolomics has now enabled the correlation of metabolites with their structural data to their respective biosynthetic gene clusters (BGCs). Combined with the automation and qualitative evolution of tools observed in recent years [4], these approaches have become increasingly appealing for new bioactive NPs discovery.

Filamentous fungi are known for their ability to produce a diverse array and a substantial amount of NPs [5]. Over the years, significant knowledge has been accumulated about their metabolism, and fungal genomes exploration has highlighted the high proportion of specialised metabolites produced by enzymes with

co-located genes. In addition to biosynthetic capabilities, such BGCs could be associated with resistance, transport, or regulatory genes. Thus, their evolutionary history may provide clues to their biological activities that could guide molecular engineering research [6]. However, despite the richness of BGCs exhibited by fungi, one of the extensive risks NP chemists face is the strong tendency to rediscover previously characterised molecules [2]. Such a trend is inconsistent with the genomic data that have been acquired, showing a large number of gene clusters associated with unknown specialised metabolism. Indeed, the differences observed between theoretical *in silico* models and *in vitro* cultures indicate that gene clusters not expressed in the laboratory are numerous and form an untapped resource for novel bioactive NPs [7,8].

Penicillium rubens, formerly known as *Penicillium chrysogenum* [9], is a fungus used in the industry to produce *β*-lactam antibiotics, including penicillin. Consequently, both industrial and wild terrestrial strains of *Penicillium* have been extensively studied over the years. After decades of classical strain improvements using the wild strain NRRL1951 [10,11], all industrial strains with high penicillin yields are now derived from the Wisconsin 54-1255 strain, which has therefore become a model species within the filamentous fungi [12]. Thus, the availability of its genome [13] makes it a worthy candidate for studying its metabolism. In 2009, 34 BGCs were identified in *P. rubens*, only 4% of which were associated with a known and isolated compound. Nine years later, 28% of the compounds from the now-identified 50 BGCs were characterised [10]. Thus, understanding the conditions under which silent, orphan or cryptic gene clusters could be activated remains of great interest. Following the

example of what has been achieved for bacterial NPs [14], decoding the genome is the crucial first step toward understanding how to unlock such cryptic genes.

In systems biology, the biochemical and physiological properties of metabolism organisms can be described by Genome-Scale Metabolic Networks (GSMNs) studies [15]. Reconstruction of such models is based on computational methods using both *in silico* and experimental data. Indeed, integrative biology principles applied to genomics and metabolomics allow us nowadays to link the high-quality data obtained by analytical technologies to the corresponding models. Moreover, increasing model complexity aims to integrate and visualise heterogeneous knowledge-related metabolism more and more efficiently [16], thereby enhancing our metabolism understanding at the system level [17]. However, sustainability, evolution, and exploitation of models are intrinsically linked to their adoption by the scientific community, as illustrated by the work performed on the *Caenorhabditis elegans* metabolic network reconstruction [18]. Apart from well-studied model organisms, whose non-exhaustive list is presented in Gu *et al.* [19], few examples of individual network reconciliation exist [20,21]. This phenomenon can be attributed to various factors, including the massive generation of data associated with its quality and its updating, the lack of inter- and intra-operability between platforms and software, the multiplication of reconstruction tools linked to the current facility to reconstruct GSMNs, and the lack of transparency in the acquisition of previous networks [22–25]. Moreover, a GSMN represents a platform of organised and summarised resources intended to reflect the optimal metabolic capabilities of the target organism at the reconstruction time (*e.g.* knowledge snapshot). In this respect, given the constant and

rapid evolution of data, the need to maintain these models up-to-date and standardised is increasingly becoming pressing.

Therefore, since the first *P. rubens* GSMN (*i.e. i*AL1006) was published in 2013 [26], the work presented here proposes a new GSMN reconstruction for *P. rubens* Wisconsin 54-1255, considering the points mentioned above (S1 file). The present study is primarily concerned with the reconstruction process, with particular emphasis on providing informative outcomes regarding a specific model generation. Additionally, particular attention was paid to specialised metabolism. Thus, the provided model could be compatible with future research on NPs pathways regulation, which could furnish insight into cryptic pathways. Thus, the *i*Prub22 model, composed of 5,897 reactions and 5,192 unique metabolites, combines data from previous reconstructions, an updated functional annotation, a homology search with various phylogenetically close and distant organisms, and manual curation based mainly on literature.

Results

Draft generation: reconstruction and reconciliation

*i*Prub22 GSMN reconstruction started with the automatic generation of two drafts generated using genome functional annotation and homology searches with pre-existing models.

Functional annotation subnetwork

Usually, three annotation labels are required to provide a source of ‘annotation-based’ metabolic reactions. Thus, Enzyme Commission (EC) numbers, Gene ontology terms (GOT) [27,28], and protein domains (PFAM) were sought among the

P. rubens sequences. Of the 12,556 gene sequences that constitute the *P. rubens* genome [13] 8,948 (71%) possess at least one annotation (*i.e.* EC number, GOT and/or PFAM), and 2,270 (25%) are annotated by all three annotations (Fig 1 in S2 file). The EC numbers are associated with genomic sequences based on KEGG orthology (KO) identifiers [29]. Thus, the joint use of KAAS [30] and gene information stored in KEGG allows an 11% enrichment in EC numbers compared to the only benefit of Trinotate [31] results (Fig 1 in S2 file, Table 1 in S2 file). The specificity and complementarity of these three annotation sources are illustrated in Fig 2.A in S2 file. As a result, these annotations allowed the generation of the functional annotation subnetwork composed of 3,390 genes (*i.e.* 27% of the genome), 1,359 pathways, 3,205 enzymatic reactions and 3,577 metabolites.

Orthology subnetwork

In parallel, following the search for homology between species, a second subnetwork was reconstructed. Orthologous genes between *P. rubens* and seven other reconstruction templates were searched to achieve the creation of this subnetwork. Orthology templates were chosen according to the quality of their GSMNs, their phylogenetic proximity to *P. rubens*, or their reconstruction procedure (see the selected network's topology in Fig 3 in the S2 file).

Only the genomic sequences initially present in the GSMNs are queried using OrthoFinder [32]. Respectively, the templates *Arabidopsis thaliana* [33], *Aspergillus nidulans* [34,35], *Aspergillus niger* [34,35], *Neurospora crassa* [36], *Penicillium chrysogenum* species complex [34,35], *Saccharina japonica* [37] and *Schizosaccharomyces pombe* [34,35] are composed of 2,330, 1,279, 1,299, 836, 1,352, 5,016 and 810 sequences of which only 0.04%, 1.9%, 0.15%, 1.7%, 11%,

0.04% and, 0.49% were not found in the constitution of the queried proteomes. The 8,519 homologous sequences between *P. rubens* and the different templates *A. thaliana*, *A. nidulans*, *A. niger*, *N. crassa*, *P. rubens* species complex, *S. japonica*, and *S. pombe* are distributed according to 478, 942, 866, 635, 1,003, 1,273 and, 667 orthogroups respectively (Table 2 in S2 file and S3 file). Sequence distribution among the different species templates and orthogroups is displayed in Fig 4 in the S2 file. Of these homologous sequences, 2,903 are paralogs and will not be considered for reconstruction. Respectively, there are 1,494, 1,144, 1,049, 766, 1,174, 2,302, and 764 orthologs between the templates studied (*i.e. A. thaliana*, *A. nidulans*, *A. niger*, *N. crassa*, *P. rubens* species complex, *S. japonica*, and *S. pombe*) and *P. rubens*. Complementarily, the number of *P. rubens* genes being orthologous to the different templates are 866, 1,818, 1,620, 1,104, 2,368, 2,329, and 1,114, respectively.

Orthologous genes to the template ones were used to obtain their corresponding reactions related to *P. rubens* Wisconsin 54-1255. As the selected template networks were mostly reconstructed between 2010 and 2018 *via* the KEGG database [29], a mapping step on the MetaCyc database [37] was necessary for the interoperability between the OrthoFinder results and the subnetworks inference. The draft generation indicates that less than 50% of the detected and potentially informative reactions were lost during this mapping (Fig 2.B in the S2 file). Mapped reactions correspond to 46% of the total reactions for *A. thaliana*, 49% for *A. nidulans*, 49% for *A. niger*, 49% for *P. rubens* species complex, 49% for *S. pombe* and 46% for *N. crassa*. The best mapping rate (87%) is obtained with the *S. japonica* network, whose GSMN was reconstructed following a similar protocol to the one presented here and directly *via*

the MetaCyc database [37]. In terms of gene number, these losses represent 18% of the total number of genes detected by OrthoFinder (*i.e.* 5,616 genes). However, 70% of the genes (*i.e.* 716 genes) were finally included in the GSMN as they possess correspondence *via* the annotation or with external sources (Fig 5 in S2 file). The remaining unmapped 307 sequences were lost and represent a set to be preferentially explored for future network improvements. Thus, the orthology subnetwork is composed of 4,615 genes, 1,550 pathways, 2,946 enzymatic reactions and 3,238 metabolites.

Enrichment with external sources

Penicillium rubens is a widely studied model organism, but the available data heterogeneity makes conciliation a delicate task. Therefore, the choice to add external data was made on consistent sources, namely PchCyc, the Pathway/Genome Database (PGDB) from BioCyc [38], and the latest version of the organism's network, called Prubens [39]. This reconstruction, resulting from a large-scale automatic reconstruction process, takes advantage of a mapping on MetaCyc from the original version of the *i*AL1006 network [26]. Thus, data were added when reactions, or in default, all their metabolites, had compatible MetaCyc identifiers and were supported by at least one Gene-Protein-Reaction (GPR) association. Respectively, Prubens and PchCyc are composed of 2,533 and 1,291 reactions of which 650 are shared. Of the 3,824 total reactions queried, 2,418 had a compatible MetaCyc identifier, and 2,112 were associated with at least one gene. Furthermore, of the 1,725 reactions already present in the draft, 313 had identical GPRs associations, 123 had no genomic information in the external sources (*e.g.* in the *i*Prub22 draft, these reactions are henceforth annotated between 1 and 39 genes),

and 1,289 possessed different GPR associations. Finally, the draft was completed by 510 reactions (*i.e.* 440 reactions belonging exclusively to Prubens, 43 reactions belonging exclusively to PchCyc and 27 reactions coming from both sources). Moreover, 263 supplementary reactions from *i*AL1006 were also included since all the reactants/products of those reactions possess a MetaCyc identifier. To ensure data traceability, the original identifiers of these reactions were kept and the selection is shown in Fig 6 in the S2 file.

The Initial draft

All the data obtained were then merged to produce a first version of the network (*i.e.* draft). The complementarity of each approach, displayed in Fig 1 and in Supporting Information (Figs 2.C, 9 and 10 in the S2 file), makes it possible to question the meaning and relevance of the added data. These figures trace the origin of each reaction and, therefore, of the genes and metabolites, within the draft. Thus, it is easier to disentangle the information from biological reality or computational bias. For instance, in the orthologous subnetwork, 17 and 1,237 reactions (Fig 5.C in the S2 file) were exclusively from the most phylogenetically distant templates (*i.e. A. thaliana* and *S. japonica*, respectively). In the case of the 17 *A. thaliana* reactions, 8 (47%) of them are also supported by the annotation source, and for *S. japonica*, the number amounts to 967 (78%) reactions. Similarly, the addition of external sources (*i.e. i*AL1006, Prubens and PchCyc) supported 773 reactions (17% of the draft reactions) which were not retrieved with the annotation and orthology subnetworks. Therefore, reactions supported only by one source should be given special attention in the future.

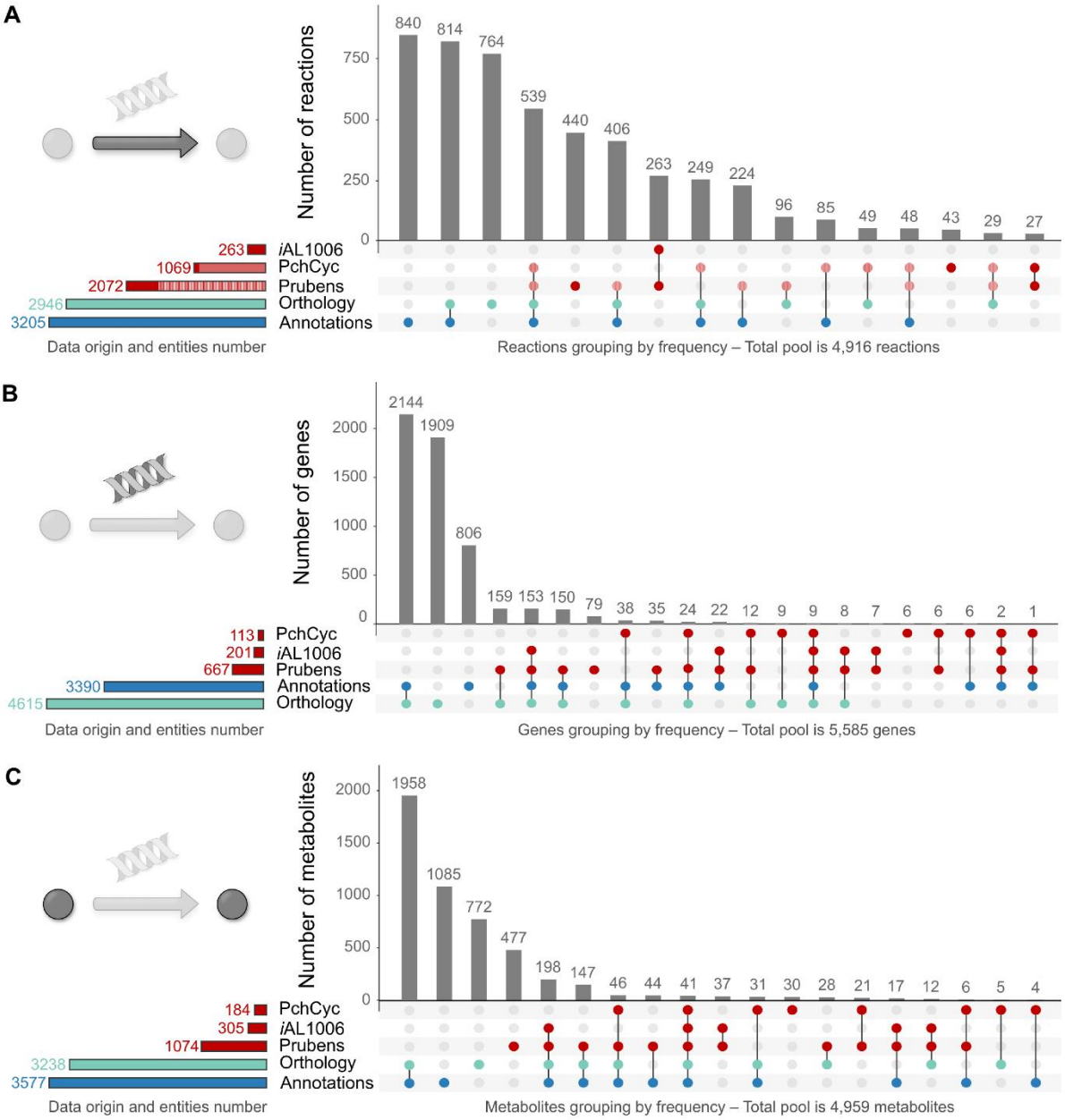


Fig 1. UpSet diagram representing the origin of each of the reactions (A), genes (B), and metabolites (C) added to the *Penicillium rubens* Wisconsin 54-1255 draft. This traceability underlines the complementarity of the approaches. Information comes from annotation (■) orthology (■) subnetworks and external sources (■). The red hatched area (▨) corresponds to reactions shared between the external sources and the draft from the annotation and/or orthology subnetworks. For a further view and details see Figs 9 and 10 in S2 File.

Moreover, according to the nomenclature of EC numbers, reactions are divided into the following eight categories: oxidoreduction 28%, transfers reactions 24%, hydrolyses 18%, lyses 7.9%, isomerisation 2.8%, reactions involving ligases 3.4%, translocation 0.38% and undetermined reaction type 16%. Nevertheless, this distribution is different within the genes since the most frequent classes are: transferases (29%), hydrolases (26%) and oxidoreductases (24%). However, it should be noted that only 42% of the genes have a clearly identified EC number, predominantly as a functional annotation result (Fig 7 in the S2 file).

Furthermore from the 4,959 metabolites present at this reconstruction stage, 1,085 (22%) come exclusively from the annotation's subnetwork, 772 (16%) from the orthology subnetwork and 565 (11%) from external sources (Fig 1.C). Consequently, 2,537 (51%) metabolites are shared between at least two different sources (Fig 8 in the S2 file). According to the MetaCyc compounds' ontologies, 349 (*i.e.* 7% of draft metabolites) belong to the specialised metabolism (*i.e.* 41 (12%) exclusively from the annotation, 92 (26%) exclusively from the orthology, 153 (44%) exclusively from external sources and the remaining 63 (18%) are supported by at least two of these sources). MetaCyc v23.0 contains approximately 12% of compounds annotated "secondary".

In fine, the reconstructed draft includes 5,585 genes which support 4,916 reactions included in 1,817 pathways and involving 4,959 metabolites.

Reconstruction improvements

Once the initial draft is produced, several modifications must be performed to obtain a functional and searchable model compatible with the experimental observations. The first step is to establish a list of metabolites known to be produced by the studied

organism. These target compounds' presence in the draft and their topological producibility were then investigated. When these compounds are either absent or not topologically producible, gap-filling steps should be performed to improve the model connectivity. Therefore, the following section deals with the necessary prerequisites for setting up a high-quality GSMN ready for subsequent flux analyses.

Targets selection for model enhancement

As it is not possible to bring the same confidence weight to all available data, the targets selected to study and improve the metabolic network of *P. rubens* were grouped into three distinct sets of targets (*i.e.* set 'Targets1' with high confidence, set 'Targets2' with medium confidence and set 'Targets3' with low confidence – S4 file for further details).

The first target set corresponds to information from the literature. The target metabolites belong indifferently to specialised or constitutive metabolism in that particular case. Two publications, in particular, caught our attention, one allowing the accurate modelling of the sugar pathway in fungi [40] and the other resulting from the penicillin biosynthesis pathway manual curation [41]. This list was completed by metabolites related to the biomass function [26]. In addition, the SMASH tool suite [42] was used to highlight 16 specialised metabolite pathways through gene cluster search. Although, only the biosynthetic pathways of penicillins, roquefortines and patulin can be exploited due to the lack of MetaCyc identifiers for compounds of the other pathways. However, knowing that in *P. rubens*, the patulin biosynthetic pathway is incomplete [7], this compound is discarded from 'Targets1', and its pathway curation was later performed in this sense. Consequently, 11 metabolites were supported by genome mining sources in the first list of targets to be monitored. During

this process search, a list of 47 compounds lacking MetaCyc identifiers was also generated (*i.e.* orphan metabolites). Moreover, even if Melanin and PR-Toxin are not strictly speaking orphan metabolites since they are referenced on MetaCyc, they cannot be produced topologically since no reactions were associated with them. At that stage, those latter compounds cannot be monitored within the network unless focusing on their closest referenced precursor. Finally, set 'Targets1' is thus composed of 243 targets.

The second list is obtained by querying specific NP databases. Using a taxonomy-oriented query from the naturaL prOducTs occUrrence databaSe (LOTUS) [43], 240 compounds for *P. chrysogenum*/*P. rubens* are retrieved along with their InChIKeys which are used to search within the MetaCyc database. Based on an exact InChIKey match, followed by an analogue of the first two sections of the identifier and finally on the first part (*i.e.* corresponding to planar chemical structure search), only 8, 11 and 47 metabolites were found, respectively. Depending on these criteria, between 4, 7 or 13 of these metabolites were already present in the draft. Therefore, in the best case, only 20% of the information in LOTUS can be included in the reconstruction. The assignment verification to the studied strain allows the selection of 35 metabolites which constitutes the set 'Targets2'.

Finally, the third set of targets was established based on the metabolites present in the reconstruction (*i.e.* at the step intermediate C – Table 1) and that have annotation with the term 'Secondary-Metabolites' in the MetaCyc compounds ontologies when they were searched with SmartTables. At that stage, the model contains 400 metabolites classified as "secondary". They are divided into 149 compounds that are at least produced and consumed by a reaction, 90 that are only consumed and

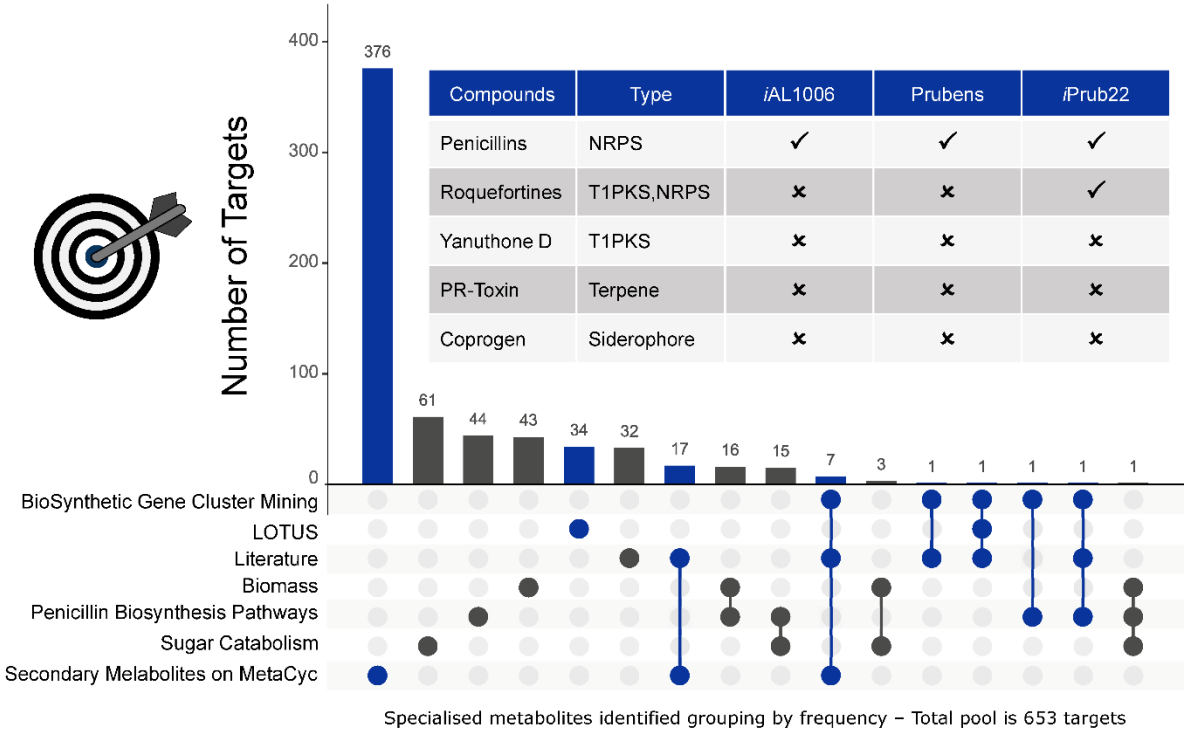
161 that are only produced (*i.e.* 251 topological dead-ends). Thus, Set ‘Targets3’ was composed of 400 targets.

Table 1. Potential topological producibility expressed in the different curation steps of the reconstruction.

	Reconstruction progress						
	Draft	A	B	C	D	E	GSMN
Number of entities	--●--	--●--	--●--	--●--	--●--	--●--→	
Reactions	4,916	5,364	5,452	5,513	5,528	5,800	5,895
Active reactions	-	2,670 (50%)	2,760 (51%)	2,930 (53%)	2,947 (53%)	3,504 (60%)	3,683 (62%)
Metabolites	4,959	4,974	5,021	5,039	5,048	5,167	5,192
Producible metabolites	-	1,893 (38%)	1,953 (39%)	2,090 (41%)	2,102 (42%)	2,609 (50%)	2,748 (53%)
Metabolites only consumed	1,274 (26%)	1,248 (25%)	1,229 (24%)	1,221 (24%)	1,220 (24%)	1,109 (21%)	1,076 (21%)
Metabolites only produced	1,416 (29%)	1,406 (28%)	1,394 (28%%)	1,390 (28%)	1,390 (28%)	1,328 (26%)	1,308 (25%)
Metabolites consumed and produced	2,269 (46%)	2,320 (47%)	2,398 (48%)	2,428 (48%)	2,438 (48%)	2,730 (53%)	2,807 (54%)
Producible metabolites (targets 1)	-	193/237 (81%)	198/237 (84%)	226/237 (95%)	226/237 (95%)	226/237 (95%)	234/243 (96%) ▲
Producible metabolites (targets 2)	-	5/35 (14%)	6/35 (17%)	6/35 (17%)	14/35 (40%)	14/35 (40%)	14/35 (40%)
Producible metabolites (targets 3)	-	87/400 (22%)	95/400 (24%)	147/400 (37%)	147/400 (37%)	368/400 (92%)	368/400 (92%)

The main objective of gap-filling was to ensure that the known metabolites in *Penicillium rubens* were present before using the reconstruction as a model for analysis. As gap-filling is an iterative process, it was linearised, and results were grouped for clarity's sake. Thus, between the draft and the GSMN, intermediates A, B, C, D and E were generated. First of all, to test the metabolites' producibility, 233 transport and 217 exchange reactions were incorporated (draft to A), followed by 86 spontaneous reactions (A to B) and then 61 (B to C), 15 (C to D), 272 (D to E) and 75 (E to GSMN) reactions from the four gap-filling runs. Minor corrections (not described) were made between E and the final reconstruction. The topology analyses were carried out with the seed list defined from the metabolites present in the extracellular medium. The six metabolites specific to the biomass reaction were only monitored from their inclusion in the reconstruction at the GSMN step (▲).

Altogether, 653 metabolites were used to obtain a high-quality GSMN by seeking the network for their presence and connectivity. Based on the MetaCyc annotations, BGCs mining and LOTUS database, this list is divided into 215 and 438 compounds belonging to constitutive and specialised metabolism, respectively (Fig 2).



Compounds	Type	*i*AL1006	Prubens	*i*Prub22
Penicillins	NRPS	✓	✓	✓
Roquefortines	T1PKS,NRPS	×	×	✓
Yanuthone D	T1PKS	×	×	×
PR-Toxin	Terpene	×	×	×
Coprogen	Siderophore	×	×	×

Fig 2. Origin of the 653 targets used for reconstruction enhancement. Example of secondary metabolites produced by *Penicillium rubens* according to the main natural product classes: NRPS (Non-Ribosomal Peptides Synthetase), T1PKS (Type I Polyketide synthase), hybrid form, terpenes and siderophores. The selection was based on data from the literature (*i.e.* Biomass [26], Sugar Catabolism [40], Penicillin Biosynthesis Pathways [41] and literature details on Supporting Information), genome mining [42] and LOTUS databases [43]. All targets are divided according to constitutive (■ – 215) and specialised (■ – 438) metabolism. Metabolites found (✓) or not (×) in the different versions of GSMNs are shown. Metabolites without MetaCyc-compatible information cannot be included in the networks (*i.e.* absence of compound identifier as for yanuthone D or the absence of reactions leading to compound production/degradation as for coprogen or PR-Toxin).

Modelling exchanges with the environment

To check the producibility of a compound by topological analysis and then by constraint analysis, it is necessary to define a set of initiation metabolites called seeds. Compounds selection from the extracellular medium led us to review and modify the reactions related to the metabolite transport phenomena between the various model compartments.

Although the proposed model does not include intracellular compartmentation, initially, 76 reactions, supported by a total of 204 genes, involve transport mechanisms. The location of their gene products corresponds to the cell membrane for 107 of them. The 22 reactions that do not possess one of these genes in their GPR association were either corrected or blocked in the model (*i.e.* reactions that biologically model an intracellular transfer whereas it is extracellular in the reconstruction). The performed curation allowed adding 198 metabolite transfer reactions between the extracellular and intracellular compartments. At least one gene, for a total of 220, is associated with 91 of them. For modelling purposes, 37 demand reactions were also included (*i.e.* irreversible transfer from the external to the intracellular environment).

Then, exchange reactions corresponding to 185 uptake reactions and 32 production reactions were added (Fig 11 in the S2 file and S5 file). Such exchange reactions are related to only the transfer of the same metabolites between the extracellular compartment and the environment (*i.e.* system boundaries). These reactions are entirely artificial. However, their addition will allow the simulation of the nutritional conditions of the organism (*i.e.* for further use of the model, *e.g.* tests of functionalities and production of metabolites of interest). Thus, the seeds list used for the topological

analyses corresponds to the metabolites present in system boundaries (*i.e.* having an uptake reaction).

Finally, the reconstruction is composed of 274 (4.6%) transport reactions, 37 (0.63%) demand reactions, 185 (3.1%) uptake reactions and 32 (0.54%) production reactions. These reactions, therefore, represent 9.0% of the GSMN reactions.

Gap-filling and topological producibility

Once the targets and seeds are defined, all that remains to be performed is to select available reactions for gap-filling querying. The database used for gap-filling (MetaCyc v23.0) contains 17,201 reactions. As gap-filling is a heuristic approach, these reactions are filtered into two categories to select the more relevant set of reactions to query. The filtering presented below reduces the space to be interrogated by 46%. Consequently, Gap-filling was run firstly with MetaCyc subset reactions (*i.e.* 9,281 reactions) with a high confidence weight.

The first category corresponds to reactions related to the fungi kingdom. This list was constructed based on a meta-network obtained by the reconstructions of a species' repertoires belonging to the fungi kingdom [44]. This meta-network is composed of 3,810 reactions. Interestingly, 77% of the reactions (*i.e.* 2,964 reactions) were already present in the draft. This list of reactions was completed by the BioCyc PGDBs of *Candida albicans*, *Exophiala dermatitidis* and *Saccharomyces cerevisiae*, composed of 1,320, 1,714 and 1,562 reactions, respectively. Combining these three latter networks yield a set of 2,905 reactions, among which 70% (*i.e.* 2,041 reactions) were already present in the draft. The fusion of these two lists of reactions provided a pool of 4,709 reactions, of which 64% (*i.e.* 3,036 reactions) were already included

in the draft reconstruction. Finally, this first category comprises 1,043 reactions of interest.

At this point in the reconstruction, the draft network contains only reactions supported by at least one gene and thus is composed only of reactions catalysed by enzymatic proteins. For instance, biological catalysis carried out by RNA molecules (*e.g.* intra- or inter-molecular catalysis) or spontaneous reactions are not initially present in the reconstruction. However, their importance in modelling biological systems or under experimental conditions is no longer in question [45–47]. Therefore, the second category includes reactions for which the genomic information of the enzyme is absent or unknown, always according to data on MetaCyc. They were gathered by filtering the reactions on the MetaCyc web server into “Spontaneous reactions for which no enzyme is required” and “Enzyme-catalysed reactions for which no enzyme has been identified (pathway holes)”. It allowed the selection of 705 and 4,971 reactions (downloaded in October 2021 and May 2022, respectively). However, it is noteworthy that 22 (3.1%) and 631 (13%) reactions belonging to these two sets, respectively, are already present in the network, thus questioning the relevance and accuracy of the annotations.

For the classification and traceability of the results, the process leading to the generation of the high-quality GSMN is divided into five intermediate steps (*i.e.* A to E). Concerning the draft (*i.e.* fusion of all data supported by at least one gene) to which are added the exchanges (*i.e.* A to B), 38% of the metabolites (*i.e.* 1,893 compounds) are topologically producible, and 50% of the reactions (*i.e.* 2,670 reactions) are active under the evaluated conditions. The various manual curations allow us to improve these percentages to 53% (*i.e.* 2,748 compounds) and

62% (*i.e.* 3,683 reactions) respectively. More particularly, out of the 653 metabolites monitored, 279 (43%) are producible as soon as the draft is generated, and for the GSMN, this percentage reaches 91%. The topological connectivity improvements associated with the different gap-filling steps are summarised in Table 1. For the first set of targets (*i.e.* 'Targets1'), 61 reactions were incorporated into the reconstruction (*i.e.* B to C). GPR associations were included for 20 reactions in this carefully curated set. For the second set of targets (*i.e.* 'Targets2'), 29 out of 35 metabolites were missing from the draft. The addition of 15 reactions (*i.e.* C to D) allowed for the recovery of 8 of them. The third set of targets (*i.e.* 'Targets3') was used to improve the network connectivity by targeting secondary metabolites initially present in the draft. The 272 added reactions (*i.e.* D to E) improved potential producibility for 507 metabolites and activated 557 reactions. A final gap-filling step was performed with all the metabolites present in the initial draft against reactions with unknown enzyme sequences. It resulted in the addition of 75 more reactions (*i.e.* E to GSMN). Finally, 510 reactions (available in the S6 file) were added by gap-filling; almost half of them came directly from the MetaCyc prefiltered set (Fig 12 in the S2 file). Furthermore, 86 specialised metabolite enrichment is noted as initially 360 metabolites were labelled with the annotation "secondary metabolite" against 446 in the later version.

Reconstruction transformation into a constrained model

From a reconstruction viewpoint, obtaining a consistent flux model requires some adjustments (*e.g.* reversibility, redundancy and non-reducibility being common issues). When loading the reconstruction, 62 reactions involving a compound that was both a reactant and a product were erroneous. Their lack of clarity, linked to the

use of metabolites belonging to classes or superclasses, led to the deletion of 57 reactions, and to the correction of 5 other ones (*i.e.* overly generic and unbalanced compounds inducing an unnecessary redundancy). The second point concerns the reversibility of reactions. Among the 965 reversible reactions available, 98 were corrected according to the latest available data on MetaCyc SmartTables. It is interesting to note that 165 reactions have an unknown directionality and can therefore be left reversible or not for future simulations. Finally, 79 occurrences of duplicated reactions were removed from the model. All of the above model modifications are reported in Supporting Information (S7 file) to be compliant with MIASE standards [48]. On the 5,753 remaining model reactions, 1,309 (23%) were mass imbalanced, and 921 (16%) were charge imbalanced. Concerning the 5,420 metabolites, 3,517 (65%) were exclusively involved in balanced reactions. Only 188 (3.3%) are devoid of molecular formulae.

Finally, the model quality was assessed based on its ability to simulate organism growth. Biologically, the biomass reaction models the organism synthesising essential amino acids, membrane lipids, and sugars. When a metabolite flux is null, this implies the incompleteness of the GSMNs. It results either from missing reactions in the biosynthetic pathway or an accumulation of one or more reaction products due to the absence of a degradation reaction of these metabolites. Thus, to overcome this issue, manual analysis was carried out either by adding outward transport reactions or determining the missing reactions through similar organisms' GSMNs analysis. According to previous studies, *P. rubens* has a maximum growth rate of around 0.17 h^{-1} when it grows on sucrose [49]. Wild-type *Penicillium* species were reported to grow on glucose/sucrose at a rate between 0.14 and 0.22 h^{-1} [50]. In

*i*Prub22, by forcing the maximum specific thiamine, sulphur, phosphate and iron uptake to 1 mmol.gDW^{-1}, allowing an infinite source of oxygen, fixing a nitrogen input at 10 mmol.gDW^{-1}.h^{-1} and leaving the rest of the uptakes closed, the absolute growth flux prediction for *P. rubens* is between 1.42 and 3.04 h^{-1}.

Comparisons and evolution of *Penicillium rubens* GSMN

Specialised metabolism through versions

The GSMNs *i*AL1006 and Prubens involved 1,395 and 3,058 metabolites in 5 and 14 compartments, respectively. The evolution and comparison of *i*Prub22 metabolites with those of previous networks are based on MetaCyc (v23.0) identifiers for Prubens and the first section of InChIKeys generated from InChI for *i*AL1006. Indeed, due to the identifiers incompatibility, the comparison with the 2013 network was not direct and required caution in the analysis of the results. This latter GSMN included 849 unique metabolites (*i.e.* after removing compartment information), of which 554 (62%) are annotated with an InChI. Conversion of these identifiers to InChIKeys [51] yielded 512 different first sections InChIKeys (the first section of InChIKey corresponds to the structure without stereochemistry). *i*Prub22 had InChIKeys identifiers for 3,651 (67%) of its metabolites, of which 3,382 were unique first section. Based on the InChIKeys comparison, 24 were unique to *i*AL1006, and 488 were also found in *i*Prub22. These 488 metabolites were linked to 617 compounds (first part of InChIKey - no stereoisomery considered) in *i*Prub22, among which 6 were classified as “a secondary metabolite” according to MetaCyc ontology. Additionally, Prubens contained 2,594 metabolites, of which 2,415 (93%) possess a MetaCyc identifier (v23.0). Among these compounds, 2,377 were found in *i*Prub22, and only 38 were absent. Thus, *i*Prub22 encompassed 93% of Prubens’ metabolites and was enriched

with 2,779 additional entities. In the MetaCyc compound ontology, Prubens had 218 (8.4%) metabolites classified as “a secondary metabolite”, 211 of which are shared with *i*Prub22, which itself contains 434 (8.4%) entities of this class.

Connectivity and metabolic coverage

The proposed *i*Prub22 model is composed of 5,703 genes bringing the number of *P. rubens* genes into the model (*i.e.* metabolic coverage) to 45% compared to 8% and 14% for the first and second reconstructions dating from 2013 and 2018, respectively. In addition to an increasing number of reactions associated with better metabolic coverage links to recent improvements in databases and annotation methods, the major evolution over the years has logically been better connectivity inside models (Fig 3).

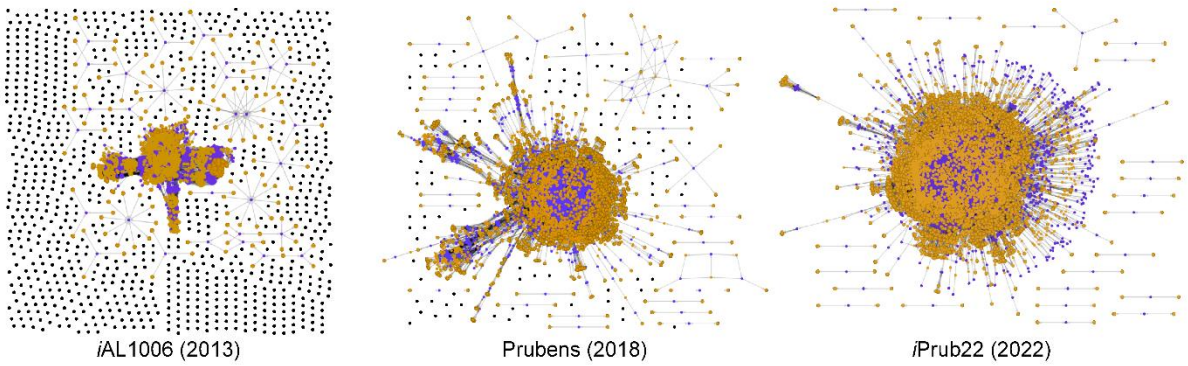


Fig 3. *Penicillium rubens* network visualisation through bipartite graphs. Evolution and improvement of the topology over the years. The purple (■) and orange (■) nodes represent the reactions and the metabolites, respectively. Black nodes (■) represent disconnected metabolites. Images were obtained with ModelExplorer (version 2.1) [52].

The GSMNs *i*AL1006, Prubens and our suggested reconstruction are composed of 1,006, 1,793 and 5,699 genes, 1,471, 1,793 and 5,895 unique biochemical reactions that connect 1,395, 2,594 and 5,192 metabolites, respectively.

Of the 5,703 genes present in the reconstruction, 3,771 (30%) new genes are included in the model, 800 (6%) are shared with the two previous versions of the network, and 939 (7%) and 193 (2%) are present only in the most recent and oldest version of the network, respectively. The 53 genes contained in previous versions and not found in *i*Prub22 are mainly involved in metabolite transport (data obtained with FungiFun, not shown). According to the GOT annotation provided by FungiFun (Fig 13 in S2 file), the genes contributing to this new model have annotations covering various biological and metabolic processes such as antibiotic responses, signal transduction, and protein phosphorylation and molecular binding. Also, the KEGG annotation indicates that processes involving DNA and RNA (*e.g.* nucleotide metabolism, transcription, translation, replication and repair) are present. These categories are reflected in the annotation provided by FunCat. These 3,771 genes are associated with a minimum of 1 reaction and a maximum of 61 reactions, for a mean and median of 4.5 and 2 reactions, respectively.

Moreover, among the 6,884 available sequences which are not included in this reconstruction, about 61% of them are of sufficient size to encode functional enzymes (*i.e.* an average of 380 amino acids) (Fig 4).

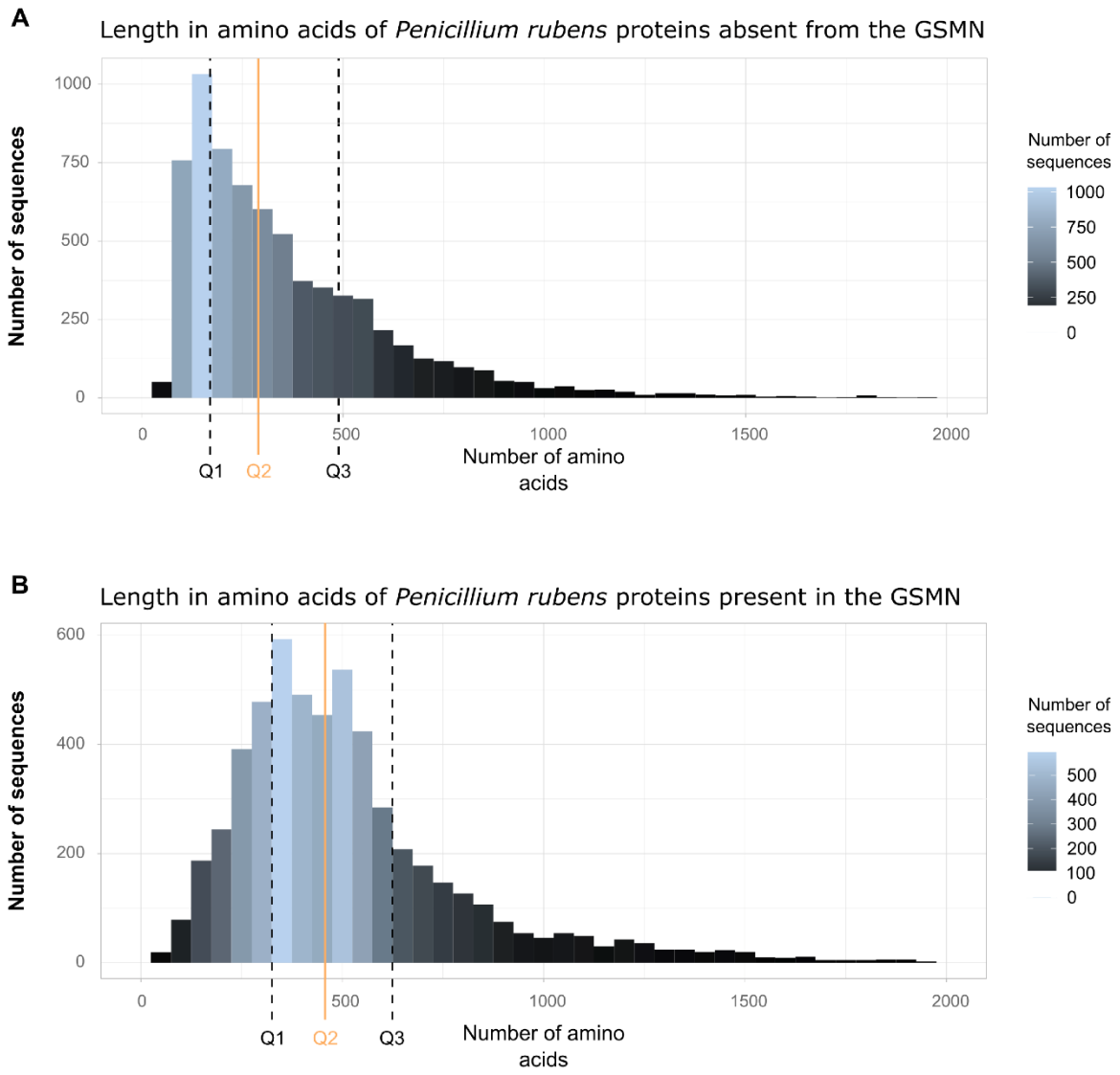


Fig 4. Amino acid length of *Penicillium rubens* sequences not included (A) and present (B) in the reconstruction. Median (continuous orange line ■) and quartiles (black dot lines) are shown. A range of 50 amino acids is displayed. (A) The average size of the 6,991 *P. rubens* sequences is 373 amino acids, and the median is 290. Measuring intervals: [15(min)-170];] 170-290];] 290-489];] 489-3,852 (max)]. (B) The average size of the 5,565 *P. rubens* sequences is 539 amino acids, and the median is 458. Measuring intervals: [28(min)-325];] 325-458];] 458-624];] 624- 7,282 (max)].

Interoperability

The identifiers of metabolites and reactions were kept consistent with MetaCyc and cross-referred through MetaNetX [53]. The focus was on various annotation

enhancements (*e.g.* identifiers interoperability of metabolites, reactions, and genes between known databases) and on the addition of Systems Biology Ontology terms [54] (SBO) to match the standards established *via* MEMOTE. SBO annotations provide semantic information that characterises the model components [55]. Concerning metabolites annotations, a relatively low match rate on the BiGG (13%) and KEGG (43%) historical reconstruction databases was noted. On the other hand, more than two-thirds of the metabolites had a chemical identification (*e.g.* ChEBI, PubChem). Details are given in Table 2, and *i*Prub22's different characteristics are available in Supporting Information in the form of a MATLAB live script (S7 file). The reconstruction presented here is rated with a MEMOTE score of 68% [54]. Finally, based on these various annotations, it remains possible to complete them with mass spectral information from the MassBank [56] and GNPS [57] mass spectral databases for further identification in real biological samples. Based on a mapping from the InChIKey, 508 metabolites were associated with at least one spectral data.

530 **Table 2. Details of the GSMN enrichment (nature and number of metadata).**

Metabolites	
Entities number	5,454
▪ **Structure identifiers**	
○ InChI	3,876 (71%)
○ InChIKey	3,877 (71%)
○ SMILES	5,266 (97%)
○ Molecular weight	5,260 (96%)
▪ **Database identifiers**	
○ BiGG	716 (13%)
○ BioCyc	5,431 (99.6%)
○ CAS	957 (18%)
○ ChEBI	3,559 (65%)
○ Chemspider	1,484 (27%)
○ DrugBank	237 (4,3%)
○ HMDB	1,547 (28%)
○ KEGG	2,367 (43%)
○ KNApSacK	96 (1.8%)
○ LIPIDSMAPS	138 (2.5%)
○ MetaboLights	1,048 (19%)
○ MetaNetX	5,426 (99.5%)
○ ModelSEED	4 (0.07%)
○ PubChem	3,683 (68%)
○ SABIORK	12 (0.22%)
○ SwissLipids	26 (0.45%)
○ UMBBD	50 (0.92%)
▪ **SBOTerm**	
○ Simple chemical SBO:0000247	5,454 (100%)
Reactions	
Entities number	5,897
▪ **Database identifiers**	
○ BiGG	4 (0.07%)
○ BioCyc	5,162 (88%)
○ Brenda	4,343 (74%)
○ KEGG	2,470 (42%)
○ MetaNetX	5,426 (92%)
○ Rhea	2,873 (49%)
○ Seed	1,329 (23%)
▪ **SBOTerm**	
○ Biochemical or transport reaction SBO:0000167	3 (0.05%)
○ Biochemical reaction SBO:0000176	5,279 (90%)
○ Translocation reaction SBO:0000185	50 (0.8%)
○ Exchange reaction SBO:0000627	218 (3.7%)
○ Demand reaction SBO:0000628	37 (0.6%)
○ Biomass production SBO:0000629	1
○ ATP maintenance SBO:0000630	1
○ Transport reaction SBO:0000655	53 (0.9%)
○ Active transport SBO:0000657	38 (0.7%)
○ Passive transport SBO:0000658	108 (1.8%)
○ Symporter transport SBO:0000659	21 (0.4%)
○ Antiporter transport SBO:0000660	2 (0.03%)
○ Spontaneous reaction SBO:0000672	86 (1.5%)

Finally, interoperability between tools was ensured by upgrading the network distribution format to comply with SBML level 3.

Discussion

The strengths and limitations of reconstruction automation

Since the publication of a reference protocol for the reconstruction of GSMNs [58] and the emergence of omics data, numerous microbial models have emerged, essentially of bacteria. Methods established primarily from microorganisms have been automated [19,59], allowing simultaneous and multiple eukaryotic GSMNs creations [34,39]. In the meantime, several tools [59,60] have also been developed, raising interoperability issues and the well-known need for standardisation. Thus, AuReMe [61] was chosen for its data traceability and network visualisation capabilities. This toolbox merges data from functional annotation, orthology search from previously published models and manual expertise. Traceability of the reaction origin has a double objective. It provides information both on evolutionary and phylogenetic concepts, thus highlighting the core genome, which mainly characterises the genes involved in essential intermediary metabolism, DNA replication and repair, and transcription and protein synthesis. Additionally, traceability provides a quality estimation of the reconstruction by giving a ‘confidence weight’ to the reactions. This manual curation can be performed in two ways, focusing either on reactions or on genes. Thus, all of the information presented in Fig 1 and Fig 9 in the S2 file are clues to guide the refinement of the draft toward obtaining a GSMN. In addition, the complementarity between functional annotation, orthology search and external sources addition during the reconstruction process is also visible in Fig 10 in the S2 file. Hence, the used approaches and data complementarity are

the heart of the GSMN reconstruction process for *P. rubens*. Therefore, is there a legitimate interest in cross-referencing all of this information which is fundamentally and originally the identical mechanism result, which is sequence homology?

The first question that can be asked concerns the choice between carrying out a reconciliation of pre-existing data or a *de novo* reconstruction. As described by Oberhardt *et al.*, the reconciliation process implies that for the two previously reconstructed networks, if there is “no discriminating biological evidence for a given reaction to being included in one reconstruction but not the other, the reactions should be identically included in both”. The struggle is real since working with reconstructions using incompatible or home-made identifiers is a significant source of error and a complicated task (Fig 6 in S2 file). However, in terms of network evolution perspectives, the consensus and systematic comparison of these data could lead to improvements (*i.e.* refining genomic information by updating the GPR associations) for future manual curation.

These comparisons provide information on the complementarity of the approaches and the sensitivity/specificity of the GPR associations. Thus, one of the limits of the automated reconstruction lies in the assignment of genes for non-specific reactions (*i.e.* a large number of genes being associated with such type of reaction). Moreover, intra-operability and especially identifiers durability as the databases are updated is sometimes not guaranteed. For example, of the 1,291 reactions from PchCyc, 79 (6%) have an incompatible identifier with MetaCyc version 23.0. For these reasons, few tools currently exist that allow automatic comparison of these networks, although efforts are being made in this direction [62]. As a result, relatively few model organism

reconstructions benefit from pre-existing data [20,21], and when they do exist, they require a significant community effort [18].

Another example of information loss is related to database mapping, which arises in particular when integrating subnetworks obtained by orthology search. During the creation of the orthology subnetwork, more than half of the reactions were discarded due to a lack of MetaCyc-compatible identifiers. These reactions, supported by genomic data, could, therefore, constitute a preferred pool for future completion of the GSMN (*i.e.* addition of reactions or refinement of existing GPRs). For example, from the 546 lost reactions during the mapping on *N. crassa*, 505 were allocated to metabolite transport and had model-specific identifiers. Only manual curation will allow for the recovery of such information. Thus, this known interoperability problem (*i.e.* lack of MetaNetX [53] identifiers or model-specific identifiers) highlights the need for uniform writing standards both at the database level and in the intrinsic writing of models. Also, this points out the issue in the relevance of reconciling the subsequently reconstructed networks. While these networks carry relevant genomic information, their integration is tricky, as shown in the section on modifications of the reconstruction to obtain a viable flux model.

The second question is whether orthology search should be worth using in addition to functional annotation. Are these two approaches redundant or complementary? As orthologous genes arise by speciation events, these groups of genes are assumed to share identical biological functions [63]. By definition, the search for orthologous sequences allows the metabolism section detection common to organisms (*i.e.* consensus), thus highlighting their evolutionary history. Constitutive metabolism will therefore be mainly targeted by this type of approach since it is shared between

various species. On the other hand, due to its specificity, specialised metabolism can only be incorporated into the models by functional annotation or, if it fails, by manual curation. The functional annotation approach should, therefore, cover the one performed by orthology search. However, 16% of the reactions in the draft still come exclusively from one or more subnetworks resulting from the orthology search. Therefore, this method also helps to benefit from the manual expertise within the models used for orthology search, as it would appear that the information they contain is not systematically referenced in the functional annotation database (*i.e.* 764 reactions are exclusively specific to the orthology subnetwork – Fig 1). In addition, one would also expect the most informative templates to be the most phylogenetically related. In the case of the *i*Prub22 reconstruction, most information obtained by orthology search came from *S. japonica*. Therefore, the most informative template for the reconstruction is not fundamentally the most biologically relevant. Since the selected models were published between 2010 and 2018, and the *S. japonica* was the most recently reconstructed network using a protocol very similar to the one used in this study, this reflects a lack of details in the past and present data rather than proper evolutionary concepts.

As mentioned above, the specialised metabolism of an organism can probably only be raised by functional annotation. What about its presence within the proposed reconstruction? This point can be illustrated by focusing on two compound families: roquefortines and penicillins. First, roquefortines and meleagrin are indole alkaloids produced by several *Penicillium* and *Aspergillus* species from tryptophan and histidine. Knowing that all the information related to the synthesis of these compounds is encoded in MetaCyc (since version 20.5) and is grouped under the PWY-7609

super-pathway, why is there no trace of them, even partial, in the draft? As described in Guzmán-Chávez *et al.* [10], roquefortines and meleagrin are catalysed by 13 reactions mediated by 6 enzymes/genes. Since their pathways are not described in previous reconstructions or orthology templates, the only possibility to incorporate this specific pathway in our reconstruction is from the functional annotation subnetwork. However, although the 6 genes involved in the biosynthesis of these compounds were present in the draft and encoded between 2 and 20 reactions, no reactions related to the pathways concerned were found in the draft. Backtracking its absence, through traceability provided by AuReMe, indicated that this absence is related to an error in the PathLogic module use. It unintentionally and automatically pruned 9 genomic sequences whose 6 belong to the roquefortines pathways. Concerning the second example, within MetaCyc, the reactions leading to different penicillins biosynthesis are listed in 3 pathways: PWY-5629 (isopenicillin-N biosynthesis – 2/2 reactions found in the draft); PWY-7716 (penicillin G and penicillin V biosynthesis – 3/4 reactions found in the draft); PWY-5630 (penicillin K biosynthesis – 1/1 reactions found in the draft). Although their names vary from one database to another and within the literature, the genes responsible for the production of these compounds were identified as pcbAB or ACV or ACVS (*Pc21g21390*); pcbC or IPN or IPNS (*Pc21g21380*); pcbDE or iAT or AAT or PenDE (*Pc21g21370*); phl or pclA (*Pc21g14900*). PenDE, ACVS and phl were discarded by PathoLogic during reconstruction like the roquefortines biosynthetic pathways genes. It would appear that discarded genes and associated reactions are deleted if, and only if, they are not supported by any other gene. For example, the following two reactions are catalysed by PenDE: RXN-1702 and RXN-17100 (*i.e.* production of penicillin G and

interconversion of penicillin G to penicillin V, respectively). For the first reaction, PathoLogic infers two sequences (*i.e.* one corresponding to PenDE based on the EC-number and another based on the GO annotation). Since this reaction is supported by two genes, the tool does not perform any pruning step. Conversely, the second reaction is only supported by PenDE and is consequently excluded from the reconstruction, as are all reactions related to the synthesis of roquefortines. Nevertheless, the reconstruction methods' complementarity allows us to recover almost all of the penicillin biosynthetic reactions, even if the GPRs are not precise enough. Moreover, the genes associated with penicillins synthesis, *Pc21g21380* (IPNS), *Pc21g21370* (PenDE), and *Pc21g14900* (phl), were present in the draft and supported between 2 and 12 reactions. However, *Pc21g21390* (ACVS) was absent from the draft even though the reaction it catalyses was found in it and supported by 10 other genes found by orthology. These results raise questions about the relevance and accuracy of the automatically deduced GPRs associations and, thus, more broadly, about the quality of the networks used for reconstruction through orthology. Therefore, in addition to the entire PWY-7609 pathway for roquefortine biosynthesis (Table 3 in S2 file), manual curation was limited to the inclusion of the RXN-17100 reaction (PenDE-mediated interconversion of penicillin G to penicillin V) and the ACVS gene for the 6.3.2.26-RXN reaction (the first reaction inducing isopenicillin-N synthesis). Even if GPRs' specificity and accuracy are essential for model simulations (*e.g. in silico* knock-out), verbose GPRs within a reconstruction remain sources of information to explain evolving concepts. Thus, GPRs associations cleaning (Table 4 in the S2 file) was not performed.

The third and last point concerns the limits of reconstruction automation, notably the gap-filling steps. Gap-filling makes it possible to complete the pathways from a structural point of view. As this process is based on heuristics and the absence of genomic data, additional results from such tools must be done sparingly and carefully. Here, sets of targets were used to perform gap-filling against proper subsets of MetaCyc depending on the purpose (*e.g.* preferential selection of spontaneous or fungi reactions). Iterative processes were performed with the repair networks and targets from most to least relevant to achieve 'reasoned gap-filling'. Nonetheless, beware that performing a complete gap-filling, *i.e.* using the set of putative metabolites present in our reconstruction against the entire MetaCyc database, is absurd from a biological and evolutionary point of view. This set was used only against controlled versions of repair networks (*i.e.* for the detection of spontaneous reactions and for the last round of gap-filling whose supporting genomic sequences are unknown in MetaCyc). By reasoning in this way, it is possible to give an additional confidence weight to almost half of the gap-filling reactions (Fig 12 in S2 file). As shown in the results presented in Table 1, gap-filling improves the connectivity of the GSMN. The semi-automated process for the draft generation allows topologically reach 86% and 22% of the selected targets belonging to the constitutive and specialised metabolisms, respectively. These numbers demonstrate the methods' efficiency for the automatic reconstruction of basal metabolism. It also points out how far we still have to go with regard to specialised metabolism. Furthermore, it should be remembered that, as described in Dusad *et al.* [64], topological approaches are limited by two well-known issues, namely hub metabolites and reactions reversibility.

This last point will be discussed later in the section on the reconstruction evolution to the flux model.

Finally, what parameters should be considered as a plausible possible model, as close as possible to biological reality (*e.g.* addition, modification, deletion of reactions and constraints)? This point should be addressed at various scales, from the most generic (*e.g.* addition of layers of information related to compartmentation, spontaneous reactions and exchange reactions) to the most specific and precise (*i.e.* refinement of metabolic pathways with a focus on the nature of GPRs associations). Due to their nature, these steps often require manual expertise and thus highlight the limits of such automation. As *i*Prub22 is intended to be improved in this sense with feedback from the scientific community, a user-friendly wiki is available to facilitate communication and improvement for future versions. The wiki associated with the GSMN offers a less cumbersome virtual alternative to the efforts developed during the nonetheless indispensable processes of community meetings over the past years (*e.g.* jamboree or workshop) [65–67].

GSMN quality

The GSMN origins date back to 1999, with the first model publication for the bacterium *Haemophilus influenzae* [68]. Thiele and Palsson published a protocol for reconstructing metabolic networks in four key steps ten years later, which is still used as a reference today [58]. However, in the history of GSMN reconstruction, the development of uniform criteria for judging the quality of a model is relatively recent, encouraging the tendency to prefer quantity over quality [69]. Except for the MEMOTE test suite [54], there are still few tools or measures available to assess the completeness of a GSMN. Thus, the GSMN presented here has been reconstructed

following, among others, these three minima and general criteria defined by the work of Monk *et al.* [69]:

- the extent of manual curation: what aspects and means are used to refine the model? This point was addressed in the previous section.
- metabolic coverage: what is the metabolic space, *i.e.* the genome proportion, covered by the data present in the GSMN?
- the use of established standard operating procedures: what are the means and standards of writing to be used to encode the information? This step is independent of biological knowledge and allows the GSMNs application scope to be extended.

With the improvement of methods and the data proliferation, the *P. rubens* GSMNs evolution underlines a trend towards improved metabolic coverage over the years that seems to be enhanced substantially. The function of an enzyme is intrinsically dependent on its three-dimensional shape, *i.e.* structure and folding, which in turn is induced by the amino acid sequence of which it is composed. It should also be noted that eukaryotic proteins are generally longer (*i.e.* median size of 360 amino acids [70]) than those of other living organisms. Therefore, the fewer amino acids a *P. rubens* sequence contains, the less likely it is to carry a functional catalytic domain. The sequence size distributions presented in Fig 4 show that of the sequences discarded from the GSMN reconstruction, almost half are less than 300 amino acids in length. The remaining half is of sufficient size to encode functional enzymes and constitute a reservoir/pool of candidates for future improvement of the network. This set of genes with unknown functions and probably specific to the studied organism offers

research perspectives for an improvement of future models (*e.g.* unlocking the topological accessibility of certain dead-ends).

Computer modelling is one of the means used by Systems Biology to understand, organise and integrate large quantities of data from diverse sources (*e.g.* molecular, cellular, *etc.*). Relying on ontologies (*i.e.* set of controlled vocabulary) allows the exchange, interoperability and reuse of the models produced. This step, independent of model functionality, can occur at all stages of its life cycle. One aspect related to computational interoperability is associated with the annotation of the model entities in terms derived from the 'Systems Biology Ontology' (SBO) [55]. This type of annotation is not specific to metabolism modelling. However, it allows, like any ontology, to generate a classification and a hierarchy of the data making them easier to interrogate for the user since controlled vocabulary use is part of good practice recommendations for computer modelling. In addition to the distribution format, network sustainability depends on its usability. It is well known that interoperability between databases is a delicate task. Therefore, the most exhaustive annotation of the entities in the model is essential and is now an undeniable criterion for increasing the quality of a network [23,25]. Firstly, when these annotations belong to specific databases such as KNApSAcK (*i.e.* a database dedicated to specialised metabolites), they constitute an entry point for model exploration. For information purposes, *i*Prub22 contains 81 metabolites annotated with an identifier from this database. Secondly, the molecule's structural identifiers allow the entities to be identified and used as an anchor for querying other databases. For instance, using an InChIKey mapping considering only the first identifier section, thus disregarding the stereochemistry and compound's charge, 9.8% of GSMN metabolites are linked

to mass spectral information retrieved from the GNPS and MassBank databases. This coverage is lower than the average 40% detected by Frainay *et al.* work [71]. This confirms the ever-limited aspect of obtaining and integrating metabolomics data acquired in given organisms within GSMNs. Such difficulty could be one more problem in validating GSMN behaviour, at the chemical level, with respect to biological observation.

However, as mentioned above, MEMOTE [54] is a suite of tests aimed at qualifying a model by providing a summary of its characteristics both in terms of topology and flux. Its quality score considers the annotation content of the model. For example, a complete annotation in SBO terms guarantees a quality of 40%. This tool provides an easily accessible resource aiming toward model standardisation for detecting blocking points and/or abnormalities, thus helping manual curation. The GSMN we propose possesses a MEMOTE score of 68% against 31% and 16% for the 2013 and 2018 networks, respectively. Nevertheless, due to the biases taken during the reconstruction, such as the creation of artificial genes to help their identification, the systematic conservation of all data supported by genomic information and the annotation of some SBO terms not currently monitored, the 68% score is slightly underestimated (the MEMOTE report is available in S8 file). Furthermore, this tool is designed and optimised to evaluate models that preferentially respond to the SBML3 FBC2 format. Since the previous networks were not encoded with the flux balance constraints (FBC) package, some characteristics cannot be evaluated properly, and thus, their MEMOTE results should be put into perspective. This is particularly true for analyses related to genes and their products, as this notation was only introduced in version 2 of the FBC package.

The recurrent non-reproducibility of results, known as the reproducibility crisis [72], affects all areas of science and *in silico* modelling is no exception. In fact, limited interoperability between software may yield incorrect models parsing, which can lead to errors or misunderstandings when analysing GSMNs [73]. Thus, the proposed model was standardised in the most recent versions of SBML and its extension package flux balance constraints (FBC). However, even if the emphasis on reconstruction has been placed on the maximum transparency and traceability of data acquisition, with the constant evolution of databases and tools, providing all the information for the strict whole model reproducibility remains challenging. For this reason, GSMNs are regarded as knowledge state snapshots about an organism at the precise moment of its reconstruction, so a particular effort to keep on watch would be necessary to ensure their sustainability. Ultimately, the main objective of this reconstruction is to propose a resource grouping of known data for *P. rubens* based on these three key complementary notions: traceability, accessibility and visibility, focussing additionally on the exploration of specialised metabolisms' presence.

From reconstruction to a model that simulates environmental exchanges

Transforming a genome-scale reconstruction into a computational model for predicting properties and behaviour is a considerably time-consuming process that requires expertise. Even if steady-state constraint analyses are neither informative on the concentration of the metabolites nor on the temporal dynamic system aspects, it is still the more appropriate tool for exploring complex biological systems. These analyses are based on linear optimisation theories and constraints implementation based on three aspects: physicochemical (*i.e.* steady-state, stoichiometry,

thermodynamics), topological (*i.e.* spatial modelling of intracellular and extracellular compartments) and environmental (*i.e.* various nutrient media simulations defined by exchange and transport reactions) [74]. The previously obtained reconstruction is used as a backbone for mathematical approach modelling, which will perform *in silico* predictive hypotheses-driven. However, the transition from the reconstruction model development to the fluxes models requires various adaptations, both computational and biological.

The uncertainty inherent in the GSMNs predictions is influenced by the numerous tools, methods and algorithms that exist for both the reconstruction phase (*i.e.* network structure) and the analysis phase (*i.e.* simulation results). However, the specific sources and magnitudes of these uncertainties remain complex to quantify, which presents challenges for evaluating the relevance and reliability of model predictions. In this respect, the recent great review by Bernstein *et al.* [24] identifies five uncertainty areas surrounding the reconstruction and analysis of GSMNS. These fields include (1) genome annotation, (2) specification of environmental conditions, (3) formulation of biomass equations, (4) network gap filling, and (5) flux simulation. Their work highlights the need to assess, communicate and understand the uncertainty sources associated with a model to discuss their impact on the relevance of predictions resulting from the model analysis. As functional annotation and gap-filling have been dealt with previously, we will focus here on the specification of the environment and the formulation of the objective biomass function. We would also like to stress that the focus of the work presented in this paper is on reconstruction considering specialised metabolism, and thus, we propose preliminary work on the

network analysis of the flux distribution. Finally, an insight into the necessary improvements to the model will be discussed in this section.

The biomass reaction is a commonly used objective function in constraint-based modelling, including FBA and FVA, that simulates the growth of a specific organism. It identifies essential growth compounds and assigns weights based on their occurrence, normalised to represent the dry-weight biomass of 1 gram. Understanding and optimising the growth of organisms requires defining the biomass reaction and its subsystems, which correspond to the synthesis of macromolecules from building blocks and precursors. Experimental values for growth-associated ATP maintenance (*i.e.* GAM) and non-growth-associated ATP maintenance (*i.e.* NGAM) are added to the reaction, representing the energy required for macromolecule polymerisation during growth and for maintaining the organism's viability, respectively [58]. However, in the absence of specific data, the biomass composition of a model organism is often used as a template, generating biases in the interpretation and aggregation of results when comparing GSMNs [24]. *Penicillium rubens* is a well-studied model organism, and experimental data indicate that its relative macromolecule content is distributed as follows: 45% proteins, 25% carbohydrates (including 22% cell wall compounds and 3% glycogen), 5% lipids (including 0.5% fatty acids, 1% sterol esters, and 3.5% phospholipids), 9% nucleic acids, and 8% each of ASH and the soluble pool [75,76]. As the biomass reaction in *i*AL1006 was constructed based on these experimental data [26], the same reaction is used in *i*Prub22. However, *i*Prub22 lacks several subsystems found in *i*AL1006 that regulate the biosynthesis of fatty acids (*i.e.* r1434), phospholipids (*i.e.* r1460), lipids (*i.e.* r1464), sterol esters (*i.e.* r1462), and glycerides (*i.e.* r1461). These subsystems

could not be included in *i*Prub22 due to the absence of compatible or relevant MetaCyc identifiers. Finally, refining or improving the biomass reaction for more accurate growth prediction can be achieved through sensitivity analysis, identifying critical reactions and key parameters for maximum biomass yield, or experimental measurements to determine the exact quantities of components needed for growth, allowing adjustment of the biomass reaction coefficients. For instance, the BOFdat workflow [77] may offer a prospective solution to incorporate lipid monitoring, which is currently not present in *i*Prub22.

Defining the chemical composition of the environment for metabolic modelling is a delicate task fraught with uncertainties, as described by Bernstein *et al.* [24] (*e.g.* inconsistent media definition in specific databases or undefined chemical input in experimental settings). However, environmental specification is a critical component of flux analyses and serves as the cornerstone for such investigations. As filamentous fungi are known to possess cellular machinery that enables extracellular nutrient absorption, uptake reaction selection was carried out by focusing on extracellular metabolites. Specifically, we integrated reactions from the well-curated *i*AL006 model and complemented them with information on the extracellular localisation of gene products (*e.g.* inclusion of reversible or non-reversible transport reactions between the intracellular and extracellular compartments, regardless of whether they are artefactual). Thus, we adopted the "seed" approach (*i.e.* metabolites occurring naturally in the environment of the organism studied and essential input for the GSMN) to generate biologically relevant models that have the potential to enhance our understanding of metabolic processes and facilitate future investigations. Indeed with 185 uptake reactions included in the

reconstruction, *i*Prub22 offers a broad range of possibilities for modelling simulations. Moreover, proposing diverse combinations will give clues on organism behaviour by identifying aspects of *i*Prub22 predictions that exhibit either high sensitivity or resilience to changes in the environmental composition. Finally, once the nutrient nature has been defined, the subsequent step entails relying on experimental data to establish proper uptake bounds for flux simulation analyses.

Then, the modifications performed to deal with problems encountered when converting the reconstruction to a flux model focus mainly on updating and correcting the directionality of reversible reactions (*i.e.* adding constraints by closing reactions whose directionality remains unknown on MetaCyc) and on the redundancy contained in the reconstruction. This point is addressed at two levels. On the one hand, automatic and manual curation of duplicated reactions is carried out (*i.e.* reactions with the same reactants and products). The presence of such reactions is explained by the duplicate's existence within the databases and by the data reconciliation contribution (*i.e.* addition of reactions whose unbalanced equations do not allow their automatic detection). On the other hand, there is redundancy within the networks linked to varying metabolite names. Indeed, the same chemical entity may have several forms, and thus identifiers, that coexist within the model without having a bridge reaction between the different forms. Examples include the case of coexisting linear or cyclised states of molecules (*e.g.* carbohydrates), the chirality encoding as a function of the different stereoisomers, or the use of more generic concepts with compounds class against single chemical species (*e.g.* molecules ontology). From a computational perspective, this identifiers multiplication, or impoverishment when the annotation is too specific, leads to ultra-connected or

under-connected network sections. Uniformity of identifiers on parent or child terms can thus reduce this artefact. As these corrections directly influence the outputs of these constraints-based analyses, all the modifications applied to the model generation are reported as additional data.

These modifications result in a nitrogen-sensitive model capable of presenting different behaviours in agreement with the experimental data [78]. Nevertheless, without additional constraints, the model seems insensitive to carbon sources, either the high metabolic robustness of the fungus or the presence of extreme pathways such as futile cycle [79]. Indeed, with the only required uptake minima (*i.e.* a source of nitrogen, thiamine, iron, sulphur, oxygen and inorganic phosphate), there is nevertheless a production of D-glyceraldehyde 3-phosphate inducing the synthesis of fructose-6-phosphate which is, then redistributed among the different sugars present in the network. As it stands, the model proposes a phenotype close to that known for growth on ethanol. The addition of artificial constraints (described in the S7 file) allows the system to be unblocked and the GSMN model to become functional (*i.e.* produce biomass). However, the *in silico* prediction of biomass flux exceeds the experimentally measured growth rate by almost 90%. Hence, the model exhibits inadequate constraints concerning carbon uptake, leading to the observed increase in growth rate. One of the most plausible hypotheses for correcting this inaccuracy is to investigate the presence of thermodynamically infeasible cycles (*e.g.* type II or III extreme pathways, also known respectively as futile cycle or internal cycle) within the model [80]. In metabolic network modelling, thermodynamically infeasible cycles are a group of reactions that violate the laws of thermodynamics when active (*i.e.* unbalanced production or consumption). As thermodynamically infeasible cycles

do not generate advantageous metabolites while consuming energy, they are not considered feasible pathways within the metabolic network. The presence of these cycles is generally attributed to inaccuracies or errors in the reconstruction process, stemming from incomplete knowledge of metabolic pathways or oversimplified assumptions regarding, for instance, reaction reversibility or the absence of regulatory processes. The most commonly used method for internal cycle detection is the II-Cobra method [81], and other algorithms have emerged over the years, such as the CycleFreeFlux algorithm [82]. GlobalFit [83] identifies erroneous energy-generating cycles (*i.e.* cycles that charge energy metabolites without a source of energy since they consume cofactors to generate motive power), which are futile cycles that are more difficult to identify and highly prejudicial to the simulations. Therefore, the presence of infeasible cycles can significantly impact the predictive capability of models, making it essential to identify and eliminate them to enhance the accuracy and reliability of the models.

Finally, a significant improvement of the proposed model will lie in GPRs verification and correction. For reconstruction, the false positive presence within a GPR carries information (*i.e.* genomic sequence encoding an enzyme with a similar mode of action). Thus, the cross-referencing of false positives with GPR and gap-filled reactions would enable the pool creation of preferred candidates to be interrogated to detect, for example, new functionalities or to highlight evolutionary concepts. On the other hand, from the computational model side, erroneous GPRs greatly complicate the analysis of knock-out results *in silico*. Incorrect predictions, linked to imperfect knowledge of the models studied, are an opportunity for biological discovery. Indeed, the abnormal behaviours of the model allow us to pinpoint the

most relevant aspects of the metabolism and are, therefore, sources of hypothesis for addressing these failures. It is a valuable help in guiding experiments that often lead to new organism functionality characterisation.

What about specialised metabolites?

The reference strain *P. rubens* Wisconsin 54-1255 is the result of a long classical strain improvement programme [11]. Various rounds of random mutagenesis have led to the selection of a strain capable of producing higher quantities of penicillins, notably at the expense of other specialised metabolites production expressed by *P. rubens* NRRL 1951 wild-type strain [84]. Proteome analysis [85] and the study of these mutations [11] revealed lower expression levels for some genes related to specialised metabolism and the formation of non-functional proteins. The proposed GSMN expresses the potential for metabolite production carried by the genome and thus provides a platform for knowledge that can be enhanced from such data. However, it should be noted while the presence of a compound in the reconstruction offers a valuable starting point for further investigation, it does not necessarily guarantee its production within the model.

According to the classification of metabolites established by the MetaCyc ontologies, 13% of *i*Prub22 compounds would be associated with specialised metabolism. However, it is not uncommon to find metabolisms building blocks relative to constitutive metabolisms such as geranyl diphosphate or myoinositol in the specialised metabolites lists. This fact illustrates that the specialised metabolites concept, as understood by biologists or chemists, diverges from what is implemented in the databases. Thus, based on the information provided in the literature and the results of genome mining, the percentage of specialised metabolites contained in

*i*Prub22 should be further minimised. It raises the question of what constitutes a specialised metabolite for these communities and highlights the constant need for annotation standardisation.

Fungal specialised metabolites' particularity lies in their biosynthetic genes being co-located and forming sets called "Biosynthetic Genes clusters" (BGCs). Today such clusters are commonly identifiable *via* genome mining, but many of their products remain unknown. The review by Iacovelli *et al.* [86] reports 33 essential biosynthetic genes encoding 10 non-ribosomal peptides synthetases (NRPSs), 20 polyketide synthetases (PKSs), 2 hybrid NRPS-PKSs, and 1 dimethyl-allyl-tryptophan synthetase (DMATS). Non-ribosomal peptides identified include fungisporin, roquefortine C, D, F, M, and N, as well as associated products such as meleagrin, glandicolins A and B, histidyltryptophanyldi-ketopiperazine (HTD) and dehydrohistidyltryptophanyldiketopiperazine (DHTD). This list of NRPSs is also enriched by the following three siderophores, coprogen, ferrichrome and fusarinin family compounds. Among the polyketides identified are compounds belonging to the sorbicillinoid family and chrysogin. All these metabolites are well documented, studied and commonly accepted in the literature [7,10,11,13,87].

However, the identification of BGCs and their end products slightly differed depending on whether FungiSMASH or AntiSMASH was used (Table 5 in the S2 file). Thus, three BGCs coding for the following five compounds were found exclusively with FungiSMASH: naphthopyrone, chrysoxanthones A, B and C and depudecin. Concerning the AntiSMASH results, the BGC related to penicillin synthesis emerged more exhaustively since isopenicillin N, phenoxymethylpenicillin (penicillin V) and the precursor *δ*-(L-*α*-aminoadipyl)-L-cysteine-D-valine (ACV) were identified. In addition,

BGCs leading to the synthesis of PR-toxin, neurosporin A, ACT-Toxin II, melanin, NG-391, aspercryptins and chaetoglobosins are also raised only with AntiSMASH results. It is also worth mentioning that the similarity percentages (*i.e.* the confidence that can be placed on the actual presence of the BGC in the strain under consideration) can also be different from one version to another, as illustrated by the sorbicillin-encoding cluster.

Of the 42 BGCs identified using the FungiSMASH tool suite, 29% have yielded at least one distinct compound. However, only three of these BGC products (*i.e.* involved in the biosynthesis of penicillins, roquefortins, and initial steps of patulin) have been integrated into GSMN. Collecting and processing the information on specialised metabolites biosynthesis raises several questions. Firstly, are the metabolic pathways leading to the biosynthesis of those NPs comprehensively elucidated and documented in the literature? Furthermore, if so, are these pathways indexed in the relevant databases such as MetaCyc? For instance, CobraMod [88] is a pathway-centric curation tool designed to enable the modification and extension of GSMN. This tool extracts metabolic information from BiGG, KEGG, and BioCyc databases. However, depending on the existence of biosynthetic pathways for the selected metabolites and their degree of completeness, what measures are envisaged to enhance the modelling accuracy? This point leads to the two lists creation, one containing the metabolites for which an identifier exists in MetaCyc, and the other composed of orphan metabolites for which, among other things, the closest known antecedents (*i.e.* precursors) will have to be determined. Subsequently, if the metabolic pathways are fully present, curation consists of verifying the actual presence or absence of the reactions in the draft and, if the information in the literature

provides it, checking the corresponding GPR associations. The accuracy of the GPR associations is pivotal for the biological and computational interpretation of the *in silico* knock-outs. Thus, to conform with the literature data, manual curation was applied to *i*Prub22 for penicillin, meleagrin, roquefortine and the last known precursor of patulin produced [7]. On the other hand, if the metabolic pathways are semi-complete, like for yanuthone D [89] or absent, like for chrysogine [90], the analyses will focus on the closest known precursors, and further work will aim to find the most accurate method for integrating and generating the missing information. These gaps could potentially be resolved by adding modules or a single reaction, balanced and supported by all the genes involved in the biosynthesis of the compounds of interest. Recently an automatic pipeline called BiGMeC (Biosynthetic Gene cluster Metabolic pathway Construction) has been developed to automatically reconstruct metabolic pathways associated with BGCs, specifically targeting PKS and NRPS [91]. Based on the analysis of antiSmash outputs, the resulting enzymatic reactions take into account redox cofactors and energy demand. The reactions determined in this way are compatible with the BiGG database, promising a facilitated integration of the biosynthesis pathways encoded in the well-characterised or not BGCs into the GSMNs. Finally, in the case of orphan metabolites, it will be necessary to create both the metabolites and the reactions underpinned by genomic sequences. To maintain optimal traceability, the addition of these entities will be supported by as much metadata as possible such as bibliographic references for the constitution of biosynthetic pathways, InChIKey, InChI, PubChem identifiers, molecular weights, charges and SMILES until their incorporation into public databases. In addition to the biological relevance and the creation of a model as close to reality as possible, the

interest in precisely recreating these pathways lies in the possibility of subsequently analysing in greater detail the distribution and flux reallocation from the precursors of all the targeted metabolites.

The last point concerns the interoperability between specialised metabolic databases and reconstruction databases. The LOTUS database [43] (*i.e.* database of specialised metabolites classified by taxonomy) is queried to target specialised metabolism more specifically. Of the 240 hits found for *P. rubens*, only 47 compounds are potentially usable (*i.e.* metabolites mapped based on their InChIKey versus MetaCyc). This low proportion of integrable data is even more marked if we focus on the Natural Product Atlas database [92], a microbial NP resource containing, among others, referenced data for structure, compound names and source organisms. Still, based on the InChIKey string, 102 compounds were reported for *P. rubens*, but only 6-aminopenicillanate was present in MetaCyc (v23.0) as in the network structure. Extending the search to the genus *Penicillium*, 2,159 NPs are reported. However, only 20 of these compounds have an exact match on MetaCyc, and 44 molecules are raised, looking only at the first section of the InChIKey. Among them, 17 are present in *i*Prub22, including the following seven exact matches: citreoisocoumarin, griseophenone C, brevinamide F, verruculogen, paxilline, 6-aminopenicillanate and *β*-10-hydroxy-12-demethyl-11,12-dehydropaspaline. These 17 metabolites are involved in 6 consumption reactions, 8 production reactions and 1 reversible transport reaction. These 15 reactions were introduced in the GSMN from either previous networks (*i.e.* 3 from *i*AL1006 and 6 from Prubens), gap-filling (*i.e.* 4 reactions) or functional annotation (*i.e.* 2 reactions). Once again, this reflects the limited compatibility and communication between databases for specific data

integration since most of the specialised metabolism in the strict sense found in *i*Prub22 comes from manual processes.

Conclusion

This work presented a revised version of the *P. rubens* Wisconsin 54-1255 GSMN, which consists of 5,897 reactions, 5,033 genes and 5,192 metabolites. The reconstruction was achieved by updating the functional annotation, searching for orthology links with various organisms and reconciling data from previous *P. rubens* GSMNs. A preliminary flux constraint model, simulating the growth of the fungi in a minimum environment, has also been made available and can be adjusted for further analysis of the specialised metabolism. The reconstruction generates multiple models, and their simulations refine and enhance the reconstruction by identifying potential avenues for improvement or correction. This iterative and cyclical process enables a comprehensive and significant improvement in the studied organism understanding, ultimately leading to increasingly accurate models. Finally, a particular effort was made to explore the specialised metabolism integrated into the model. Although the generation of more data in recent years has led to an increasing size of networks, the percentage of specialised metabolites in the models remains unchanged. Therefore the effort undertaken to update the reconstruction databases seems linear, but the proportion devoted to specialised metabolites warrants further investigation. Exploring the presence of specialised metabolism further highlights the lack of information within the databases and shared identifiers. Such problems need to be addressed before any use of GSMN at a large scale in NP studies.

Materials and Methods

Reconstruction is performed following the steps recommended in the 2010 Reference Protocol [58]. *i*Prub22 is provided in the SBML community standard format [93] with a quality score of 68% evaluated by MEMOTE (v0.13.0) [54]. To ensure transparency and reproducibility, the reconstruction process and related documents are available in Supporting Information.

The primary stages: generating the draft

Data origin

The genomic data for *P. rubens* Wisconsin 54-1255 (*i.e.* 12,557 protein and gene sequences), as well as the General Feature Format file associated, were downloaded from the Ensembl Fungi browser under accession number GCA_000226395 [13].

Functional annotation

Penicillium rubens functional annotation is performed using the Trinotate pipeline (v3.2.1) [31] for its boilerplate SQLite database. Thus, homology analyses are launched with blastx and blastp (v2.5.0) with an e-value cut-off of 0.001 against the UniProt database (download on 27 June 2020). The best hit of a sequence is considered its annotation. Search for protein domain is carried out by HMMER (v3.3) [94] against the PFAM database (download on 27 June 2020) with an e-value cut-off of 0.001 and a threefold in the per-domain output of 0.01. SignalP (v4.1g) [95] and TMHMM (v2.0c) [96] are used to search for peptide signals and transmembrane domains, respectively, with their default settings. Protein subcellular localization is determined using DeepLoc (v1.0) [97].

Data that are mandatory for the GSMN reconstruction, Gene Ontology Terms (GOTs) [27,28] and EC numbers are captured respectively using an internal Trinotate script and by querying the KEGG database [29] using its own orthology identifiers (KO). The resulting KO list is enriched by a *P. rubens* proteome analysis performed on the KAAS webserver (Automatic Annotation Server v2.1) [30] against a dataset composed of 367,616 genomic sequences exclusively selected from 37 fungi kingdoms (Best Bidirectional Hit-method and other settings by default – July 2020) and by the exploitation of pre-existing *P. rubens* data [13] on KEGG (Genome information accession – T01091). EC-numbers are then retrieved from these three selected KO sets using KEEG-API REST (REpresentational State Transfer) application programming interface in July 2020.

Annotations combined with the information contained in the *P. rubens* General Feature Format are aggregated to generate a GenBank file using the script: https://github.com/ArnaudBelcour/gbk_from_gff. This file was then used as input to the PathoLogic software from the Pathway Tools suite (v23.0 default settings) [98]. This qualitative metabolic model reconstruction module allows the inference of the reactome from the annotated genome. The database containing the information from the annotation was then exported in attribute-value flat files, which were necessary for further analysis in the AuReMe workspace [61] (see Data consensus and integration for further details).

Orthology completion

Using OrthoFinder (v2.3.12) [32] and Blastp (v.2.5.0), Reciprocal Best Normalized Hit (RBNH) strategy was applied to identify functionally identical genes. Blastp was parametrised with an e-value of 0.001 and performed against seven selected

templates: *Arabidopsis thaliana* [33], *Aspergillus nidulans* [34,35], *Aspergillus niger* [34,35], *Neurospora crassa* [36], *Penicillium* species complex [34,35], *Saccharina japonica* [37] and *Schizosaccharomyces pombe* [34,35]. Upstream, data from the different selected templates were filtered to keep only the sequences present in the associated GSMN models.

External sources

The reconstruction is enriched and completed by external data sources. First, data from the Pathway/Genome Database Concepts PchCyc (PGDB belonging to Tier 2) available on BioCyc are searched from the special SmartTables [38] provided. Second, compliant information present in the first [26] and latest [39] versions of the network are extracted and parsed to be added to the draft. Only those reactions supported by genomic information that either has a MetaCyc compatible reaction ID or reactions for which all reactants and products have a MetaCyc ID (in which case the original reaction ID is retained) are incorporated into the draft.

Data consensus and integration

AuReMe (AUtomatic REconstruction of MEtabolic models – v2.4) dedicated to GSMNs reconstruction [61] was used to reconstruct the *P. rubens* GSMN. This ToolBox encapsulates the various programs needed to create a high-quality network and to maintain a consistent steps record. Results obtained from PathwaysTools and OrthoFinder are injected into AuReMe to generate the intermediate subnetworks resulting from the functional annotation and the orthology search, respectively. Reconstruction was based on the data present in MetaCyc (v23.0) [99] As data templates for orthology refer to other databases like KEGG [29] or BIGG [100], a mapping operation using MetaNetX dictionary [53] (*i.e.* intrinsic to AuReMe MNXref

Version 2018/09/14) was performed to obtain identifiers compliant with MetaCyc. These data are then merged and completed with the information from external sources. Gene annotations are evaluated and compared using the FungiFun [101] web server (v2.2.8 Beta). The resulting draft is then analysed both qualitatively using topological analysis and quantitatively using constraint-based analysis, refined by manual curation and enriched with data extracted from the literature (See the following section).

The majority of the Figures are drawn using R (v3.5.1) and the following packages: ggplot2 (v3.3.5) [102], hrbrthemes (v0.8.0), ggpubr (v0.4.0) for visualisation and plyr (v1.8.6), dplyr (v1.0.7) and forecast (v0.5.1) for data formatting. Comparisons are made using the Venns diagram (http://bioinformatics.psb.ugent.be/webtools/Venn) for the simplest cases and upsetplot with UpSetR (v1.4.0) [103] for the most complex cases. For the visualisation of the network with KEGG maps, KEGG mapper was used [104].

From draft to high GSMN quality

Potential producibility – topological analyses and manual curation

The reconstruction is refined by examining the presence of known compounds (*i.e.* called targets) in *P. rubens*. The metabolites list to be verified is first established according to data described in the literature, molecules isolated in the laboratory and results identified by AntiSMASH/FungiSMASH [42]. This list is enriched by metabolites present in the LOTUS database (https://lotus.naturalproducts.net) [43] to focus more precisely on the specialised metabolism. Lastly, the metabolites present in the draft are filtered according to the annotations containing the term “secondary” in the MetaCyc ontologies.

Then, the potential topological producibility tests were carried out using the MeneTools suite (v3.2.0) [105]. It was necessary to define a list of initiating compounds to perform topological analyses (*i.e.* starting point called a seed). All metabolites found in the extracellular compartment were considered as seeds to model the nutritional environment impact (*i.e.* system boundaries). Although detailed intracellular compartmentation was not modelled within *i*Prub22, particular attention was given to the exchange between intracellular and extracellular environments due to the nutritional regime of the fungus (*i.e.* extracellular absorption). The added transport reactions were mainly the result of the information concatenation from *i*AL1006, PchCyc, and some literature. GPR associations were checked and cleaned up according to the annotation provided by DeepLoc [97]. The subcellular assignment of each gene product is available within the SBML in the notes tag of the gene entities. For modelling purposes, artificial exchange reactions (*i.e.* uptake for the input of a compound and production for the output) were added to the reconstruction. They were defined according to the metabolites present in the extracellular medium, which possessed a transport reaction towards the intracellular compartment. The seeds, therefore, corresponded to all the metabolites that have an uptake reaction (S5 file). Artificial genes of form t001 to t196 (*i.e.* transport), d001 to d037 (*i.e.* demand), u001 to u185 (*i.e.* uptake), and p001 to p032 (*i.e.* production) were assigned for each of them to discretise specific reactions more efficiently than those coming from a simple gap-filling.

Network gap-filling was performed using the MENECO tool (v2.0.0) [106] This topological gap-filling tool seeks to make accessible from a given list of seeds a set of targets. As the gap-filling process is based on heuristics, it was decided to use

filtered repair networks corresponding to MetaCyc subsets. They were built and queried successively, independently, and then jointly until the entire MetaCyc database (v23.0) was used. The MetaCyc subsets were divided into two main categories. On the one hand, the subset was composed of reactions reported in fungal reconstructions (*i.e.* using the following 3 PGDBs available on MetaCyc – *Candida albicans* SC5314, *Exophiala dermatitidis* NIH/UT8656 and *Saccharomyces cerevisiae* S288c – and a fungi meta-network [44]). On the other hand, the subset was composed of reactions for which no genomic data is associated (*i.e.* spontaneous reactions or unknown enzymes). Gap-filling processes are performed iteratively from the targets with the highest confidence on the most relevant repair networks. The resulting reactions are manually verified and incorporated into the network (reactions and their sources are available in the S6 file).

For instance, this process was used to enrich the reconstruction with spontaneous reactions specifically. As Palsson *et al.* [58] recommended, only reactions for which at least one of the entities (*i.e.* reactant or product) was already present in the draft were added to the reconstruction. As it was done for previous specific reactions, an artificial gene of the form s001 to s086 is assigned for each of them.

Objects such as metabolites or reactions are stored, listed, filtered and sorted using SmartTables available on MetaCyc [38]. Reconstruction curation and debugging are manually assisted by the network visualisation *via* ModelExplorer (v2.1) [52].

Model functional capabilities: biomass production

The 2013 network, *i*AL1006, proposes a biomass reaction adapted from the organism's behaviour [26]. Thus, ten subsystems that model the production of amino acids, cell wall components, cofactors, DNA, RNA, fatty acids, phospholipids,

glycerides, sterol esters, and some generic lipids define the biomass reaction (file S6). The complete adaptation of this physiologically relevant objective function to the data on MetaCyc was made impossible due to the lack of clear mapping between the identifiers and the use of highly generic terms. To test the validation of our proposed model, the biomass reaction was identical to that present in the 2018 network, Prubens [39] (file S5). Analyses related to constraint modelling (*e.g.* flux balance analysis – FBA –, flux variability analysis – FVA) were performed *via* MATLAB (v2018b) using the COBRA ToolBox (v3.0) [107]. Fluxer [108], a web application combining FBA and graph theory to offer an interactive graph visualisation, was used to help in the flux model refinement (*i.e. in silico* knockout effects were directly shown on the model flux distribution). The model was evaluated using FBA with uptakes of 1 for thiamine, ferrous ion, sulfur and phosphate, whereas oxygen was not limited. One of these six nitrogen sources: adenine, xanthine, hypoxanthine, urea, ammonium or ammoniac, must be opened to 10 units. Currently, the model is insensitive to the proposed carbon sources. Finally, to keep track of the modifications made to the model and according to the MIASE recommendations [48], the simulations were grouped in the LiveScripts file from MATLAB available in Supporting Information (S7 file).

SBML format

The network resulting from AuReMe was modified following the current writing conventions [25,93] to ensure the sustainability and viability of the model. To comply with these requirements (SBML level 3, FBC2), a format update was performed by relying on the official recommendations available on the COMBINE resource (http://co.mbine.org – community for the coordination of modelling standards in

biology). The interoperability and enrichment of the identifiers of the various entities of the model were achieved using MetaNetX (v4.1) [53] These annotations were encoded within SBML according to the standards of the MIRIAM resource [109] (Minimum Information Requirements in Biochemical Model Annotation). Each entity of the network was also associated with an SBO term [55] (Systems Biology Ontology – a nested classification scheme for grouping model components). The conversion to FBC2 was performed *via* the script available on the LibSBML application programming interface [110]. These format modifications were then checked and validated *via* the scripts made available to the community on this same application (SBML Validator – testing the syntax and internal consistency of an SBML file). Finally, the model is tested by MEMOTE (MEtabolic MOdel Tests – v0.13.0) [54] and its report is available in Supporting Information (S8 file).

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Supporting information

S1 file. *i*Prub22. The SBML format of *i*Prub22 Genome Scale Metabolic Network of *Penicillium rubens*. (SBML)

S2 file. List of additional tables and figures. Table 1: Functional annotation details; **Table 2:** Features and results of the seven templates used for the reconstruction of the intermediate subnetwork from the orthology search; **Table 3:** List of reactions involved in the synthesis of roquefortines and meleagrin added to the reconstruction (MetaCyc identifiers); **Table 4:** Lists of reactions involved in penicillins biosynthesis; **Table 5:** Identification comparison of BGCs between AntiSMASH and FungiSMASH; **Fig 1:** Overview of *Penicillium rubens* functional annotation; **Fig 2:** Overview of *Penicillium rubens* reactions performed with KEGG Mapper; **Fig 3:** Bipartite graph representing the seven templates networks' topology for the orthology subnetwork generation; **Fig 4:** Sequences number distribution per orthogroup according to species detected with OrthoFinder; **Fig 5:** Visualisation of orthologous genes detected by OrthoFinder and inference of their reactions to the orthology subnetwork; **Fig 6:** Sankey plot of the data selection from external sources; **Fig 7:** Classification of genes and reactions according to their associated Enzyme Commission number; **Fig 8:** Origin of metabolites in *i*Prub22 according to reaction reconstruction sources; **Fig 9:** Scatter plot of reconstruction sources complementarity; **Fig 10:** Classification of genes and reactions according to their source of integration in the draft; **Fig 11:** Sources of transport and exchange reactions added to the reconstruction; **Fig 12:** Distribution of the 510 reactions added to the reconstruction during the gap-filling steps; **Fig 13:** Annotations enrichment of

the 3,771 genes added to the *Penicillium rubens* GSMN reconstruction (Results from FungiFun). (PDF)

S3 file. Orthology results. Overview of OrthoFinder results presenting selected protein sequences, numbers of orthologous sequences and orthogroups between templates and statistics per species. (XLSX)

S4 file. Metabolites belonging to *Penicillium rubens*. Traceability of the three target lists used for model refinement supplemented by a list of orphan metabolites. (XLSX)

S5 file. List of transport and exchange reactions added to the model. File containing the transport reactions initially present in the draft, those added with or without gene support and the exchange reactions necessary to simulate the model (Uptake, Demand and Production). (XLSX)

S6 file. List of reactions from external sources and gap-filling needed to generate *i*Prub22. File containing reactions from external sources (with or without MetaCyc identifier), spontaneous reactions, the four sets of reactions from gap-filling and the three reactions required for biomass synthesis (*i.e.* biomass formulation, transport and exchange). (XLSX)

S7 file. Features of reconstruction and model. Livescript MATLAB that presents the reconstruction characteristics and the modifications required to generate a model. (HTML)

S8 file. MEMOTE report of *i*Prub22 reconstruction. Informative report that details MEMOTE test suite results. (HTML)