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Nonribosomal peptides in fungal cell factories: from genome mining to optimized heterologous production

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14 **Abstract**

15 Fungi are notoriously prolific producers of secondary metabolites including nonribosomal peptides
16 (NRPs). The structural complexity of NRPs grants them interesting activities such as antibiotic, anti-
17 cancer, and anti-inflammatory properties. The discovery of these compounds with attractive activities
18 can be achieved by using two approaches: either by screening samples originating from various
19 environments for their biological activities, or by identifying the related clusters in genomic
20 sequences thanks to bioinformatics tools. This genome mining approach has grown tremendously due
21 to recent advances in genome sequencing, which have provided an incredible amount of genomic
22 data from hundreds of microbial species. Regarding fungal organisms, the genomic data have
23 revealed the presence of an unexpected number of putative NRP-related gene clusters. This
24 highlights fungi as a goldmine for the discovery of putative novel bioactive compounds. Recent
25 development of NRP dedicated bioinformatics tools have increased the capacity to identify these
26 gene clusters and to deduce NRPs structures, speeding-up the screening process for novel metabolites
27 discovery. Unfortunately, the newly identified compound is frequently not or poorly produced by
28 native producers due to a lack of expression of the related genes cluster. A frequently employed
29 strategy to increase production rates consists in transferring the related biosynthetic pathway in
30 heterologous hosts. This review aims to provide a comprehensive overview about the topic of NRPs
31 discovery, from gene cluster identification by genome mining to the heterologous production in
32 fungal hosts. The main computational tools and methods for genome mining are herein presented
33 with an emphasis on the particularities of the fungal systems. The different steps of the reconstitution
34 of NRP biosynthetic pathway in heterologous fungal cell factories will be discussed, as well as the
35 key factors to consider for maximizing productivity. Several examples will be developed to illustrate
36 the potential of heterologous production to both discover uncharacterized novel compounds predicted
37 *in silico* by genome mining, and to enhance the productivity of interesting bio-active natural
38 products.

39 **Keywords:** Bioinformatics tools; Fungal cells; Genome mining; Heterologous production; Large
40 gene cluster; Multi-modular enzyme; Nonribosomal peptide; Nonribosomal peptide synthetase;
41 Secondary metabolite

42 **Abbreviations**

43	A-domain	Adenylation domain
44	ACV	d-(L- α -aminoadipyl)-L-cysteinyl-D-valine
45	BCGs	Biosynthetic gene clusters
46	C _{term} -domain	Terminal condensation domain
47	NRP	Nonribosomal peptide
48	NRPS	Nonribosomal peptides synthetase
49	ORF	Open reading frame
50	pHMMs	Profile hidden markov models
51	PKS	Polyketide synthase
52	PPTase	Phosphopantetheine transferase
53	SGS	Second generation sequencing
54	SMs	Secondary metabolites
55	TAR cloning	Transformation-associated recombination cloning
56	TGS	Third generation sequencing

57 1. Introduction

58 The era of nonribosomal peptides (NRPs) started with the brilliance of Fleming to analyze the
59 unexpected ability of a filamentous *Penicillium* strain to impair bacterial growth. These observations
60 led to the discovery of the penicillin antibiotic, a NRP derived compound which was not identified as
61 such at that time (Fleming, 1929). Indeed, the biosynthesis mechanism of this type of peptides,
62 named thiotemplate mechanism, was elucidated only 42 years later with the description of the Non
63 Ribosomal Peptide Synthetase (NRPS) of the gramicidin antibiotic from *B. subtilis* (Lipmann et al.,
64 1971). Since then, there has been growing interest in the study of this kind of compounds. As a result,
65 NORINE, the first database entirely dedicated to NRPs was established in 2007, starting with 700
66 NRPs entries (Caboche et al., 2008). At present, over a thousand of NRPs have been characterized
67 and classified (Pupin et al., 2016). These NRPs were mostly found in prokaryotes and fungal
68 eukaryotes (Demain, 2014), and are notably not present in metazoa and plantae eukaryotic clades.
69 Few exceptions were reported such as the production of astins in the plant *Aster tataricus*, latter
70 refuted and attributed to a fungal endophyte *Cyanodermella asteris* (Schafhauser et al., 2019 -
71 submitted). Likewise, polytheonamide initially isolated from the marine sponge *Theonella swinhoei*
72 (Hamada et al., 2005), was finally shown to be produced by a symbiotic bacteria (Freeman et al.,
73 2016; Wilson et al., 2014).

74 Nonribosomal peptides, as suggested by their name, are not assembled on the ribosome but by
75 large multi-modular enzymatic complexes that are encoded by gene clusters that often span over tens
76 of kilobases in the genome (**Fig. 1**). However, NRP synthetases are synthesized on the ribosome,
77 before being activated by a specific phosphopantetheinyl transferase (PPTase). This PPTase is
78 responsible for the conversion of the *apo*-NRPS into active *holo*-NRPS through the addition of a so-
79 called phosphopantetheine arm onto thiolation domains (Beld et al., 2014). NRPSs can be divided
80 into multiple modules, each of them responsible for the incorporation of one peptide monomer. These
81 modules comprise multiple enzymatic domains exhibiting specific functions in the NRP assembly
82 line (Caboche et al., 2008). The adenylation (A) domain is able to recognize and activate a specific
83 monomer, which is then tethered onto a thiolation (T) / peptidyl carrier protein domain through the
84 phosphopantetheine arm. The flexibility of the multi-enzymatic complex enables the presentation of
85 monomers to the condensation (C) domain which catalyzes the formation of a peptide bond between
86 monomers from two subsequent modules (Winn et al., 2016). In NRPSs involved in lipopeptide
87 biosynthesis, a major NRPs class, a starter C domain priming the synthetase enables to acylate the
88 first peptide monomer with the fatty acid part (Rausch et al., 2007). Some NRPSs also exhibit

89 additional domains responsible for monomer modifications, such as methylation, oxidation and
90 epimerization.

91 NRPSs predominantly operate according to a linear synthesis, with an initiation module (A-T)
92 able to recognize the first monomer, followed by as many modules (C-A-T) as monomers required to
93 complete the peptide. Nonetheless, some NRPSs rather operate with an iterative or nonlinear
94 organization, and then may not follow the colinearity rule between the number of modules and the
95 size of synthesized peptides (Richter et al., 2014; Schwecke et al., 2006). The amonabactin
96 synthetase found in *Aeromonas* sp. is a prime example of an atypical assembly line which displays an
97 iterative, alternative and optional mode of synthesis. In this NRPS, the AmoG A-domain activates
98 alternatively a Trp or a Phe, on which are bonded two dipeptides iteratively generated by the AmoE
99 and AmoF domains, and optionally in two out of four cases, an AmoH domain incorporates a glycine
100 between the 2,3-dihydroxy benzoic acid and the lysine of one of the dipeptides (Esmaeel et al.,
101 2017). NRPS assembly lines usually end with a termination module comprising a thioesterase
102 domain (TE) responsible for the release, and the cyclization, in most cases, of the final compound
103 (Du and Lou, 2010; Kopp and Marahiel, 2007). In fungi, thioesterase domains can be replaced by
104 condensation terminal domain (C_{term}) ending the NRPS and leading to cyclic NRPs (Gao et al.,
105 2012). Alternatively, in NRPSs involved in peptaibols biosynthesis (i.e., NRPs harboring a C-
106 terminal alcoholic group instead of carboxyl group), TE is substituted by a reductase domain
107 releasing the peptide with a C-terminal alcohol (Manavalan et al., 2010).

108 Filamentous fungi are considered as prolific producers of NRPs. A large number of clusters
109 involved in NRP synthesis has been detected for example in *Penicillium thymicola* (17 clusters),
110 *Aspergillus fumigatus* (14 clusters), *Talaromyces islandicus*, *Beauveria bassiana* (13 clusters), and
111 *Pestalotiopsis fici* (12 clusters) (Chiang et al., 2014; Gibson et al., 2014; Schafhauser et al., 2016;
112 Tang et al., 2018; Wang et al., 2015). The high potential of fungal organisms for NRPs production
113 can be exploited for the discovery of novel compounds of interest. The search for novel compounds
114 in fungi is typically achieved following two different approaches: one starting from the biological
115 activities and the other, more recent, from genome sequences. The principle of the former consists in
116 screening samples originating from various environments for their biological activities. Once the
117 targeted bioactivity is detected, the active natural product is purified and characterized (Kildgaard et
118 al., 2017; Kunakom and Eustáquio, 2019). Usually bioactivities are first screened at small-scale and
119 then upscaled to reach higher production rates. However, this strategy is very restrictive as only
120 compounds displaying the screened activity and being produced in sufficient amount to induce an

121 observable activity, will be detected (Luo et al., 2014). In contrast, the second approach is based on
122 the use of bioinformatics tools in order to identify the SM gene clusters of interest (Luo et al., 2014;
123 Mushtaq et al., 2018). Frequently, the associated compound is not produced because the related
124 cluster is cryptic and the cultivation conditions were probably not optimal to succeed in its
125 awakening) (Anyaoagu and Mortensen, 2015; Lazarus et al., 2014). Therefore, the second approach
126 often requires genetic engineering either to activate the expression in the native host (homologous
127 production) or to transfer the biosynthesis pathway in a heterologous host (heterologous production)
128 (Luo et al., 2016).

129 In the past few years, microbial genome sequencing and annotation methods have made an
130 incredible leap forward, providing the scientific community a wealth of publicly available fungal
131 genomes (Grigoriev et al., 2014). This technological progress has opened new perspectives for the
132 discovery of novel compounds through the bioinformatic approach. The development of novel
133 bioinformatics tools enables, at present, the analysis of multi-modular complexes such as NRPSs, but
134 also polyketide synthases (PKS) or hybrid complexes PKS-NRPS (Weber and Kim, 2016). The case
135 of polyketides, assembled from acyl- and malonyl- coenzyme A by PKSs, is already well
136 documented and is beyond the scope of this review (Chooi and Tang, 2012; Schümann and
137 Hertweck, 2006; Zhang et al., 2011). Nonetheless, this study presents the multiple bioinformatics
138 tools developed to predict the modular organization of NRPSs, and the substrate specificity of their
139 adenylation domains, with an emphasis on the specificities of fungal systems. Bearing in mind that
140 these NRPs identified by genome mining are frequently poorly synthesized under laboratory culture
141 conditions, and taking into account for most of these fungi that adapted molecular tools are lacking,
142 the heterologous expression of their biosynthetic pathways is often a necessary step (Nah et al., 2017;
143 Sung et al., 2017; Tsunematsu et al., 2013a). The heterologous production of NRPs in bacterial
144 organisms such as *Escherichia coli* and *Streptomyces* spp. have been widely reviewed (Beites and
145 Mendes, 2015; Nah et al., 2017; Weber et al., 2015b; Zhang et al., 2011), but only few studies have
146 investigated the potential of fungal organisms to produce such foreign compounds. Therefore, this
147 review discusses, through multiple successful examples, the possibility to use fungal cell factories to
148 heterologously express NRPS-encoding genes identified by genome mining. Finally, the multiple key
149 factors to consider obtaining an efficient NRPs heterologous producer are described and then
150 illustrated onto the example of the NRP-derivative penicillin, a multi-decades case of study.

151 **2. Computational tools and methods for genome mining**

152 Biosynthetic gene clusters (BGCs) involved in the synthesis of secondary metabolites (SMs)
153 usually contain core synthase genes, regulatory and transport-related elements, and sometimes genes
154 conferring a resistance to the related SM. Most of the developed bioinformatics tools can be applied
155 to the analysis of BGCs related to multiple type of SMs (e.g. NRPS, PKS) coming out from either
156 bacterial or fungal genomic data. Regarding to NRPS analysis, bioinformatics methods have been
157 developed based on the high conservation of the NRPS thiotemplate mechanism and decades of
158 experimental characterization (Weber, 2014; Ziemert et al., 2016). Applications of these algorithms
159 can be used to identify NRPS cluster genes, their subsequent annotation, the prediction of the
160 backbone of the putative NRP produced, and the search for related NRPs. The *in silico* annotation of
161 BGCs relies on the algorithm efficiency, but also on the genome assembly quality, as the quality and
162 the completeness of the annotation depend directly on the input sequences (Yandell and Ence, 2012).
163 These two aspects will be further discussed in the following sections.

164 **2.1 Recent advances in genome sequencing and annotation**

165 From the mid-2000s, Second Generation Sequencing (SGS) technologies, also named Next
166 Generation Sequencing (including Illumina and Ion Torrent technologies), revolutionized genomics
167 and many other biological fields, by offering low-cost genome sequencing (Loman et al., 2012).
168 These developments generated an exponential growth in the genome sequences available in public
169 databases (more than 190,000 prokaryotic genome assemblies available on NCBI databases as of
170 March 2019, <https://www.ncbi.nlm.nih.gov/genome/browse/>) (Tatusova et al., 2014). SGS
171 technologies are based on the principle of a high-throughput method, with a short read length up to a
172 few hundred bases (e.g., 250 bp for Illumina HiSeq), and usually a low error rate. The drawbacks of
173 short read genome assemblies are related to low contiguity and poor gene completeness (Heather and
174 Chain, 2016; Koren and Phillippy, 2015). As NRPS biosynthetic gene clusters can span several tens
175 of thousands bases - even more when considering “accessory genes” -, BGCs full sequence might be
176 spread into several contigs (Conway and Boddy, 2013; Vesth et al., 2016). This causes complex
177 issues for reconstructing BGCs from draft genomes, as well as determining if several unknown gene
178 clusters in an organism are related to only one or several NRPSs.

179 The recent emergence of Third Generation Sequencing (TGS), with PacBio and Oxford
180 Nanopore technologies, brings new interesting features for BGC annotation. These two methods
181 yield long read length (from a few tens of kb for PacBio, up to 1 Mb for Oxford Nanopore,
182 depending on the quality of the DNA extraction (Jain et al., 2018)), hence generating high contiguity

183 assemblies (Lu et al., 2016). This long read length may present a real advantage in the case of BGC
184 studies, allowing for obtaining complete sequences, and therefore the entire set of involved genes.
185 Yet, in contrast to SGS, TGS displays a lower throughput and a higher error rate (Rhoads and Au,
186 2015). Several software packages, such as Flye (Kolmogorov et al., 2019) and Canu (Koren et al.,
187 2017), provide efficient methods for TGS data assembly. When the read depth coverage is not
188 sufficient, hybrid strategies taking advantage of the low error rate of SGS can be used for polishing
189 TGS reads or contigs, allowing for obtaining a high-quality genome assembly (Bashir et al., 2012).
190 For instance, SPAdes (Antipov et al., 2016) offers hybrid assemblies, while Pilon (Walker et al.,
191 2014) allows the user to polish TGS assemblies.

192 Regarding the NRPS identification and annotation, these steps are driven by fast and
193 reasonably accurate bioinformatics methods, which have now become one of the most predominant
194 approaches to support the discovery of novel SMs. This emerging field is promising, as a result of
195 constant effort in software development (Chavali and Rhee, 2018; Weber and Kim, 2016; Ziemert et
196 al., 2016) and the availability of genomic data. Most methods for annotating NRPSs rely on
197 similarity searches, which is a robust approach as these multi-enzymatic systems are composed of
198 repeated, well conserved domains. The detection of core biosynthetic components is commonly
199 conducted by protein signature searches (Chavali and Rhee, 2018). These signatures, also called
200 pHMMs (profile Hidden Markov Models) are probabilistic models capturing the versatile
201 information contained in a multiple sequence alignment (i.e., amino acid composition and
202 insertions/deletions per site) to perform specific and sensible annotations (Eddy, 2011, 1998). Hence,
203 from the prediction of synthetase genes, it is then possible to deduce the NRPS architecture (i.e.,
204 domain composition), and search for additional genes surrounding this core (e.g., regulation and
205 transport related genes, tailoring enzymes).

206 **2.2 Bioinformatics tools dedicated to NRPS study**

207 Publicly available bioinformatics tools for annotating NRPS started to be devised a decade ago
208 (Blin et al., 2017a), since then, a large number of software packages and methodologies for genome
209 mining analyses has been released (**Fig. 2**). The Secondary Metabolite Bioinformatics Portal
210 represents a very useful online resource (<http://www.secondarymetabolites.org/>) facilitating the
211 exploration of these numerous software packages. It gathers information about the majority of
212 genome mining and annotation tools, as well as databases applied to secondary metabolism
213 investigation (Weber and Kim, 2016). The portion of these tools implying specific functionalities for

214 NRPS and closely related PKS is mostly constituted with generic algorithms applicable for both
215 bacteria and fungi, even if the training of their algorithm was mainly based on bacterial data. For
216 instance, NRPS annotation: NP.searcher (Li et al., 2009), ClustScan (Starcevic et al., 2008), and
217 antiSMASH (Blin et al., 2017a) are generic tools for the overall annotation of NRPS. The latter is
218 currently the most comprehensive and up-to-date pipeline for BGC analysis, providing a great deal of
219 available functionalities for refining NRPS annotations (Blin et al., 2017a; Weber and Kim, 2016).
220 Among the small portion of tools dedicated to Fungi, SMURF (Khaldi et al., 2010) is also a well-
221 known software performing the basic identification of putative synthase and auxiliary enzyme genes.
222 It is also noteworthy that antiSMASH possesses a version for fungal data (i.e., fungiSMASH -
223 <http://fungismash.secondarymetabolites.org>) (Blin et al., 2017c) which adapts the internal gene
224 predictor for an eukaryotic one (GlimmerHMM). In addition, fungiSMASH also offers to apply
225 CASSIS (Wolf et al., 2016), an algorithm aiming the improvement of NRPS/PKS BGC boundaries
226 delimitation by searching the presence of common regulation pattern in BGC promoters.

227 In addition to these general NRPS annotation tools, some are entirely devoted to the annotation
228 of NRPS biosynthetic pathways, focusing on specific features of NRPSs and their products. Indeed,
229 these tools aim to predict the peptidyl backbone of the putative NRP produced by analyzing two
230 elements: substrate specificity of adenylation domains and condensation domain subtypes classes.

231 Substrate selection is based on the specificity of adenylation domains. Determining this
232 specificity is a key element in the structural characterization of the putative synthesized compound.
233 The specificity-conferring code, known as “Stachelhaus or NRPS code”, was the first prediction
234 method based on the identity of ten amino acid residues located in the phenylalanine-binding pocket
235 of PheA (adenylation domain of the first module of the gramicidin synthetase) (Stachelhaus et al.,
236 1999). These critical residues were demonstrated to be involved in the substrate specificity as they
237 define the binding-pocket recruiting specifically the various monomers. Therefore, with multiple
238 alignments of the already known NRPS A-domains, it was possible to establish a code that correlate
239 these residues to specifically recognized amino acid substrates (Challis et al., 2000). Therefrom,
240 several substrate specificity prediction methods were developed such as the pHMM-based approach
241 analyzing the specific protein signatures in the active site (Minowa et al., 2007). This method was
242 integrated and further developed in NRPSsp (<http://www.nrpsp.com/>) and NRPS-PKS-substrate-
243 predictor (<http://www.cmbi.ru.nl/NRPS-PKS-substrate-predictor/>) (Khayatt et al., 2013; Prieto et al.,
244 2012). Another method, based on a Support Vector Machine approach, is exploited in
245 NRPSpredictor2. This software package provides predictions according to the physicochemical

246 properties of the 34 residues in a radius of 8 angstrom around the PheA binding pocket (Rausch et al.,
247 2005; Röttig et al., 2011). Substrate prediction obtained through the above-mentioned methods are
248 usually accurate when dealing with bacterial data but are still not entirely reliable with fungal data.
249 Three reasons can justify these limitations regarding substrate prediction : (1) low sequence
250 conservation between A-domains (10-40% identity) (Agüero-Chapin et al., 2016), (2) the existence
251 of different A-domain patterns for a same recognized amino acid (Khayatt et al., 2013), or on the
252 contrary, (3) A-domains displaying a relaxed selection of more than one substrate (Belshaw et al.,
253 1999; Esmael et al., 2016; Richter et al., 2014). Therefore, new strategies have been developed to
254 address the still unresolved challenge of substrate specificity prediction. One recently developed
255 approach, SEQL-NRPS (<http://services.birc.au.dk/seq1-nrps/>), enables to predict substrate
256 specificities based on an alignment-free method detecting occurrences of motifs in domains primary
257 structure (Knudsen et al., 2016). Recently, prediCAT, a phylogenetic-based algorithm enabling to
258 estimate the degree of predictability of A-domain, has been developed and integrated in
259 SANDPUMA (Chevrette et al., 2017). SANDPUMA is a prediction framework combining three
260 methods: (1) a retrained version of the Support Vector Machine models applied in NRPSpredictor2,
261 (2) a search for exact matches from active site motifs as in the Stachelhaus et al. (1999) approach and
262 (3) a pHMMs analysis like the Khayatt et al. (2013) approach. The last antiSMASH version (4.0)
263 modified its previous substrate predictors (Stachelhaus code, Minowa's pHMMs and
264 NRPSpredictor2) to implement SANDPUMA (Blin et al., 2017c).

265 Condensation domains also exhibit substrate specificity during the elongation reaction, as well
266 as some possible additional activities such as cyclization or epimerization. These domains can be
267 divided in different subtypes (**Table 1**). The identification of the C domain-subtype is a crucial
268 element for inferring the modifications introduced into the amino acids constituting the final peptide
269 (e.g., identifying _D-amino acids or peptide cyclization) (Caradec et al., 2014). Analysis of
270 condensation domains can be achieved with the NaPDoS web application (<http://napdos.ucsd.edu>),
271 which uses a phylogenetic approach to classify the subtypes of condensation domains and
272 ketosynthase domains from PKS (Ziemert et al., 2012). Each subtype of condensation domain
273 exhibits specific conserved motifs enabling to efficiently classify them in phylogenetic trees (Caradec
274 et al., 2014). According to its position into the phylogenetic tree, it is possible by inference to
275 determine the subtype of the analyzed C-domain.

276 When this additional information regarding core domains specificities has been collected, a
277 quite precise determination of a putative NRP compounds architecture is possible by combining the

278 predicted substrates of adenylation domains, with the modifications related to the predicted
279 condensation classes. Florine, a workflow dedicated to the annotation of NRP compounds, presents a
280 detailed decision diagram to carry out this annotation process (Caradec et al., 2014). Nonetheless, the
281 mechanism for substrate selection in NRPS is not fully elucidated and bioinformatics tools are being
282 continuously improved as a result of new discoveries. For instance, the short amino acid regions
283 connecting adjacent modules, called linkers, have been very recently highlighted to also play a role in
284 the determination of substrate specificity. Subsequently to this discovery, a web application just has
285 been released to enable the identification of these inter-modular NRPS linkers ([https://nrps-
286 linker.unc.edu](https://nrps-linker.unc.edu)) (Farag et al., 2019).

287 **2.3 Linking NRPS to related clusters or known products**

288 Once the NRPS architecture is elucidated thanks to the above described bioinformatics tools, it
289 is important to determine if the compound is already described and associated to a characterized NRP
290 (**Fig. 2**). A lot of useful tools are available to perform this dereplication steps, but only few of them
291 will be described thereafter. The standard approach for analyzing BGC is to use the Basic Local
292 Alignment Search Tool (BLAST) search program hosted on the NCBI website
293 (<http://www.ncbi.nlm.nih.gov/blast>). This application enables to perform a sequence similarity search
294 relative for instance to sequences listed in the “nucleotide” (BLASTn) or the “non-redundant protein
295 sequences” (BLASTp) collections of the NCBI (McGinnis and Madden, 2004; NCBI Resource
296 Coordinators, 2016). Alternatively, it is possible to query dedicated databases for BGCs, NRPSs and
297 their products. Regarding BGC pathways, ClusterMine360 is a PKS/NRPS database mainly updated
298 by crowdsourcing, which offers a two-way approach where nearly 1000 gene clusters can be browsed
299 directly from a cluster list or following a search from more than 200 compound families (Conway
300 and Boddy, 2013). The antiSMASH database is another relevant resource, encompassing currently a
301 collection of antiSMASH results for almost 25,000 genomes - but mainly of bacterial origin - and
302 offering the possibility to build precise queries (Blin et al., 2017b). MultiGeneBlast tool enables
303 identifying homologs of multigene modules, such as NRPSs, on the basis of similarity and synteny
304 criteria (Medema et al., 2013). MultiGeneBlast is also integrated in antiSMASH, where it suggests
305 some potentially related clusters (or the identical cluster itself) taken from different BGC databases
306 of varying levels of curation. For example, the “KnownClusterBlast” option refers to a curated
307 database containing experimentally characterized clusters, while the “ClusterBlast” option uses an
308 extended database composed mostly of only hypothetical clusters (Weber et al., 2015a).

309 The only database fully dedicated to NRP compounds, Norine, currently comprises 1190
310 peptides, including 346 NRPs of fungal origin distributed in 46 families. Norine offers different
311 search modes: (1) an “annotation search” engine, which includes queries with all annotations (e.g.,
312 biological activity, molecular weight, monomer count, or producing organism), and (2) and a
313 “structure mode” allowing searches based on monomer composition fingerprints, sequence similarity
314 or whole structures. The analysis of predicted peptides permits searching for related analogous
315 compounds already characterized, or to check if some amino acid patterns involved in some
316 biological activities can be spotted (Pupin et al., 2016).. Only a few main databases were mentioned
317 in this section, but a more exhaustive list is provided on the Secondary Metabolite Bioinformatics
318 Portal (Weber and Kim, 2016).

319 **2.4 Fungal NRPS analysis challenges**

320 Most of the methods and bioinformatic tools available so far were designed both for the
321 analysis of NRPS of fungal and bacterial origins. However, these organisms are taxonomically
322 distant and some differences at the genomic level can be readily identified. Indeed, in comparison
323 with prokaryotic genomes, fungal genomes are characterized by a larger size (i.e., several tens of
324 millions bases), a higher number of chromosomes, a lower gene density, and the presence of introns
325 (Elliott and Gregory, 2015; Setubal et al., 2018). BGC analysis pipelines, such as antiSMASH, offer
326 an internal gene prediction before applying protein signature searches. Structural annotations (i.e., the
327 prediction of whole gene structure) performed by automated internal gene predictors (e.g., Glimmer3
328 in antiSMASH (Medema et al., 2011)) are sufficiently accurate for bacteria, whereas annotation of
329 genes from eukaryotic genomes is usually more complex. Dedicated gene prediction pipelines for
330 eukaryotes often need some external data (gene expression and/or homologous protein data) to
331 support *ab initio* predictions and provide higher quality annotation (Yandell and Ence, 2012). As it is
332 essential to have coding sequences correctly annotated (i.e. with intron-exon and intergenic structures
333 well resolved) to accurately predict NRPS gene clusters, fungal genomes are likely to benefit from a
334 preliminary gene prediction, before being processed by BGC analysis tools. The available tools,
335 dedicated to eukaryotic genome analysis, to perform this preliminary gene prediction are depicted in
336 a relevant review (Yandell and Ence, 2012).

337 When pioneering predictive models for the substrate specificity of adenylation domains were
338 developed a decade ago (Minowa et al., 2007; Rausch et al., 2005), very few fungal data was
339 available. Therefore, these models provide predictions based mainly on monomers identified as a

340 substrate for the few well-characterized bacterial NRPS at that time. Consequently, these
341 bioinformatics tools cannot predict with high accuracy the substrate specificity of most of the A-
342 domains of eukaryotic NRPSs. Furthermore, based on the Norine database, it appears that NRPSs of
343 fungal and bacterial origin display only a moderate range of overlapping substrate and incorporate a
344 different set of monomers into the NRPs (**Fig. 3**). For example, Aib (2-aminoisobutyric acid) and
345 amino alcohols such as Pheol (phenylalaninol) and Leuol (leucinol) seem to be specific to peptaibols
346 produced mainly by fungi. Consequently, some fungal monomers are simply not recognized as no
347 predictive models exist for these substrates. However, predictive models are only applicable if the
348 monomer is directly selected by an adenylation domain and not resulting from the modification of a
349 precursor amino acid. While Aib seems to be directly loaded (Chutrakul et al., 2008), amino alcohols
350 in NRPs result from a modification of the amino precursors during synthesis of the peptide (Du and
351 Lou, 2010; Manavalan et al., 2010). Determining if the monomer is loaded as such or modified
352 during the synthesis is a complex task as no generalized rules can be applied. For example, *D*-amino
353 acids are generally the result of an epimerization domain activity on *L*-amino acids, but, in some
354 cases, *D*-Ala was reported to be directly loaded (McErlean et al., 2019).

355 Even if a few software packages, such as NRPSpredictor2 (Röttig et al., 2011), have previously
356 developed models to predict the substrate specificity of adenylation domains, there is still an
357 important need for devising tools enabling to predict more accurately which monomers are
358 incorporated in fungal NRPs. Therefore, future challenges will be to go further in the elucidation of
359 fungal NRPS mechanisms, and to establish novel tools allowing for increased the accuracy of
360 substrate predictions in specific fungal systems, especially regarding to exotic- and *D*- amino acids.

361 **3. Heterologous production of NRPS in fungal hosts**

362 The number of available fungal genomic sequences in databases is continuously expanding
363 thanks to individual or large-scale collaborative efforts, such as the “1000 fungal genomes project”
364 (Araujo and Sampaio-Maia, 2018; Stajich, 2017). These available fungal genomes represent a gold
365 mine for novel NRP compounds discovery. Unfortunately, in many cases, the newly sequenced fungi
366 do not (or only poorly) produce these natural products under usual laboratory conditions in synthetic
367 media, which are not always suitable to develop the filamentous fungi or to produce NRPs (Sung et
368 al., 2017). One option is to activate or enhance the homologous production in the native host through
369 promoter exchange for instance (Bode et al., 2015; Soukup et al., 2016). If this is not possible, due to
370 a lack of molecular tools for example, the heterologous production is one of the best approaches to

371 reach a sufficient yield of production, and enable further characterization of the NRP (Alberti et al.,
372 2017). In this part, the methodology commonly used for heterologous production of NRP is
373 presented, taking into account different parameters such as the choice of the heterologous host, the
374 genetic tools required for the transformation, the different expression systems used and the methods
375 used for large scale heterologous production of NRP.

376 **3.1 Heterologous host choice**

377 The choice of the host is one of the most critical parameters to consider for heterologous gene
378 expression and product synthesis. The selected strain has to present some desirable characteristics,
379 such as accessible and efficient genetic manipulation tools, capabilities to grow at high cell density,
380 ability to secrete the produced compounds, and above all, a codon compatibility with the foreign
381 genes (Beites and Mendes, 2015). The well-known bacterial cellular platforms *Escherichia coli*,
382 *Bacillus* sp., and *Streptomyces* sp., which display most of these characteristics, are commonly used
383 for heterologous expression of NRPs. Nonetheless, bacteria exhibit a different codon usage from
384 eukaryotic genes, which must be considered. One option for addressing this difficulty is to provide
385 additional tRNA sequences for missing and rare codons, such as the AUA codon for Ile in *E. coli*
386 (Skiba et al., 2018). Alternatively, as they are more closely related to filamentous fungi at a
387 taxonomic level, yeasts constitute appropriate hosts to express foreign fungal NRPS genes. Different
388 studies reporting NRP heterologous production in yeasts are presented in **Table 2**. Among yeasts,
389 *Saccharomyces cerevisiae* displays both a high codon usage compatibility towards foreign fungal
390 genes (Tsunematsu et al., 2013b), and a great potential for the secretion of the produced compounds
391 (Mattanovich et al., 2012). This host also displays a certain tolerance towards foreign bacterial
392 sequences, as was recently demonstrated with the heterologous production of a NRP initially
393 produced by *Streptomyces lavendulae* (Wehrs et al., 2018). As it is possible in this yeast to express a
394 NRPS-encoding gene coming from bacteria, which is taxonomically very distant to the host, its
395 potential is even more promising for the heterologous production of fungal NRP. Moreover, the
396 poorly developed secondary metabolism of *S. cerevisiae* is an asset, as it minimizes the competition
397 with homologous SM pathways, and promotes the detection/purification of the targeted compound.
398 Genetic manipulations are also made straightforward by a plethora of molecular tools, and the
399 aptitude of the yeast to efficiently perform transformation-associated recombination (TAR) enabling
400 the cloning of large DNA fragments (Anyagou and Mortensen, 2015). However, *S. cerevisiae* lacks a
401 functional PPTase encoding gene, which thus must be introduced into the host (Barajas et al., 2017).

402 In accordance with its advantages, and despite the risk of obtaining a low rate of production, *S.*
403 *cerevisiae* is by far the most widespread yeast host for NRP heterologous production. Nevertheless,
404 the non-conventional yeast *Hansenula polymorpha* (also belonging to the Saccharomycetales) can be
405 an alternative choice. This methylotrophic yeast is also easily cultivable, even at large-scale, and
406 strongly regulated promoters are available (Saraya et al., 2014). Moreover, *H. polymorpha* was
407 successfully used to produce the NRP d-(L- α -aminoadipyl)-L-cysteinyl-D-valine (ACV), a precursor
408 of penicillin and cephalosporin (Gidijala et al., 2009). Another Saccharomycetales, *Pichia pastoris*
409 (now *Komagatella pastoris*) is also an appropriate host to heterologously express large SM clusters.
410 In this host, multiple examples of large multi-modular PKS expression have been reported (Kasahara
411 et al., 2010; Xue et al., 2017). Nonetheless, to date, no NRP heterologous production has been
412 reported, despite the obvious potential of *P. pastoris* for SM production. Finally,
413 *Schizosaccharomyces pombe* is the only yeast reported to produce naturally a NRP (ferrichrome),
414 which indicates that it expresses a functional PPTase (Bushley et al., 2008; Schwecke et al., 2006).
415 This characteristic would establish this yeast as a suitable host, but the limited genetic toolbox for
416 Schizosaccharomycetales (only distantly related to Saccharomycetales such as *S. cerevisiae*)
417 currently restricts its scope of usage (Erler et al., 2006).

418 Despite yeasts different filamentous fungi, taxonomically close to the original host, can be used
419 for the heterologous expression of foreign NRPS genes. Numerous filamentous fungi display
420 interesting intrinsic properties that might make them potentially convenient hosts for heterologous
421 production of NRPs. First of all, they display a highly developed secondary metabolism, with the
422 expression of a large number of NRPs/PKs, and therefore contain a functional PPTase. In addition,
423 filamentous fungi were numerous times demonstrated to be able to recognize and correctly splice
424 introns from foreign fungal mRNA sequences, bypassing the necessity to purify and clone large
425 introns-free cDNAs from NRP gene cluster (Lazarus et al., 2014; Unkles et al., 2014). Usually, the
426 choice of a taxonomically closely-related host is preferable, since it generally exhibits a good
427 compatibility regarding codon usage, thus facilitating translation (Zhang and Liu, 2016). Indeed, in
428 native hosts, mRNA sequences harbour mainly preferred codons, interspersed with scarcer codons in
429 specific zones, so as to slow down the ribosome and enable the pre-folding of the translated proteins
430 (Ongley et al., 2013). Among the potential fungal hosts, *Aspergillus* spp., such as *A. nidulans*, *A.*
431 *niger*, and *A. oryzae*, are the more widely used for NRP heterologous production (**Table 3**). These
432 species are relatively easy to grow on cheap carbon sources in submerged cultures. Moreover, they
433 have been already used for decades at industrial-scale for food and pharmaceutical applications

434 (Mattern et al., 2015; Pickens et al., 2014). Conversely, *Aspergillus* spp. exhibit an extensively
435 developed secondary metabolism, which may constitute a drawback. Indeed, the subsequent
436 detection and purification of heterologous compounds is complicated due to the presence of a
437 multitude of natural products. Among those species, *A. oryzae* displays the most limited endogenous
438 secondary metabolism, providing a trade-off to the drawback just described (He et al., 2018).
439 Moreover, this species is GRAS (generally recognized as safe), especially as concerns the absence of
440 production of aflatoxin, a common mycotoxin found in most of *Aspergillus* species (Sakai et al.,
441 2008). Lastly, numerous genetic tools, such as selectable markers and various strong promoters, are
442 available to easily manipulate *A. oryzae* (Pahirulzaman et al., 2012). This is partly because of its
443 close relatedness with the genetic model *A. nidulans*, thus resulting in the compatibility of genetic
444 tools (Alberti et al., 2017).

445 **3.2 Genetic toolbox for the cloning of genes involved in NRP biosynthesis**

446 Once an appropriate host has been selected, the genes involved in the biosynthesis of the NRP
447 have to be transferred from the natural producer to the heterologous host. In the case of NRPs
448 heterologous production, this step can be particularly challenging for two reasons: (1) NRPS-
449 encoding genes can encompass tens of kilobases which are not easily amplifiable by classical PCR
450 operation, (2) NRPs synthesis often relies not only on the expression of the NRPS encoding-gene but
451 required to transfer a set of genes which are generally grouped within the same cluster in the genome
452 of the natural producer. This section will depict some of the solutions to overcome these issues.

453 **3.2.1 Cloning of NRPS long sequences**

454 As cloning NRPS genes encompassing up to tens of kilobases can be challenging, several
455 methods have been developed to enable the reconstitution of a long fragment from multiple amplified
456 PCR fragments (e.g., overlap extension PCR, Gibson assembly, USER cloning,) (**Fig. 4**). It is
457 advisable to first perform a reverse transcription to generate a single-strand NRPS cDNA, which
458 comprise the intron-free open reading frame, from a pool of total mRNA. The overlap extension PCR
459 approach (**Fig. 4 -A**) permits then the assembly of multiple overlapping fragments, obtained via
460 standard PCR, into a full length double-strand NRPS DNA (Bryksin and Matsumura, 2010; Horton et
461 al., 1989). The Gibson assembly method (**Fig. 4 -B**) is another approach enabling the fusion of
462 multiple DNA fragments. To set-up this method, the multiple PCR fragment are first amplified by
463 standard PCR with primers displaying homologous sequences (approximately 40 bp). The isothermal
464 one-step assembly of the fragments exhibiting terminal overlapping sequences is possible through the

465 joint action of a proofreading DNA polymerase, a Taq DNA ligase and a 5' T5 exonuclease. First, the
466 5' T5 exonuclease generates single-stranded 3' DNA overhangs by withdrawing nucleotides from the
467 5' ends. The generated homologous overhangs can then anneal, and the full-length double-stranded
468 DNA be synthesized through the action of the DNA polymerase and the Taq DNA ligase. This
469 system can generate very large DNA fragments, up to several hundreds of kilobases, with high
470 efficiency (Gibson et al., 2009). Likewise, the Uracil-Specific Excision Reagent (USER) cloning
471 (**Fig. 4 -C**) is also an efficient approach to assemble multiple DNA fragments. To implement USER,
472 a deoxyuridine residue (U) is first included through the designed primers in each PCR fragment.
473 Subsequently, the U residue is cleaved by a DNA glycosylase-lyase Endo VIII and a uracil DNA
474 glycosylase, thus generating a 3' overhangs, enabling a directional assembly of PCR fragments in a
475 linearized target plasmid (Cavaleiro et al., 2015; Smith et al., 1993).

476 Once the long fragment is isolated (e.g. gDNA, cDNA) or reconstituted through one of the
477 above-mentioned approaches, TAR cloning is the most commonly used method for its transfer into
478 cosmids or plasmids. Yeasts, especially *S. cerevisiae*, have the ability to perform homologous
479 recombination resulting in the incorporation of the long fragment into the vector. The TAR cloning
480 approach (**Fig. 4 -D**) implies first the cloning of two fragments homologous to the 5' and 3' parts of
481 the long-length sequence, separated by a restriction site, into the vector. The vector comprising 5'-3'
482 parts is then linearized on the above-mentioned restriction site and co-transformed in yeast with the
483 full-length fragment (Kouprina and Larionov, 2016). The denomination "direct TAR Cloning" is
484 used when gDNA or cDNA, eventually prior enzymatically digested, are directly used as a full-
485 length fragment in the co-transformation (Zhang et al., 2019). On the contrary, if the full-length
486 fragment was assembled by overlap extension PCR before to be inserted in the vector through
487 homologous recombination, the method is named ExRec (overlap extension PCR – yeast
488 homologous recombination) (Schimming et al., 2014; Tsunematsu et al., 2013b).

489 However, the insertion of long NRPS sequences into plasmids lead to a raise in the overall
490 plasmid size, which is dramatically affecting the subsequent cloning efficiency. The maximum insert
491 size can vary from one plasmid to another, but it is generally accepted that the insert size can reach
492 up to 25 kb. Beyond this size, the use of cosmids, which are plasmids containing a phage sequence,
493 enables to clone larger sequences (30-50 kb) in a more stable way (Carter and Shieh, 2015; Collins
494 and Brüning, 1978). In filamentous fungi, an alternative method for cloning large SM clusters via
495 Fungal Artificial Chromosomes (FACs) has been developed (Bok et al., 2015; Clevenger et al.,
496 2017). An equivalent artificial chromosome system is also available in yeasts (YACs) (Hughes et al.,

497 2015). The use of artificial chromosomes has enabled the direct cloning of DNA fragments up to 300
498 kb (Zhang et al., 2019).

499 Alternatively, a very efficient way to isolate a pre-identified NRPS gene is to take advantage of
500 *de novo* synthesis technology to re-assemble the constituting oligos. This method was already applied
501 to synthesize the bottromycin NRPS gene with the purpose of expressing it in *Streptomyces* species
502 (Huo et al., 2012), and could be similarly implemented in a fungal host. Nonetheless, even if the cost
503 of this technology is continuously decreasing, it remains the most expensive way to obtain a long
504 DNA sequence (Kosuri and Church, 2014).

505 **3.2.2 Cloning of a large number of genes**

506 Beside NRPS genes, numerous genes such as those encoding a PPTase, tailoring enzymes, or
507 transporter-associated genes, are required to successfully produce a bioactive NRP or NRP
508 derivative. The cloning of many genes can imply the synthesis of several plasmids which is
509 laborious, costly, and problematic in case only few selection markers are available for the chosen
510 host (Kumakura et al., 2019; Li et al., 2017). Several solutions particularly well-suited to NRP
511 heterologous production have been proposed to alleviate this issue. One possible strategy consists of
512 assembling the genes in the same plasmid to create a multiple-gene expression plasmid (Tsunematsu
513 et al., 2013b). This approach was successively applied to heterologously express the aspyridone PKS-
514 NRPS gene and its tailoring enzymes in *A. oryzae*. The aspyridone cluster identified in *A. nidulans*
515 comprises the *adpA* PKS-NRPS gene and six genes encoding tailoring enzymes (*adpB*, *adpC*, *adpD*,
516 *adpE*, *adpF*, and *adpG*). A plasmid was synthesized to enable the simultaneous expression of
517 combinations of four genes under the control of four promoters (P_{amyB} , P_{adh} , P_{gpdA} and P_{eno}) in a
518 single plasmid (Wasil et al., 2013). The golden gate cloning method can facilitate to a considerable
519 extent the process of cloning multiple transcription units (promoter – gene – terminator) in a single
520 expression vector. This strategy relies on the use of type II restriction enzymes that can generate a
521 specific 5' or 3' DNA overhangs of 4 nucleotides depending of the enzyme considered. Subsequently
522 to the enzymatic digestion, two fragments with appropriate cleavage site can be ligated in a one-step
523 restriction-ligation (Engler et al., 2008). Based on the golden gate method, easily tunable vectors
524 have been designed with proper cleavage sites enabling to introduce and interchange DNA elements
525 (e.g. promoters, coding sequences, terminators, protein tag sequences, resistance genes) as mere
526 building blocks. These kinds of DNA assembling platforms have already been designed for several
527 potential host such as the filamentous fungus *Ustilago maydis* (Terfrüchte et al., 2014), and the yeasts

528 *S. cerevisiae* (Agmon et al., 2015; Mitchell et al., 2015), *S. pombe* (Kakui et al., 2015), and *Y.*
529 *lipolytica* (Celińska et al., 2017). Alternatively, viral 2A peptide sequences can also be used to
530 express several genes under the control of the same promoter in a single plasmid. These sequences,
531 interspaced between each ORF, are cleaved during protein translation, hence releasing the proteins
532 from each other (Kim et al., 2011; Unkles et al., 2014).

533 However, if the use of multiple plasmids and genomic integrations is required, methodologies
534 allowing for recycling of selection markers have been developed in some common fungal hosts. The
535 loss of *URA3* selection gene in *S. cerevisiae*, can be achieved by cultivation on 5-fluoroorotic acid
536 plates (Akada et al., 2006; Boeke et al., 1987). The cre-lox system also enables selection marker
537 recycling in yeast such as *S. cerevisiae* and *P. pastoris* (Pan et al., 2011; Sauer, 1987). A recycling
538 system is also operational in *A. nidulans*, where it is possible to replace the marker of a previous
539 transformation by cloning into the first selection gene, a second construction with a different marker
540 (van Dijk and Wang, 2016; Yaegashi et al., 2014).

541 **3.3 Fungal expression systems**

542 **3.3.1 Yeast heterologous expression systems**

543 The first study reporting a successful heterologous expression of a fungal NRPS in yeast was
544 related to the production of ACV in *H. polymorpha* (Gidijala et al., 2008). This example will be
545 discussed in detail in section 4. In contrast, for all subsequently reported heterologous expressions of
546 NRPS in yeast, *S. cerevisiae* was selected as a host (**Table 2**), for example with the fumitremorgin
547 biosynthetic pathway from *A. fumigatus* (Tsunematsu et al., 2013a). The precursor of fumitremorgin
548 is the NRP brevianamide F, resulting from a condensation of L-tryptophan and L-proline by FtmA
549 NRPS. This compound is then prenylated by FtmB to form tryprostatin B, before being further
550 modified by FtmC, FtmD, FtmE, and FtmF leading to spirotryprostatin A or B. The adopted
551 approach for the cloning of the 8-kb *ftmA* gene was to take advantage of the capability of *S.*
552 *cerevisiae* to perform TAR cloning (Kouprina and Larionov, 2016). With the purpose of removing
553 introns, sometimes not or mis-spliced by heterologous yeasts, the total mRNA from the native
554 producer was extracted and converted into cDNAs by a reverse transcriptase. After homologous
555 recombination, the intron-free *ftmA* was inserted into a plasmid designed for expression in *S.*
556 *cerevisiae* under the control of the strong galactose-inducible GAL1 promoter. After the cloning of
557 *ftmB*, the NRP derivative tryprostatin B was detected at 100-fold higher concentration than in the
558 native producer *A. fumigatus* (35.6 and 0.383 mg.l⁻¹, respectively) (Tsunematsu et al., 2013a). This

559 significant increase in tryprostatin B titers is a prime example for illustrating the potential of the
560 heterologous expression approach to improve NRP productivity.

561 A heterologous expression approach has also been used to experimentally assign an NRPS
562 cluster identified on the basis of genome mining to the synthesis of a given NRP. In *A. fumigatus*, 14
563 gene clusters were identified to be involved in the biosynthesis of NRP compounds (Frisvad et al.,
564 2009). Among them, Afu6g12080 was identified and predicted to be involved in fumiquinazoline
565 biosynthesis (Ames et al., 2010). Using a similar approach to that taken with *ftmA*, total mRNA was
566 extracted from *A. fumigatus* and converted into cDNAs by a reverse transcriptase. From this cDNA,
567 multiple overlapping PCR fragments with homologous ends (<3 kb) of the introns-free Afu6g12080
568 NRPS gene were amplified and assembled together using overlap extension PCR methodology. The
569 resulting 11.8-kb fragment was cloned under the control of the strong constitutive P_{tef1} promoter in
570 pKW1810, a *S. cerevisiae* expression plasmid, by TAR cloning. The coding sequences for Flag and
571 6xHis were added, respectively, to the 5' and 3' ends of the NRPS gene. The presence of tags attached
572 to protein ends facilitates the further detection and purification of the produced NRPS. In this study,
573 the authors were able to confirm the expression of Afu6g12080 by Western blot using antibodies
574 directed against 6xHis and Flag tags. addition of the gene *npgA* encoding a PPTase, fumiquinazoline
575 F (Anthranilic acid [Ant] - LTrp - LAla), was purified from the culture broth of the constructed strain
576 (0.4 mg.l⁻¹). Moreover, an analog compound containing a hydroxylate-Ant was also identified,
577 highlighting the plasticity of the first A-domain of the NRPS encoded by Afu6g12080 (Ishiuchi et al.,
578 2012).

579 The Afu6g12080 NRPS from *A. fumigatus* is very similar to TqaA, another trimodular NRPS
580 identified in *Penicillium aethiopicum*. With the purpose of devising a ready-to-use host for
581 NRPS/PKS heterologous production, Gao and coworkers have introduced the *npgA* PPTase encoding
582 gene into the genome of *S. cerevisiae* (Strain BJ5464-NpgA). Meanwhile the *tqaA* cDNA, devoid of
583 introns, was obtained by reverse transcription on mRNA extracted from *P. aethiopicum*.
584 Subsequently, TAR cloning enabled the integration of the 5'-Flag modified NRPS gene in the yeast
585 expression plasmid pXW55 under the control of the strong constitutive ADH2 promoter. The
586 heterologous expression of *tqaA* was demonstrated through successful purification of the 450-kDa
587 synthetase with anti-Flag antibody affinity chromatography followed by gel filtration. Afterward, the
588 activity of the synthetase was confirmed with the detection of fumiquinazoline (Gao et al., 2012).

589 The strategy developed to express *tqaA* (Gao et al., 2012) was repeated several times to
590 heterologously express NRPS genes in the BJ5464-NpgA strain with the same plasmid. The *ardA*
591 NRPS gene from *Neosartorya fischeri*, was in such manner expressed in this strain, leading to
592 tricyclic ardeemin FQ production (Haynes et al., 2013). From the same filamentous fungi, *anaPS*,
593 involved in the biosynthesis of a precursor of acetylaszonalenin, was heterologously expressed to
594 investigate the role of the terminal condensation domain in NRP cyclization (Gao et al., 2012). The
595 expression of the 276-kDa bimodular synthetase AspA from *Aspergillus alliaceus* in *S. cerevisiae*
596 revealed the iterative mode of synthesis of the first module as it led to the synthesis of a tri-peptide
597 Ant-Ant-Trp (Gao et al., 2013). Likewise, the production of fungal anticancer cyclooligomer
598 depsipeptides, initially produced by *B. bassiana*, was reconstituted in *S. cerevisiae* through cloning of
599 the gene encoding the bassianolide synthetase (348 kDa) and the beauvericin synthetase (352 kDa)
600 (Yu et al., 2017, 2013).

601 Despite the opportunities offered by TAR cloning methodology, the insertion and correct
602 expression of large sequences, such as NRPS genes, remain difficult in numerous cases. One
603 potential approach to address this challenge is to dissect and clone the synthetase gene into separate
604 modules. The feasibility of module dissection was first been highlighted with the aspyridone
605 synthetase AdpA, encompassing a single NRPS module fused to the C-terminal part of a PKS. The
606 sequence encoding this module was isolated from *A. nidulans* and expressed in *S. cerevisiae*.
607 Subsequently the heterologous AdpA module was purified, prior to confirming the correct enzymatic
608 activity by *in vitro* production of acyltetramic acid in co-incubation with the PKS part (Xu et al.,
609 2010). Conversely, several mono-module NRPS-encoding genes from *Aspergillus terreus* were
610 expressed in the multi-copy pESC-URA plasmid: *apvA-mela*, *pgnA*, and ATEG_03090, respectively
611 involved in the biosynthesis of aspulyinone E, phenguignardic acid, and atromentin (Hühner et al.,
612 2018). The cryptic mono-module NRPS encoding gene ATEG_03630 from the same filamentous
613 fungi was similarly awakened by heterologous production, leading to the production of 2,4-
614 dihydroxy-5,6-dimethyl benzaldehyde (Wang and Zhao, 2014).

615 NRPSs encompassing more than one module can be dissected and each module cloned
616 separately. Nonetheless, some key factors must be considered. Primarily, the position where the
617 NRPS can be dissected is crucial. In the case of the bimodular AspA, the A₁-T₁-C₂ tridomain can be
618 solubly expressed unlike the bidomain A₁-T₁. Similarly, the second module was soluble in the four
619 domains C₂-A₂-T₂-C_t form and not in the A₂-T₂-C_t form (Gao et al., 2013). Recently, Bozhüyük et al.
620 (2019) have suggested an alternative fusion site area between the two sub-domains of the C-domain,

621 where NRPSs can be dissected. Authors postulated that the self-contained catalytically active unit
622 can be redefined as $C_{Asub}\text{-A-T-}C_{Dsub}$, where C_{Asub} is the sub-domain accepting the growing peptide
623 chain in the N-terminal C-domain and C_{Dsub} is the sub-domain providing the peptide chain in the C-
624 terminal C-domain. Genetic engineering enables, by exchanging these functional units between
625 different NRPSs with similar domain composition, to generate chimeric NRPSs assembling novel
626 NRPs. Up to now, these units have been employed only to reconstitute entire chimeric NRPSs, and
627 there is no evidence that they can be expressed separately in a soluble form (Bozhüyük et al., 2019).
628 A second point to consider for the dissection of NRPSs is related to the presence of intermodular
629 linkers, which are short amino acid regions connecting covalently subsequent modules. The
630 importance of these short sequences has recently been highlighted between modules one and two of
631 the beauvericin and bassianolide synthetases (Yu et al., 2013). The dissection of these synthetases by
632 cloning module 1 and module 2+3 into two distinct plasmids without disrupting the pre-identified
633 linker sequence led to an effective production of beauvericin and bassianolide. In contrast, the same
634 experiment without the linker did not lead to any production of cyclooligomer depsipeptides. In
635 addition to linkers, there are also short amino acid sequences, called COM domains, enabling
636 noncovalent association of the different proteins when the NRPS is composed of several proteins
637 such as in the tyrocidine synthetase or the surfactin synthase. These COM domains are essential
638 when different fractions of the NRPS are expressed separately but can also be exploited to generate
639 chimeric NRPS systems. This was demonstrated with the co-expression of the first module of the
640 tyrocidine NRPS (TycA) from *Brevibacillus parabrevis* with the third module of the surfactin
641 synthase (SrfAC) from *Bacillus subtilis* (Siewers et al., 2010). TycA was expressed with its native
642 COM^D domain, whereas SrfAC was fused with the COM^A domain, compatible with COM^D , isolated
643 from TycB module. Co-expression in *S. cerevisiae* of these two complexes into distinct pESC
644 plasmids lead to the expected production of ${}_D\text{Phe-}_L\text{Leu}$.

645 3.3.2 Filamentous fungi heterologous expression systems

646 With the exception of the *pcbAB* NRPS gene (involved in ACV synthesis), expressed in
647 *Neurospora crassa* (Smith et al., 1990), *Aspergillus* spp. were predominantly used to heterologously
648 express NRP compounds (**Table 3**). One of the earliest reported NRP heterologous expressions in
649 filamentous fungi was related to brevianamide F from *A. fumigatus*. The brevianamide NRPS gene,
650 also later expressed in *S. cerevisiae* as described above, had previously been identified through
651 heterologous expression in *A. nidulans* (Maiya et al., 2006). In order to prevent mis-amplification,

652 the 8.7-kb *ftmA* gene encoding the bimodular NRPS was amplified from *A. fumigatus* as two distinct
653 fragments (5' and 3'). The fragments were assembled together, cloned under the control of the alcohol
654 dehydrogenase promoter (P_{alcA}), and integrated in the genome of the host strain using *pyrG* as a
655 selection marker. In *A. nidulans*, P_{alcA} is a very strong promoter induced by ethanol or L-threonine,
656 and repressed in the presence of glucose (Felenbok et al., 2004). Cultivation under induction
657 conditions led to brevianamide F synthesis with a titer of 45 mg.l⁻¹, which represents a 3,500 fold
658 increase as compared to native strain *A. fumigatus* Af2936. Later, *ftmA* gene, assembled through
659 ExRec overlap-extension PCR-yeast homologous recombination (**Fig. 4**), was also expressed in *A.*
660 *niger* (Tsunematsu et al., 2013a). The FtmA synthetase from *N. fischeri*, displaying 88% identity at
661 the amino acids level with that from *A. fumigatus*, was also successfully expressed in *A. nidulans*
662 (Wunsch et al., 2015). *FtmA* gene under the control of the constitutive glyceraldehyde-3-phosphate
663 dehydrogenase promoter (P_{gpdA}) was inserted into the genome of *A. nidulans*, resulting in a
664 production of brevianamide F ranging between 9.7 and 36.9 mg.l⁻¹, depending on the mutant
665 considered. Interestingly, the productivity from one positive mutant to another displayed large
666 variations (from 1 to 4 magnitudes) (Maiya et al., 2006; Wunsch et al., 2015). This can be due to the
667 ectopic integration of the gene in variable regions of the genome.

668 Numerous fungal heterologous expression studies were conducted to decipher the mode of
669 synthesis of NRPSs. For example, the typical mode-of-operation of two siderophore synthetases has
670 been highlighted by heterologous expression in filamentous fungi. For this purpose, *FSNI* from
671 *Fusarium sacchari* and *CsNPS2* from *Ceriporiopsis subvermispora* were respectively expressed in *A.*
672 *oryzae* and *A. niger* (Brandenburger et al., 2017; Munawar et al., 2013). The *FSNI* gene is encoding
673 a NRPS involved in the synthesis of ferrirhodin, whereas *CsNPS2* is related to the biosynthesis of a
674 type VI siderophore. In both cases, the NRPSs were operating through an iterative mechanism with
675 three additional thiolation-condensation domains receiving the same monomer (N₅-acetyl-N₅-
676 hydroxy-L-ornithine) from a single upstream located adenylation domain. Likewise, through
677 heterologous expression in *A. nidulans*, the synthesis of asperphenamate has been demonstrated to be
678 dependent on the joint action of two distinct NRPSs: ApmA and ApmB. Indeed, the NRP produced
679 by ApmA, N-benzoylphenylalaninol, binds onto the monomer attached to ApmB to generate
680 subsequently the asperphenamate compound (Li et al., 2018). On the other hand, there are some
681 studies reporting identification and confirmation of NRP biosynthetic pathways through heterologous
682 expression. In the case of gliotoxin, a toxin synthesized by *A. fumigatus*, the related gene cluster was
683 known but the role of each gene had not been elucidated (Chang et al., 2013). NRPS gene *gliP*

684 expressed under the control of P_{alcA} in *A. nidulans* was identified to be involved in the first step of
685 gliotoxin biosynthesis with the assembly of NRP-type precursors: cyclo-(L-phenylalanyl-L-seryl) and
686 cyclo-(L-tryptophanyl-L-seryl). Likewise, by heterologous expression in *A. oryzae*, *pscyA* and *pscyB*
687 genes from *Penicillium sopii* have been demonstrated to be the only two genes required for the
688 biosynthesis of cycloaspeptide. Methylated amino acids constituting this NRP are provided through
689 the activity of an N-methyltransferase encoded by *pscyA*, while the peptide assembly is performed by
690 a 5-modules NRPS encoded by *pscyB* (De Mattos-Shipley et al., 2018). Another reported example
691 was the case of KK1, a NRP natively produced by *Curvularia clavata*, displaying anti-fungal activity
692 against the plant pathogen *Botrytis cinerea*. The 40-kb gene cluster potentially involved in KK1
693 synthesis was split into two fragments and cloned into *A. oryzae*. The transformed strain was able to
694 produce KK1, demonstrating the role of this gene cluster in the NRP synthesis (Yoshimi et al., 2018).

695 Some mono-modular NRPSs have also been heterologously expressed in filamentous fungi.
696 The *mela* gene from *A. terreus* was cloned into *A. nidulans* (Guo et al., 2015) and *A. niger* (Geib et
697 al., 2016; Geib and Brock, 2017) resulting in the production of aspulvinone E. Similarly, following
698 the cloning of *micA* obtained from *A. nidulans*, the transformed *A. niger* strain was able to produce
699 microperfuranone (Yeh et al., 2012).

700 *A. niger* was also used to heterologously express several cyclodepsipeptides, high value NRP
701 compounds, with anticancer, antimicrobial and anthelmintic properties (Sivanathan and
702 Scherkenbeck, 2014). Enniatin, beauvericin, and bassianolide belonging to this group of compounds
703 are assembled by iterative bimodular synthetases able to link D- α -hydroxy acid with L- amino acids.
704 The *esyn1* NRPS gene from *Fusarium oxysporum* was expressed in *A. niger*, leading to the
705 production of enniatin at initial un-optimized titer of 1 mg.l⁻¹ (Richter et al., 2014). Beauvericin and
706 bassianolide were also heterologously produced after the cloning of the corresponding *bbBeas* and
707 *bbBsIs* synthetase genes from *B. bassiana* (Boecker et al., 2018). In these two studies, a promising *A.*
708 *niger* heterologous expression host was developed based on the Tet-on bacterial-fungal hybrid
709 expression system. Tet-on system encompassed an rtTA2S-M2-dependent promoter controlling the
710 expression of the NRPS gene and a tetracycline-dependent transactivator rtTA2S-M2. Addition of a
711 tetracycline derivative, doxycycline (Dox), in the medium activated the system and induced the
712 expression of the gene. The expression strength is directly correlated to the Dox concentration added,
713 thus making Tet-on tuneable and very efficient, with expression levels equivalent to those obtained
714 with the widely used strong constitutive promoter P_{gpdA} (Meyer et al., 2011). In addition, the impact
715 of the number of *bbBeas* gene copies integrated in *A. niger* genome on the beauvericin productivity

716 was also investigated (Boecker et al., 2018). The strain harbouring a tandem gene copy produced
717 approximately two-fold higher titers of beauvericin than the strain harbouring a single copy, under
718 similar culture conditions (628 versus 294 mg.l⁻¹).

719 The typical iterative mechanism and the high homology of bimodular cyclodepsipeptide
720 synthetases were also exploited to assemble hybrid NRPSs. In the case of closely related synthetases,
721 inter-module swapping is one of the most effective approaches for altering the incorporated
722 monomer, and thus generating a novel putatively bioactive compound (Brown et al., 2018). For this
723 purpose, the first module of a synthetase is combined with the second module of another system in
724 order to generate new non-natural nonribosomal peptides. Studies reporting this inter-module
725 swapping between cyclodepsipeptide synthetases have employed the above described Tet-on
726 expression system in *A. niger* (Steiniger et al., 2017; Zobel et al., 2016). Module 1 of PSYN NRPS
727 (from *Rosellinia abscondita*), activating either _D-Lac or _D-Phe, was fused to module 2 of EnSYN
728 (from *F. oxysporum*) and BeSYN (from *B. bassiana*) activating _L-Val and _L-Phe, respectively. The
729 subsequently transformed *A. niger* colonies were able to produce six novel compounds, but with
730 lower yields than for native cyclodepsipeptides. The lower yields observed can also be related to a
731 bottleneck in substrates for different derivatives (Zobel et al., 2016). Using a similar approach, after
732 screening hybrid NRPS resulting from several combinations of modules from different
733 cyclodepsipeptide NRPS (i.e., EnSYN, BeSYN, and BaSYN) in *E. coli*, the most promising
734 combinations were expressed in *A. niger* (Steiniger et al., 2017). The combinations EnSYN-
735 BaSYN.T.C₃, BeSYN-BaSYN.T.C₃, BaSYN-EnSYN.T.C₃, and BaSYN-BeSYN.T.C₃ were
736 successfully expressed, leading to high titers of hybrid cyclodepsipeptides: octa-enniatin B (4 mg.l⁻¹),
737 octa-beauvericin (10.8 mg.l⁻¹), and hexa-bassianolide (1.3 g.l⁻¹). Module and domain swapping
738 between different PKS/NRPS is an innovative approach to generate new compounds, difficult to
739 obtain by chemical synthesis, and displaying interesting biological activity (Süssmuth and Mainz,
740 2017). With this approach, 6 novel compounds were also produced in *Fusarium heterosporum*, after
741 57 swapping combinations with 34 modules from various fungal PKS-NRPS (Kakule et al., 2014).
742 Other notable examples of chimeric compounds include: niduchimaeralin A/B in *A. nidulans* by
743 swapping the PKS-NRPS CcsA from *Aspergillus clavatus* with the Syn2 from *Magnaporthe oryzae*
744 (Nielsen et al., 2016), and predesmethylbassianin A / pretenellin A in *A. oryzae* by swapping between
745 the PKS-NRPS DMBS and TENS from *B. bassiana* (Fisch et al., 2011).

746 Likewise, hybrid PKS-NRPS genes have also been heterologously expressed in filamentous
747 fungi to produce compounds of interest. For example, tennelin was produced in *A. oryzae* after

748 expression of *tenS* PKS-NRPS from *B. bassiana* under the control of the starch-inducible *amyB*
749 promoter (P_{amyB}), leading to up to 5-fold higher yields than in the native fungus (Halo et al., 2008;
750 Heneghan et al., 2010). Under the control of the same promoter, a PKS-NRPS from *Chaetomium*
751 *globosum* was expressed in *A. nidulans*, enabling the production of the potential anti-HIV Sch
752 210972 (Sato et al., 2015). Once again, P_{amyB} was employed to express the 12.4-kb *ACE1* PKS-
753 NRPS from *M. oryzae* in *A. oryzae*, leading to magnaporthepeyrone production (Song et al., 2015).
754 Similarly, the aspyridone biosynthetic pathway (*adpA* gene cluster) was reconstituted in *A. oryzae* by
755 cloning the genes of *AdpA* cluster from *A. nidulans* (Wasil et al., 2013). Additionally, two successive
756 PKS and NRPS genes (*glpks3-glnrps7*) in one cluster of *Glarea lozoyensis* were also successfully
757 expressed as a polycistronic mRNA in *A. nidulans* to produce xelolozoyenone (Yue et al., 2015). The
758 biosynthetic pathways of astechrome and macrolactone valactamide were reconstituted also in *A.*
759 *nidulans* through fungal artificial chromosomes.

760 **3.4 Key factors to scale up heterologous production of NRPs**

761 Multiple key factors have to be considered to succeed in NRP heterologous production and to
762 increase the productivity. Each step, from the cloning and the transcription of the long heterologous
763 gene to the functional synthetase, can be a barrier to the production (**Fig. 5**). Some of these limiting
764 steps, and the possible solutions to overcome them, will be discussed in this section.

765 **3.4.1 Incompatibility of the host with foreign NRPS nucleic and amino acid sequences**

766 The incompatibility of the foreign nucleic acid sequences with the host machinery is a major
767 cause of failure of heterologous production. This incompatibility is more pronounced when the native
768 producer is taxonomically distant from the host organism. It can mainly be related to a difference in
769 codon usage slowing down or ending the mRNA translation. As mentioned above, codons of the
770 foreign sequence can be optimized by replacing rare codons by more common ones for the host
771 (Ongley et al., 2013). This approach also enabled, in *Aspergillus* species, a stabilization of the
772 transcripts of heterologous genes by preventing premature polyadenylation and subsequent
773 degradation of mRNA (Tanaka et al., 2014). However, this methodology is time consuming in the
774 design of the optimized sequence and relies on still expensive DNA synthesis technology.

775 At the protein level, the large size of NRPSs increases the possibility to display recognition
776 sequences for host proteases. In particular, the amino acid sequences of inter-module sequence
777 regions, which are largely unfolded (i.e., limited number of α -helix and β -sheet) and display a high

778 degree of flexibility, are therefore more vulnerable to protease activity (Samel et al., 2007; Tarry et
779 al., 2017). Yeasts have been described to express multiple vacuolar proteases, such as
780 endoproteinases A and B, potentially released during cell lysis. To prevent the degradation of the
781 newly-synthesized NRPS, a *S. cerevisiae* strain has been mutated in *pep4* and *prb1* genes encoding
782 the previously mentioned proteases (Jones, 1991; Kealey et al., 1998). The resulting BJ5464 strain
783 has been widely used for heterologous production of various NRPs (Gao et al., 2013, 2012; Haynes
784 et al., 2013; Yu et al., 2013).

785 In addition, non-native large multi-modular complexes may undergo a complete or partial mis-
786 folding, leading to a loss of enzymatic activity due to rapid degradation of the complex or an
787 inappropriate tertiary structure of catalytic sites. This can be solved or attenuated by co-expressing
788 foreign chaperone encoding genes, which are likely to be involved in the folding of the synthetase in
789 the native host (Ongley et al., 2013). Alternatively, it was reported that a lower growth temperature
790 could promote the folding of heterologously produced proteins. For example, an increase in the
791 stability of pcbAB synthetase was demonstrated in *H. polymorpha* grown at 25°C instead of 37°C
792 (Gidijala et al., 2008). Nonetheless, this effect was not observed in *S. cerevisiae* with the same NRPS
793 (Siewers et al., 2009). Moreover, in the enniatin heterologous producer *A. niger*, the effect of the
794 temperature was also investigated among other parameters. It appears that reducing the temperature
795 from 30°C to 26°C did not significantly improve the productivity, especially compared to the impact
796 of the culture medium composition (Richter et al., 2014).

797 **3.4.2 Required enzymes for the biosynthesis of active nonribosomal peptides**

798 In most cases, obtaining the properly folded synthetase is not sufficient to enable the complex
799 to synthesize the compound of interest. Indeed, to be active, NRPSs have to be converted from their
800 *apo* into their *holo* forms through the addition of a flexible phosphopantetheine arm on each
801 thiolation domains by a phosphopantetheinyl transferase. This cofactor is crucial because it carries
802 the monomers previously specifically recognized by the different A-domains. In contrast to
803 filamentous fungi, yeasts (except *S. pombe*) lack a functional PPTase (Schwecke et al., 2006). An
804 endogenous *lys5* PPTase gene, involved in lysine biosynthesis, is expressed in several yeasts but the
805 corresponding enzyme was demonstrated to be unadapted for NRPS activation (Gidijala et al., 2009;
806 Mootz et al., 2002). To complement the lack of a functional PPTase, *sfp* from *B. subtilis* or *npaA*
807 from *A. nidulans*, are generally co-expressed with NRPS genes. NpaA and Sfp have been reported to
808 display a very broad specificity on various NRPSs in diverse fungal and bacterial organisms (Ishiuchi

809 et al., 2012; Mofid et al., 2002; Quadri et al., 1998; Tobias et al., 2016). The efficiency of these two
810 enzymes was demonstrated in *S. cerevisiae*, and the same order of magnitude of NRP titers was
811 obtained with both of them (Siewers et al., 2009). Conversely, in *E. coli*, the productivity in terms of
812 cyclodepsipeptides could be increased by 4.8- and 14-fold with *npgA* gene instead of *sfp* and
813 endogenous PPTase, respectively (Steiniger et al., 2017). The *npgA* PPTase gene has even been
814 integrated in the genome of a *S. cerevisiae* strain to generate a cellular platform ready to produce
815 NRP compounds (Gao et al., 2013, 2012; Haynes et al., 2013; Yu et al., 2013).

816 NRPs synthesized by functional synthetases are not necessarily active compounds, and are
817 often only early intermediates of the targeted bioactive product. Therefore, to obtain the final
818 functional product, addition of gene(s) encoding the tailoring enzyme(s) responsible for NRP
819 modification step(s) is required. This is the case for example, for ACV, which is sequentially
820 modified by three tailoring enzymes to give isopenicillin G (**Fig. 6 -B**). To heterologously produce
821 isopenicillin G in yeasts, and not only ACV, addition of the isopenicillin N synthase, isopenicillin N
822 acyl transferase and phenylacetyl CoA ligase genes was required (Awan et al., 2017; Gidijala et al.,
823 2009, 2008). Moreover, the two last above mentioned tailoring enzymes are located in the
824 peroxisome in the native producer *Penicillium chrysogenum*. This parameter can be crucial, as the
825 efficiency of these enzymes is impacted by their location and environment. An absence of
826 compartmentalization of these enzymes in peroxisomes decreased the productivity by more than 60%
827 in heterologous hosts (Gidijala et al., 2009). This trend was confirmed later in *S. cerevisiae* with an
828 increased productivity gained by tagging these enzymes with PTS1 peroxisome addressing signal
829 (Awan et al., 2017).

830 Gliotoxin produced by *A. fumigatus*, is also a bioactive product resulting from multiple
831 modifications of an NRP diketopiperazine encoded by *gliP* synthetase gene. The first step of the
832 biosynthetic pathway from the NRP to gliotoxin was elucidated through a heterologous expression in
833 *A. nidulans* (Chang et al., 2013; Gardiner and Howlett, 2005). Two tailoring enzymes candidates,
834 namely *gliC* and *gliF* were co-expressed separately with the *gliP* synthetase gene. The disappearance
835 of the precursor NRP in favour of an L-Phe hydroxylated derivative, was only observed in the strain
836 harbouring the cytochrome P450 monooxygenase encoded by *gliC*. This result demonstrated the role
837 of this tailoring enzyme in the first step of gliotoxin biosynthesis.

838 Similarly, brevianamide F nonribosomal compound, assembled by FtmA (or FtmS) in *A.*
839 *fumigatus* and *N. fischeri*, is a platform molecule, precursor to multiple compounds. Wunsch and co-

840 workers have co-transformed *ftmS* in *A. nidulans* with three different prenyltransferase genes from
841 the two previously mentioned fungi. The resulting strains were able to produce, in addition to
842 brevianamide F, up to three supplementary derivative compounds, such as deoxybrevianamide E
843 (Wunsch et al., 2015). With co-expression of *ftmA* with *ftmB*, *ftmC*, *ftmD*, *ftmE*, *ftmF* tailoring
844 enzyme-encoding genes, production of derivatives of brevianamide F was also observed. The NRPS-
845 encoding gene and five tailoring enzyme genes were assembled successively in the same single
846 plasmid, through multiple homologous recombinations in *S. cerevisiae*. All ORFs were positioned in
847 the plasmid under the control of distinct promoters and terminators. After transformation in both *S.*
848 *cerevisiae* and *A. nidulans*, the enzymatic activity of tailoring enzymes enabled the synthesis of
849 multiple precursors of spyrotriprostatin starting from brevianamide F: tryprostatin B, tryprostatin A,
850 and fumitremorgicin (Tsunematsu et al., 2013a).

851 **3.4.3 How to address NRP precursors deficiency?**

852 In the classical ribosomal pathway, only 20 proteinogenic α -amino acid residues are
853 incorporated into proteins. In addition to the proteinogenic α amino acid residues, up to 500 distinct
854 monomers were reported to constitute NRPs building blocks (Caboche et al., 2010). Among this
855 tremendous biodiversity of monomers, some are directly recognized and incorporated as such, while
856 others are modified during the peptide synthesis by the activity of a NRPS domain. This incredible
857 variety of precursor monomers is the root of the wide diversity of structure and activity of NRPs
858 (Challis and Naismith, 2004; Schauwecker, 2003). On the other hand, this feature raises a serious
859 issue regarding heterologous production, as hosts lack the ability to synthesize most of these non-
860 common monomers. A possible approach to complement monomers deficiency relies on the cloning
861 of the genes involved in the monomer biosynthesis pathway. The monomer D -hydroxyisovaleric acid
862 (D -Hiv) is for instance not synthesized by *A. niger* and poorly produced by *S. cerevisiae*. D -Hiv is a
863 precursor engaged in the biosynthesis of cyclodepsipeptides, such as enniatin, beauvericin and
864 bassianolide. It can be obtained by the modification of α -ketovaleric acid by α -ketoisovalerate
865 reductase encoded by *kivR* gene in *B. bassiana* or *F. oxysporum*, among others (Richter et al., 2014;
866 Yu et al., 2013). A two-fold increase of productivity was obtained with the expression of *kivR* gene in
867 a *S. cerevisiae* strain heterologously producing beauvericin (61.7 and 33.8 mg.l⁻¹ with or without
868 *kivR*, respectively) (Yu et al., 2013). In *A. niger*, the enniatin heterologous production has also been
869 made possible with the co-expression of the missing *kivR* encoding α -ketoisovalerate reductase with
870 *esyn1* NRPS gene (Richter et al., 2014).

871 On the other hand, a precursor deficiency can also be solved by feeding the corresponding
872 compound directly into the culture medium. This approach avoids time consuming gene cloning steps
873 but relies on the host strain being able to take up the precursor from the medium. The
874 complementation of the above mentioned *A. niger*, expressing foreign enniatin synthetase, was also
875 achieved by feeding it with _D-Hiv instead of co-expressing *kivR*. The feeding experiment was quite
876 successful, as it yielded a high enniatin B titer of 950 mg.l⁻¹ after optimization of feeding conditions
877 and culture medium composition. Under the same conditions, the strain fed with 20 mM _D-Hiv was
878 produced 25% more than the strain transformed with *kivR* (Richter et al., 2014). Nonetheless, the
879 cloning approach remains more cost effective due to the high cost of _D-Hiv (Yu et al., 2013).
880 Moreover, some adenylation domains display a broader specificity that can be leveraged to generate
881 alternative final products by feeding with alternative precursors. For instance, enniatin B is preferably
882 produced by feeding _L-valine in the medium, whereas the alternative enniatin A production is
883 enhanced by feeding with _L-leucine or _L-isoleucine (Richter et al., 2014). In another filamentous
884 fungus, *A. nidulans*, asperphenamate production was knocked down by mutating the *apmA* gene
885 responsible for N-benzoylphenylalaninol biosynthesis. This production was subsequently restored by
886 feeding the same strain with the deficient precursor (Li et al., 2018). In yeast, the relevance of
887 precursor supplementation in the culture medium has been highlighted in *H. polymorpha* expressing
888 *pcbAB* NRPS gene. The production of ACV was not possible due to the incapacity of *H. polymorpha*
889 to synthesize the precursor aminoadipic acid. Supplementation of the culture medium with this
890 monomer enabled to unlock the production of ACV (Gidijala et al., 2009). Nonetheless, the use of a
891 precursor feeding strategy is not always a guarantee of success as some negative feedback
892 mechanisms can occur and hinder the compound production. For instance, the feeding of the NRP-
893 precursor 2-aminobutyric acid in the culture medium of *C. asteris*, was recently demonstrated to
894 negatively impact the production of the astin C compound as well as the development of the fungus
895 (Vassaux et al., 2019).

896 Furthermore, the highly developed secondary metabolism of most of the filamentous fungi can
897 hogged available nutrients and precursors. This can lead to a decrease in availability of some
898 monomers and even become a limiting factor for the production of heterologous NRPs. The
899 modification of the metabolic fluxes and the deletion of SM biosynthetic pathways consuming large
900 amount of cell nutrients and energy can be favourable for the production of the foreign compound of
901 interest (Luo et al., 2016). Moreover, reducing the secondary metabolite metabolisms of the selected
902 host can facilitate the detection and the purification of the targeted compound by decreasing the

903 presence of unwanted products (He et al., 2018). Multiple methodologies have been developed to
904 knock out entire SM gene clusters in *A. nidulans*. Among the 71 BGCs predicted in *A. nidulans*
905 (Inglis et al., 2013), the clusters related to the biosynthesis of two major native metabolites of this
906 fungi, the emericellamide and the sterigmatocystin, have been deleted to generate a “cleaner”
907 expression host for PKS/NRPS heterologous production (Chiang et al., 2013).

908 **3.4.4 Heterologous NRP toxicity mitigation**

909 A further challenge facing NRP heterologous production is related to the toxicity of some
910 compounds towards the selected host. Native producers have developed various strategies to prevent
911 this toxicity, such as specific secretion mechanisms, compartmentalization of the toxic products, or
912 expression of genes encoding enzymes able to induce a resistance (Bond et al., 2016; Chanda et al.,
913 2009; Martín et al., 2005). These mechanisms are often specific to the toxic NRP product and
914 therefore may be only present in the native producer and not in the chosen host (Nielsen and Nielsen,
915 2017). The most straightforward strategy to overcome this issue consists to express the NRPS gene
916 under the control of an inducible promoter. This approach enables the choice of the period of
917 production of the toxic compound and to dissociate it from the cell growth period. Hence, GAL1-10,
918 amyB and Tet-on inducible promoters were used to express NRPS genes, respectively in *S.*
919 *cerevisiae* (Siewers et al., 2009), *A. nidulans* (Fujii et al., 1996), and *A. niger* (Richter et al., 2014).

920 Another common approach consists of transferring into the host not only the NRP biosynthesis
921 related genes, but also the specific resistance genes pre-identified into the genome of the native
922 producer. The mechanisms of self-resistance to a produced toxic compound can be classified into
923 three categories of mode-of-operation: (1) by inducing the compound efflux, (2) through enzymatic
924 deactivation of the toxic product, or (3) with a targeted alteration of the drug receptors (Cundliffe and
925 Demain, 2010). Considering that the latter are quite uncommon, and that the deactivation of the toxic
926 product can also impair the targeted activity of the compound, the implementation of an efflux
927 system is often the most straightforward approach to confer a resistance (Keller, 2015). NRPs are
928 likely to be small structures and accordingly possibly naturally secreted by some organisms.
929 Nonetheless, numerous studies reported a poor secretion of NRPs by heterologous strains such as *H.*
930 *polymorpha* (Gidijala et al., 2009), *S. cerevisiae* (Awan et al., 2017), and *A. niger* (Zobel et al.,
931 2016). Therefore, expression of a foreign NRP specific transport protein can be required to improve
932 the secretion procedure (do Valle Matta et al., 2001). However, the identification of resistance gene
933 (regardless of their mode-of-operation) in the native producer is not always easy. In filamentous

934 fungi, genes conferring resistance to toxic secondary metabolites have been highlighted multiple time
935 within the corresponding gene cluster. For instance, in the gliotoxin BGC of *A fumigatus*, the *gliA*
936 and *gliT* genes respectively encode an efflux pump and a gliotoxin oxidoreductase which can reduce
937 the toxicity of the compound (Dolan et al., 2015; Sharma and Chowdhary, 2017). Nonetheless, these
938 resistance genes are not systematically located within the corresponding gene cluster, and the
939 annotation methods are often far to be sufficiently efficient to identify precisely resistance genes,
940 especially in fungal organisms (Tran et al., 2019).

941 There are further options to protect the heterologous host from the toxicity of the foreign
942 compound, such as extracting the product from the culture medium. The toxic compound can for
943 instance be extracted by introducing a layer of organic solvent on the top of the culture, where the
944 product can diffuse (Bond et al., 2016; Rodriguez et al., 2014). Some absorber resins are also able to
945 bind the toxic compound when they are added to the culture broth. This was for instance
946 demonstrated with the Diaion HP20 resin binding the mycotoxin cercosporamide contained in a
947 fungal fermentation broth, thus enabling to increase by 100-fold the final product yield (Singh et al.,
948 2010).

949 **3.4.5 From the heterologous host to the *in vitro* production assays: a viable approach?**

950 **3.4.5.1 Approach and methodologies**

951 The *in vitro* NRP synthesis constitutes a cell-free production system where the enzymes
952 involved in the biosynthetic pathway (NRPS gene, tailoring enzymes) are purified and subsequently
953 incubated with the precursor monomers to generate the desired compound. The *in vitro* approach is a
954 suitable solution to address most of the issues described above. Indeed, it addresses the deficiency in
955 NRP precursors, but also circumvents issues of NRP toxicity towards the host strain. In order to
956 perform *in vitro* assays, the synthetase and/or associated tailoring enzymes need to be purified in
957 soluble form and in sufficient quantity from the heterologous host. Addition of a tag sequence, either
958 on the C-terminal or on the N-terminal part of the heterologously expressed synthetase, is highly
959 recommended to facilitate further extraction-purification steps. The use of 6xHis tag (Brandenburger
960 et al., 2017; Gao et al., 2013; Haynes et al., 2013) or Flag tag (Gao et al., 2012), respectively purified
961 through metal or antibody affinity chromatography, have been reported in previous *in vitro* NRPS
962 studies. Alternatively, NRPSs were also purified by size exclusion chromatography as their high
963 molecular weights facilitates their separation from other host proteins (Fickers et al., 2008; Lawen
964 and Zocher, 1990).

965 Thereafter, it is possible to assess the presence of the phosphopantetheine arm on thiolation
966 domains through three approaches. The most recently developed method consists to generate a
967 peptide mixture by a trypsin digestion of the proteins contained in the cell extract, before to separate
968 fractions from the peptide mixture through a strong cation exchange chromatography. Subsequently,
969 a MS/MS analysis enables to detect into the different fractions the presence of unique marker ions
970 derived from the NRPS-bonded phosphopantetheine arm (Bumpus et al., 2009). Alternatively, the
971 presence of the phosphopantetheine arm on the *holo*-NRPS can also be evaluate through the
972 thioester-binding assay measuring the consumption of radiolabeled [¹⁴C] amino-acids incorporated in
973 the synthesized NRP (Schwarzer et al., 2001). An older technic consists to perform an alkaline
974 phosphatase treatment to hydrolyze the phosphopantetheine part from the *holo*-NRPS. This treatment
975 releases a pantothenic acid which can be quantified through a microbiological assay against
976 *Lactobacillus plantarum* (Lawen and Zocher, 1990). Regarding to A-domains, the most common
977 approach to confirm their enzymatic activity consists to quantify the incorporation of ³²PPi into ATP
978 in presence of the precursor amino acids (ATP pyro-phosphate exchange radioassay) (Brandenburger
979 et al., 2017; Fickers et al., 2008). Alternatively, a nonradioactive method has also been developed
980 enabling to evaluate the A-domains activity through a MS/MS analysis. This technic relies on the
981 measurement of the isotopic back exchange of the pyrophosphate into a [¹⁸O]-labeled ATP (Phelan et
982 al., 2009). Recently, Duckworth and coworkers (2016) have developed another method based on the
983 measurement of the pyrophosphate released during the adenylation reaction. The pyrophosphate
984 released subsequently to the action of an active A-domain is first cleaved into an inorganic phosphate
985 by an inorganic pyrophosphatase. Subsequently, a purine nucleoside phosphorylase uses this inorganic
986 pyrophosphate in a reaction converting 7-methylthioguanosine into 7-methylthioguanine which can
987 be monitored with a spectrometric-based method at 360 nm (Duckworth et al., 2016). Finally, the
988 functionality of the *holo*-NRPS complex can be confirmed with the identification of the final NRP by
989 using appropriate analytical method (LC-MS and/or NMR).

990 **3.4.5.2 Successful *in vitro* assays for reconstitution/production of fungal NRPs**

991 Genome mining and *in silico* approaches have enabled, in *C. subvermispora*, to assign the
992 basidioferrin siderophore synthesis to the *CsNPS2* NRPS-encoding gene. Experimental validation of
993 these predictions has been performed through heterologous production coupled with *in vitro* assays.
994 *CsNPS2* was expressed in *A. niger* and subsequently purified through metal affinity chromatography.
995 The single A-domain of *CsNPS2* displayed specificity for two substrates: N₅-acetyl-N₅-hydroxy-L-
996 ornithine (L-AHO) and N₅- cis-anhydromevalonyl-N₅-hydroxy-L-ornithine (L-AMHO). Nonetheless,

997 basidioferrin was only detected after 24h of *in vitro* assay with 1 mM L -AHO and not with L -AMHO.
998 These results confirmed the role of *CsNPS2* in the basidioferrin biosynthesis (Brandenburger et al.,
999 2017).

1000 Several NRPSs were also heterologously expressed and purified from *S. cerevisiae* for the
1001 purpose of *in vitro* assays: AnaPS (Gao et al., 2012), TqaA (Gao et al., 2012), AspA (Gao et al.,
1002 2013), ArdA (Haynes et al., 2013). The presence of the *npgA* gene in the genome of the yeast was
1003 compulsory to produce the PPTase enabling to convert the synthetase from the *apo* to the *holo*
1004 activated form. *In vitro* assays with 10 μ M pure AspA with its monomer Ant (1 mM) and L -Trp (1
1005 mM) generated a tripeptide, the iterative mode-of-operation of this bimodular NRPS (Gao et al.,
1006 2013). The TqaA synthetase is to date the largest NRPS protein (450 kDa) whose activity has been
1007 reconstituted *in vitro* in its active form (Gao et al., 2012). The ArdA synthetase from *N. fischeri*,
1008 displaying high homology with TqaA, was also used to perform *in vitro* assays after being purified
1009 from a heterologous *S. cerevisiae* producer. This 430-kDa bimodular NRPS was demonstrated *in*
1010 *vitro* to sufficiently assemble Ant, L -Ala, and L -Trp into ardeemin FQ (Haynes et al., 2013).

1011 In another *in vitro* study aiming at obtaining NRP derivatives, Tsunematsu et al. (2013) first
1012 produced and purified the different intermediates of the spirotryprostatin biosynthetic pathways in a
1013 heterologous *S. cerevisiae*. In the meantime, tailoring enzymes FtmG and FqzB were also purified
1014 from heterologous yeast. With the purpose of deciphering the role of these tailoring enzymes in the
1015 final step of spirotryprostatin synthesis, *in vitro* assays were performed with the purified precursors
1016 mentioned above. As a result of this work, *ftmG* encoding a cytochrome P450 has been shown to be
1017 involved in spiro-ring formation in spirotryprostatin B, while the FAD-dependent monooxygenase
1018 encoded by *fqzB* was shown to be responsible for the spiro-carbon formation in spirotryprostatin A
1019 (Tsunematsu et al., 2013a).

1020 The *in vitro* NRP assembling strategy is especially useful as a complement to heterologous
1021 production by facilitating the elucidation of synthetase mode-of-operation or tailoring enzymes
1022 function. Nonetheless, due to the very low amounts of NRPS obtained through affinity
1023 chromatography purification, this approach is not promising for increasing NRP yields. Moreover,
1024 the cost incurred by the addition of precursor monomers and ATP largely prevents its applications for
1025 large scale NRP production.

1026 **4. Penicillin heterologous production: a NRP model system**

1027 Due to the clinical and commercial importance of β -lactam antibiotics, researchers have
1028 attempted for years to develop their heterologous production in hosts renowned for being readily
1029 cultivate at industrial scale, and easy to handle through genetic engineering. This multi-decade work
1030 has positioned the NRP-derived penicillin compounds as a model system regarding NRP
1031 heterologous production. These efforts have enabled both to better understand the ins and outs of the
1032 penicillin biosynthetic pathway and to develop various strategies to implement heterologous
1033 production in fungal cell factories.

1034 Penicillin β -lactam antibiotics are naturally produced by some *Penicillium* species, especially
1035 *P. chrysogenum* and to a lesser extent by some *Aspergillus* species such as *A. nidulans* (R W
1036 Newbert et al., 1997; Suárez and Peñalva, 1996). In *P. chrysogenum*, the elucidated penicillin
1037 biosynthetic pathway relies on the joint action of several enzymes, whose corresponding genes are
1038 mainly located within the same gene cluster (**Fig. 6 -A**). The *pcbAB* gene encodes a 3-module NRPS
1039 that is responsible for assembling ACV (**Fig. 6 -B**). This nonribosomal peptide precursor of
1040 penicillin, is then modified into isopenicillin (IPN) by an isopenicillin N synthase. This intermediate
1041 is further converted by an isopenicillin N acyl transferase (IAT) and a phenylacetyl CoA ligase (PCL)
1042 into penicillin G (Gidijala et al., 2009). The penicillin V in which the benzyl group is replaced by a
1043 phoxymethyl group is a commercial analog of the penicillin G displaying an enhanced stability in
1044 acidic environment. This analog is preferably produced by *P. chrysogenum* when phoxymethyl
1045 is added in the fermentation medium (Ball et al., 1978). This appears reasonable because the
1046 production of low amounts of penicillin compounds, and the presence of enzymes sharing a high
1047 level of sequence identity with those from *P. chrysogenum*, had been reported in (Suárez and
1048 Peñalva, 1996). An associated positive impact of these native enzymes cannot be ruled out to explain
1049 the success of this heterologous production.

1050 The first transfer of the penicillin biosynthetic pathway in a heterologous host was achieved
1051 into *N. crassa* and *A. niger*. The cosmid pCX3.2 containing the ACVS encoding gene cluster was
1052 cloned into both heterologous hosts, leading directly to production of penicillin. The presence of the
1053 produced antibiotic was successfully assessed through a bioactivity assay against *B. subtilis*. *A. niger*
1054 was reported to produce much more penicillin V than *N. crassa* (up to 2.3 $\mu\text{g}\cdot\text{ml}^{-1}$ versus 0.170
1055 $\mu\text{g}\cdot\text{ml}^{-1}$) (Smith et al., 1990). One explanation could be the lack of ACV precursor availability in *N.*
1056 *crassa*. A second hypothesis rests on the fact that *A. niger* and *P. chrysogenum* are closely related
1057 species both belonging to the order Eurotiales, unlike *N. crassa* which is associated to Sordariales.

1058 A few years later, the penicillin biosynthetic pathway was also reconstituted into the
1059 methylotrophic yeast *H. polymorpha*, which is well-known for its superior fermentation properties
1060 even at industrial scale (Stöckmann et al., 2009). The 12-kb *pcbAB* NRPS gene was inserted, under
1061 the control of the strong methanol-inducible promoter from alcohol oxidase gene (P_{AOX}) in pHIPZ4
1062 plasmid. *H. polymorpha* was transformed with the linearized plasmid in order to integrate the gene at
1063 the AOX locus. Subsequently, heterologous expression of ACV synthetase (ACVS) was assessed
1064 directly at the protein level by Western blot with antibodies directed to ACVS amino-acid sequence.
1065 As described in the section 3, the homologous PPTase Lys5 of the methylotrophic yeast was not
1066 adapted to activate ACVS, and an additional *sfp* PPTase encoding-gene was inserted. Subsequently,
1067 IPNS, IAT and PCL-encoding genes from *P. chrysogenum* were integrated into the genome step by
1068 step. Penicillin G productivity of the resulting yeast was similar to those obtained in the native
1069 producer NRRL1951 (1.1 and 1.0 $\mu\text{g}\cdot\text{ml}^{-1}$, respectively). In the same yeast, deletion of the native
1070 *PEX3* gene, responsible for the peroxisome proliferation, led to a 64% reduced yield (0.4 $\mu\text{g}\cdot\text{ml}^{-1}$)
1071 (Gidijala et al., 2009). The importance of the peroxisomal location of IAT and PCL had previously
1072 been highlighted in *P. chrysogenum* and *A. nidulans* (Spröte et al., 2009).

1073 In subsequent work, *S. cerevisiae* was also selected to heterologously produce ACV (Siewers et
1074 al., 2009). *pcbAB* was inserted into a replicative multi-copy pESC by TAR cloning under the control
1075 of the galactose inducible promoter GAL1/GAL10 ($P_{GAL1/GAL10}$). The resulting construct bearing an
1076 *URA3* selection gene was used to transform a *S. cerevisiae* Δura3 strain. One of the assets of pESC is
1077 the presence of the bi-directional $P_{GAL1/GAL10}$ enabling the cloning of a second gene. This allows the
1078 cloning of the PPTase encoding gene in the same vector as the NRPS gene (if the NRPS is encoded
1079 by a single ORF). Under galactose induction, the transformed strain carrying this plasmid was able to
1080 express functional ACVS (transcription assessed by qPCR and detection of the NRP by LC-MS
1081 analysis). A codon optimization of the 5' portion of the ACVS gene was also performed leading to a
1082 slight, but not significant, increase of the productivity. On the other hand, *pcbAB* was also integrated
1083 into a long terminal repeats (LTR) retrotransposons locus of *S. cerevisiae*. LTR retrotransposon are
1084 transposable elements present in high number and spread all over the yeast genome, thus making
1085 these sites suitable for multi-copy genomic integration (Maury et al., 2016). Yields obtained with
1086 genomic integration were 6-fold lower than in the multi-copy PESC plasmid, demonstrating the
1087 benefits of the latter expression system. A few years later, another ACV producing *S. cerevisiae* was
1088 obtained with the cloning of *pcbAB* and *npgA* gene into a low-copy centromeric plasmid (Awan et
1089 al., 2017). Multiple pairs of promoters were tested to enhance the expression of both the *pcbAB* and

1090 *npgA* genes. The combination of *pcbAB* and *npgA*, respectively under the control of P_{TDH3} and P_{PGK1}
1091 constitutive promoters, led to a 4-fold higher ACV productivity than with $P_{GAL1/GAL10}$ in PESC
1092 plasmid (≈ 0.28 versus $\approx 0.07 \mu\text{g}\cdot\text{ml}^{-1}$). This demonstrated the importance of promoter choice for each
1093 gene to be expressed, and the underestimated requirement for compatibility of each promoter/gene
1094 pair. Subsequently, the complete biosynthetic pathway to the penicillin G was reconstituted by
1095 addition of tailoring enzymes IPN, IAT and PCL. Expression of the tailoring enzymes were also
1096 evaluated under multiple combinations of promoters for the sake of optimizing the production. The
1097 resulting strain was able to efficiently produce and secrete penicillin G, but with yields still two fold
1098 less than in heterologous *H. polymorpha* (Gidijala et al., 2009).

1099 With the purpose of transferring the multiple genes involved in the penicillin synthesis from *P.*
1100 *chrysogenum* into *A. nidulans* SAA248 lacking a native penicillin gene cluster, Unkles and
1101 coworkers have exploited the previously described properties of the viral 2A peptide. By inserting
1102 viral 2A peptide between each single ORF, it is possible to express from the same plasmid a
1103 polycistronic mRNA under the control of a single strong/inducible promoter (Kim et al., 2011). Viral
1104 2A sequences were inserted between *pcbAB*, IPNS gene (*pcbC*), and IAT gene (*penDE*). The
1105 resulting 15.5-kb sequence was cloned by *in vivo* homologous recombination in *S. cerevisiae*, into
1106 pJET1.2 plasmid under the control of xylose inducible promoter. The obtained plasmid was then
1107 transferred to *A. nidulans*, leading to the transcription of the 15.5-kb polycistronic mRNA.
1108 Subsequently, each individual enzyme and especially the massive NRPS were cleaved and their joint
1109 action enabled the synthesis of penicillin G. Notably, the 331-T25 mutant having integrated multiple
1110 copies of the genes, was able to produce 3-fold more penicillin G than the wild type *A. nidulans*
1111 strain 0760 (0.687 versus $0.234 \mu\text{g}\cdot\text{ml}^{-1}$) (Unkles et al., 2014). Interestingly, in addition to the
1112 heterologous IAT enzyme, a second native IAT was identified in the transformed SAA248 strain.
1113 Even if *A. nidulans* SAA248 has lost its ability to synthesize penicillin like other *A. nidulans* strain,
1114 the presence of genes encoding enzymes involved in the penicillin biosynthetic pathway seems to
1115 persist in its genome (Suárez and Peñalva, 1996). An associated positive impact of these native
1116 enzymes cannot be ruled out to explain the success of this heterologous production.

1117 It is not straightforward to compare the penicillin production yields obtained with the different
1118 heterologous strains described in this section. Nonetheless, authors have each time compared these
1119 yields with those observed either in the *P. chrysogenum* NRRL1951 or *A. nidulans* 0760 native
1120 penicillin producers, enabling some cross-study comparisons. Regarding to yeast heterologous
1121 systems, *H. polymorpha* was reported to produce similar penicillin G amount to *P. chrysogenum*

1122 NRRL1951 strain ($\sim 1 \mu\text{g}\cdot\text{ml}^{-1}$) (Gidijala et al., 2009). In contrast, *S. cerevisiae* was half efficient,
1123 probably due to the considerably less developed peroxisomal system compared to the methylotrophic
1124 yeast (Awan et al., 2017). Regarding to filamentous fungi heterologous systems, the 331-T25 mutant
1125 of *A. nidulans* SAA248 expressing penicillin biosynthetic genes coming from *P. chrysogenum* was 3-
1126 fold more efficient than the native penicillin G producer *A. nidulans* 0760 ($\sim 0.69 \mu\text{g}\cdot\text{ml}^{-1}$), but still
1127 30% less productive than *P. chrysogenum* NRRL1951 (Unkles et al., 2014). In terms of heterologous
1128 penicillin V production, wide variations have been observed between the two described hosts with up
1129 to 13-fold higher productivity in *A. niger* compared to *N. crassa*. The highest penicillin V
1130 concentrations reported in the heterologous *A. niger* strain was likely due to the close taxonomic
1131 relatedness of this strain with *P. chrysogenum* (Smith et al., 1990).

1132 Over many years, the heterologous production of penicillin has been a long-standing test case
1133 of study. It enabled researchers to deal with most of the key factors limiting NRP heterologous
1134 production and to unlock some of them. Unfortunately, in terms of productivity, the heterologous
1135 production approach is decades behind on current industrial penicillin G production systems. Indeed,
1136 several studies were conducted to generate and screen thousands of *P. chrysogenum* mutants, with
1137 the aim of improving production yields. Industrial production, with these selected strains, has been
1138 optimized for years in terms of strain productivity and fermentation process (R. W. Newbert et al.,
1139 1997; Vournakis and Elander, 1983). The analysis of the genome of the strains with an improved
1140 penicillin production revealed the presence of multiple copies of the penicillin biosynthetic genes,
1141 which is likely to contribute in the increased productivity (Fierro et al., 2006; Müller et al., 2018; R
1142 W Newbert et al., 1997). Another characteristic of high-penicillin producing strains is the loss of
1143 several gene clusters involved in secondary metabolite synthesis which were detected in the wild-
1144 type *P. chrysogenum* NRRL195. For instance, the improved penicillin producer *P. chrysogenum*
1145 Wisconsin 54-1255 lacks the ability of producing sorbicillin and penitric acid which are present in
1146 the genome of the parental NRRL195 strain. The deletion of secondary metabolite biosynthetic
1147 pathways also contributed to improve the strain productivity for β -lactam antibiotics (Salo et al.,
1148 2016, 2015). Although the current penicillin G industrial production yields are not available in the
1149 literature, these yields were reported few years ago to be already more than 1000-fold higher than
1150 those obtained with the wild-type *P. chrysogenum* NRRL1951 (Harris et al., 2009).

1151 **5. Concluding remarks**

1152 The recent development of high-throughput sequencing technologies combined with substantial
1153 drop in sequencing costs have driven an impressive increase in the number of available fungal
1154 genomes. This factor coupled with the improvement of genome annotating methodologies has
1155 partially unlocked the SM production potential of fungi. Hence, efficient bioinformatics tools now
1156 enable a more accurate analysis in terms of nonribosomal pathway identification and substrate
1157 specificity predictions. Nonetheless, the prediction robustness is lowered because already elucidated
1158 fungal NRPs and NRPSs remain under-represented in dedicated databases as compared to bacterial
1159 ones. Consequently, the predictions based on genomic sequences need to be validated by structural
1160 elucidation of the NRPs which implies its efficient production.

1161 The production without genetic manipulation of NRP compounds identified *in silico* is not
1162 systematically possible in native hosts due to the existence of cryptic biosynthetic gene clusters
1163 and/or incompatibility between the strain and the laboratory cultivation conditions. Therefore, the
1164 heterologous production approach is often the most appropriate strategy to deal with this issue.
1165 Multiple fungal cellular platforms have been developed to heterologously produce foreign fungal
1166 nonribosomal peptides in particular. Among others, the yeast *S. cerevisiae* and filamentous fungi
1167 related to *Aspergillus* spp. have been widely used. Heterologous production of NRPs is particularly
1168 challenging due to a plethora of potential limiting steps. Combining multiple strategies to optimize
1169 each of them can greatly increase the chances of successful production. Especially, the transfer of
1170 large NRPS sequences encompassing tens of kb is one of the main hurdles to overcome. Several
1171 methodologies, such as TAR cloning, ExRec, Gibson assembly, USER cloning, and overlap
1172 extension PCR, have been developed to enable the reconstruction of single large NRPS genes or gene
1173 clusters. This laborious and time-consuming engineering of large NRPS biosynthetic pathways can
1174 be avoided by *de novo* DNA synthesis, but this remains prohibitively costly in many cases. However,
1175 considering that costs of gene synthesis are continuously decreasing, an intensification of the use of
1176 this rapid methodology for transferring foreign NRPS genes and gene clusters can be expected in the
1177 upcoming years.

1178 The correct transfer of the targeted gene in the heterologous host is not a guarantee of success
1179 for the production of the related NRP. For instance, Harvey et al. (2018) have transferred 41 cryptic
1180 fungal gene clusters to *S. cerevisiae* but only 54% have been processed by the yeast to lead to a
1181 detectable compound. This result, while promising, demonstrates the challenges related to the
1182 heterologous expression of large fungal gene clusters. Nonetheless, the efficiency of the heterologous
1183 production of NRPs can be greatly improved by taking into account some key factors. First, the

1184 transcription rate can be improved by using an appropriate promoter, and sometimes with adequate
1185 initiation factors. Translation and proper folding of the protein are also crucial factors to consider.
1186 Optimization of this can be achieved by expression of foreign chaperones enzymes or with different
1187 cultivation conditions. It is also important to protect the foreign NRPS from the proteolytic activity of
1188 the selected host. Once the properly folded NRPS is obtained and activated via a homologous or
1189 heterologous phosphopantetheinyl transferase, the assembly of the NRP can be considered. At this
1190 last stage, NRP precursor deficiency or NRP toxicity against the selected hosts can also become
1191 limiting factors. The first one can be overcome by feeding or cloning of the missing genes related to
1192 precursor biosynthesis, while the second may be prevented by conferring secretion ability to the host
1193 with the cloning of transporter-associated genes, for example.

1194 Despite these drawbacks, heterologous production strategies were successfully employed to
1195 identify novel NRP compounds, to decipher NRPS modes-of-operation and in some cases, to
1196 improve productivity. This approach is especially relevant to producing compounds for which the
1197 corresponding genes are cryptic or weakly expressed in native organisms for which no genetic tools
1198 are available yet. Conversely, transferring an NRP biosynthetic pathway to a heterologous host is
1199 often not sufficient to overcome production yield obtained with a native producer already optimized
1200 and well industrially established strain as in the penicillin case. Overall, the NRP production rates
1201 currently obtained with heterologous expression do not yet reach levels suitable for industrial
1202 production. Chemical synthesis remains in most of the cases, the more competitive way to obtain
1203 peptides and especially regarding linear peptides including only proteinogenic amino acids. A
1204 possible exception are certain high-value compounds, which are cyclic, or include unusual building
1205 block and/or monomers which are further modified by tailoring enzyme. Indeed, these specific
1206 characteristics are often difficult to obtain via chemical synthesis and should be obtained more easily
1207 through a heterologous expression of the corresponding NRPS-encoding gene.

1208 **6. Conflict of Interest**

1209 Authors of the manuscript declare there is no conflict of interests.

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- 1969

1970 **Table 1:** List of condensation domain subtypes and their associated reactions

Condensation domain subtype	Reaction description	Reference
^L C _L	Bind _L -amino acids to _L -amino acids	
^D C _L	Bind _D -amino acids to _L -amino acids	
Starter C	Load a fatty acid as first substrate of the synthesis	(Rausch et al., 2007;
Dual C/E	Combine condensation and epimerization activities	Ziemert et al., 2012)
Epimerization	Invert an _L -form amino acid to its _D -form enantiomer	
Heterocyclization	Catalyse peptide bond formation, followed by cyclization	
C _{term}	Replace thioesterase activity for the final step of peptide release	(Gao et al., 2012)
Hybrid C	Bind an amino acid to an aminated polyketide in hybrid PKS-NRPS systems	(Ziemert et al., 2012)

1971
1972

1973 **Table 2:** Previous studies reporting NRP heterologous production in yeasts

Natural product (NRP)	NRPS	NRPS MW (kDa)	Native producer	Heterologous host	Expression system	Reference
ACV	ACVS (or PcbAB)	450	<i>P. chrysogenum</i>	<i>H. polymorpha</i>	Plasmid pZ4 Plasmid pG4U	Gidijala, 2008
				<i>S. cerevisiae</i>	Plasmid pESC Genomic integration	Siewers, 2009
					Plasmid pESC	Awan, 2017
Fumiquinazoline F	Afu6g12080	450	<i>A. fumigatus</i>	<i>S. cerevisiae</i>	Plasmid pKW1810	Ishiuchi, 2012
	TqaA		<i>P. aethiopicum</i>		Plasmid pXW55	Gao, 2012
Tricyclic ardeemin F-Q	ArdA	432	<i>N. fischeri</i>	<i>S. cerevisiae</i>	Plasmid pXW55	Haynes, 2013
Beauvericin	BeSYN	352	<i>B. bassiana</i>	<i>S. cerevisiae</i>	Plasmid pXW55	Yu, 2013, 2017
Bassianolide	BaSYN	348	<i>B. bassiana</i>	<i>S. cerevisiae</i>	Plasmid pXW55	Yu, 2013, 2017, 2019
Brevianamide F	FtmA	319*	<i>A. fumigatus</i>	<i>S. cerevisiae</i>	Plasmid pKW1250	Tsunematsu, 2013
Acetylaszonalenin	AnaPS	280	<i>N. fischeri</i>	<i>S. cerevisiae</i>	Plasmid pXW55	Gao, 2012
Asperlicin C/D	AspA	276	<i>A. alliaceus</i>	<i>S. cerevisiae</i>	Plasmid pXW55	Gao, 2013
Indigoidine	BpsA	141	<i>S. lavendulae</i>	<i>S. cerevisiae</i>	Genomic integration	Wehrs, 2018
^D Phe- ^L Leu	TycA	124	<i>B. parabrevis</i>	<i>S. cerevisiae</i>	Plasmid pESC	Siewers, 2010
	SrfAC	144	<i>B. subtilis</i>			
2,4-dihydroxy 5,6-dimethyl benzaldehyde	ATEG_03630	120	<i>A. terreus</i>	<i>S. cerevisiae</i>	Plasmid pRS414	Wang, 2014
Phenguignardic acid	PgnA	104*	<i>A. terreus</i>	<i>S. cerevisiae</i>	Plasmid pESC	Huhner, 2017
Atromentin	ATEG_03090	101*	<i>A. terreus</i>	<i>S. cerevisiae</i>	Plasmid pESC	Huhner, 2017
Aspulyinone E	Apva & MelA	92*	<i>A. terreus</i>	<i>S. cerevisiae</i>	Plasmid pESC	Huhner, 2017

1974 *Estimated on the basis of the amino acid sequence length (with an average of 110 Da per amino acids)

1975 **Table 3:** Previous studies reporting NRP heterologous production in filamentous fungi

Natural product (NRP)	NRPS	NRPS MW (kDa)	Native producer	Heterologous host	Expression system	Reference
KK-1	KK-1 NRPS	1,430*	<i>C. clavata</i>	<i>A. oryzae</i>	Genomic integration	Yoshimi, 2018
Cycloaspeptide A/E	PscyB	623*	<i>P. sopii</i>	<i>A. oryzae</i>	Plasmid pTYGS-arg	Mattos-Shippely, 2018
Ferrirhodin	FSN1	495*	<i>F. sacchari</i>	<i>A. oryzae</i>	Plasmid pTAYAGSarg	Munawar, 2013
ACV	ACVS (or PcbAB)	450	<i>P. chrysogenum</i>	<i>N. crassa</i>	Cosmid Pcx3.2	Smith, 1990
				<i>A. niger</i>	Plasmid pJET1.2	Unkles, 2014
Bassianolide	BaSYN	350	<i>B. bassiana</i>	<i>A. niger</i>	Genomic integration	Boecker, 2018 Steiniger, 2017
CDP hexa-bassianolide	BaSYN-EnSYN TC ₃	350	<i>B. bassiana</i> <i>F. oxysporum</i>	<i>A. niger</i>	Genomic integration	Steiniger, 2017
Beauvericin	BeSYN	350	<i>B. bassiana</i>	<i>A. niger</i>	Genomic integration	Boecker, 2018 Steiniger, 2017
Octa-beauvericin	BeSYN-BaSYN TC ₃	350	<i>B. bassiana</i>	<i>A. niger</i>	Genomic integration	Steiniger, 2017
[PheLac]-Beauvericin	PSYN M ₁ -BeSYN M ₂	350	<i>R. abscondita</i> <i>B. bassiana</i>	<i>A. niger</i>	Plasmid pVG2.2	Zobel, 2016
Enniatin	EnSYN	350	<i>F. oxysporum</i>	<i>A. niger</i>	Genomic integration	Richter, 2014 Steiniger, 2017
Octa-enniatin	EnSYNΔC ₃ -BaSYN C ₃	350	<i>F. oxysporum</i> <i>B. bassiana</i>	<i>A. niger</i>	Genomic integration	Steiniger, 2017
[PheLac]-Enniatin	PSYN M ₁ -EnSYN M ₂	350	<i>R. abscondita</i> <i>F. oxysporum</i>	<i>A. niger</i>	Plasmid pVG2.2	Zobel, 2016
Brevianamide F	FtmA	319*	<i>A. fumigatus</i>	<i>A. nidulans</i>	Genomic integration	Maiya, 2006
				<i>A. niger</i>	Plasmid pKW20142	Tsunematsu, 2013
				<i>A. nidulans</i>	Genomic integration	Wunsch, 2015
Basidioferrin	CsNPS2	275	<i>C. subvermispora</i>	<i>A. niger</i>	Genomic integration	Brandenburger, 2017
Asperphenamate	ApmA ApmB	275* 275*	<i>P. brevicompactum</i>	<i>A. nidulans</i>	Genomic integration	Li, 2018
Gliotoxin	GliP	236	<i>A. fumigatus</i>	<i>A. nidulans</i>	Genomic integration	Chang, 2013
Microperfuranone	MicA	103*	<i>A. nidulans</i>	<i>A. niger</i>	Plasmid pSM565	Yeh, 2012
Aspulvinone E	ApvA MeIA	92*	<i>A. terreus</i>	<i>A. nidulans</i>	Genomic integration	Guo, 2015
				<i>A. niger</i>	Genomic integration	Geib, 2016, 2017

1976 *Estimated on the basis of the amino acid sequence length (with an average of 110 Da per amino acids)

1977 **Figure legends**

1978 **Figure 1:** Multiple steps involved in NRP biosynthesis pathway. **A-** Transcription of the NRPS
1979 encoding gene. **B-** Translation of the NRPS mRNA. **C-** Folding of the NRPS in multi-enzymatic
1980 domains modular organization and addition of phosphopantetheine arm (*holo*-NRPS). Several
1981 domains are presented: A: adenylation; T: thiolation; C: condensation; C_{term}/Te: thioesterase or
1982 terminal condensation domain (only found in fungal NRPSs); and an example of a potential
1983 additional epimerization (E) domain (converting an L-form amino acid to its D-form). **D-** Operating
1984 way of NRPS assembly line to generate NRP (Mm: monomer). *Epimerization reaction can occur
1985 either before or after condensation reaction.

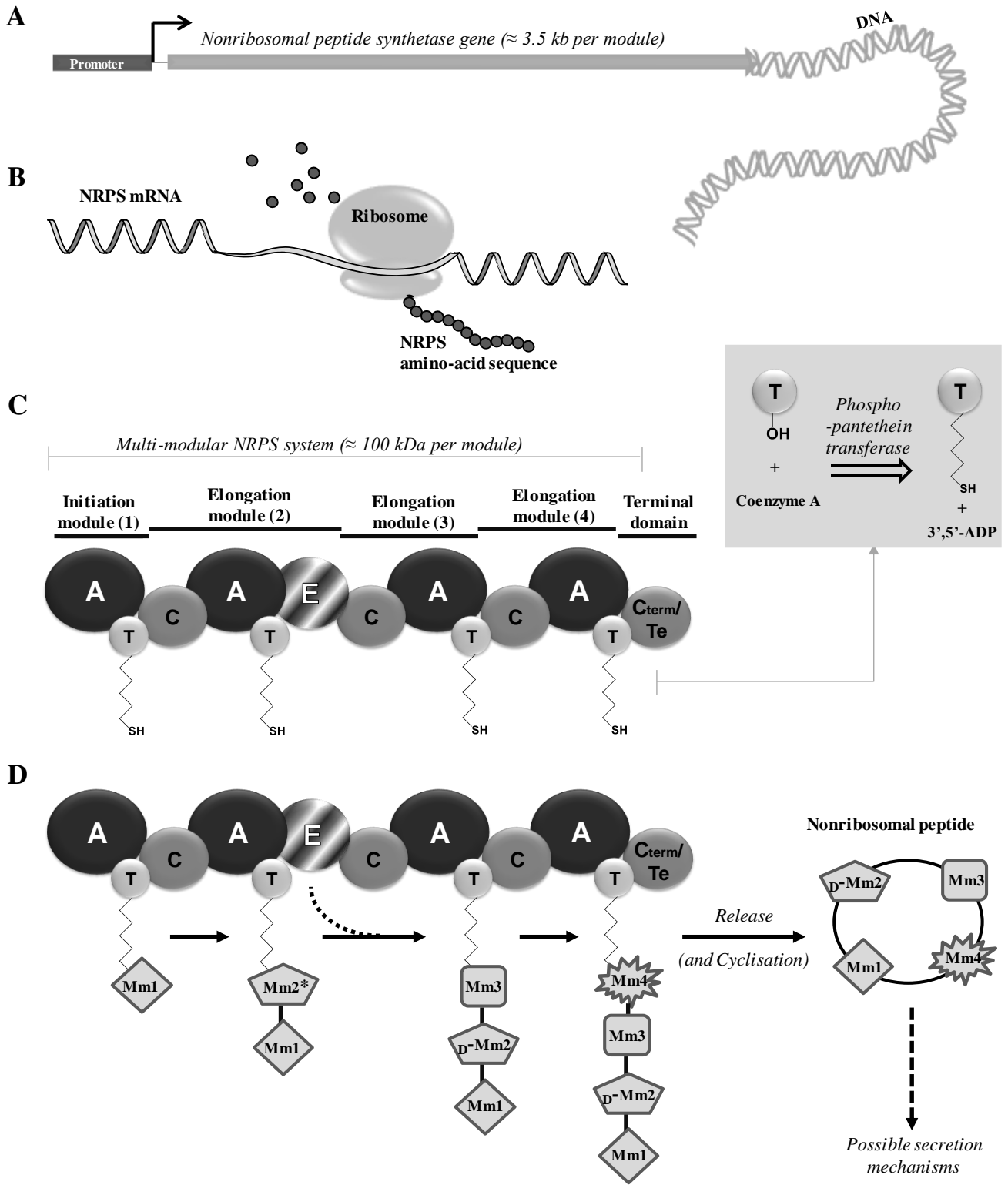
1986 **Figure 2:** Simplified workflow for novel NRP discovery by genome mining with an overview of the
1987 main bioinformatics tools available (accessible through hyperlinks).

1988 **Figure 3:** Analysis of the 50 most frequent monomers constituting the nonribosomal peptides
1989 classified in the Norine database. Each bar is subdivided into colored parts corresponding to the
1990 taxonomic clade from which the resulting NRPs was identified (i.e., white: bacteria, dark grey:
1991 fungi). The monomers specifically activated by A-domains which display pHMMs signatures
1992 enabling specificity predictions are highlighted in green.

1993 **Figure 4:** Description of the four main cloning methods enabling the reconstitution, from multiple
1994 amplified PCR fragments, of long-length NRPS sequences into a targeted vector: **A.** Overlap
1995 extension PCR approach, **B.** Gibson assembly, **C.** USER Cloning, and **D.** TAR Cloning (homologous
1996 recombination).

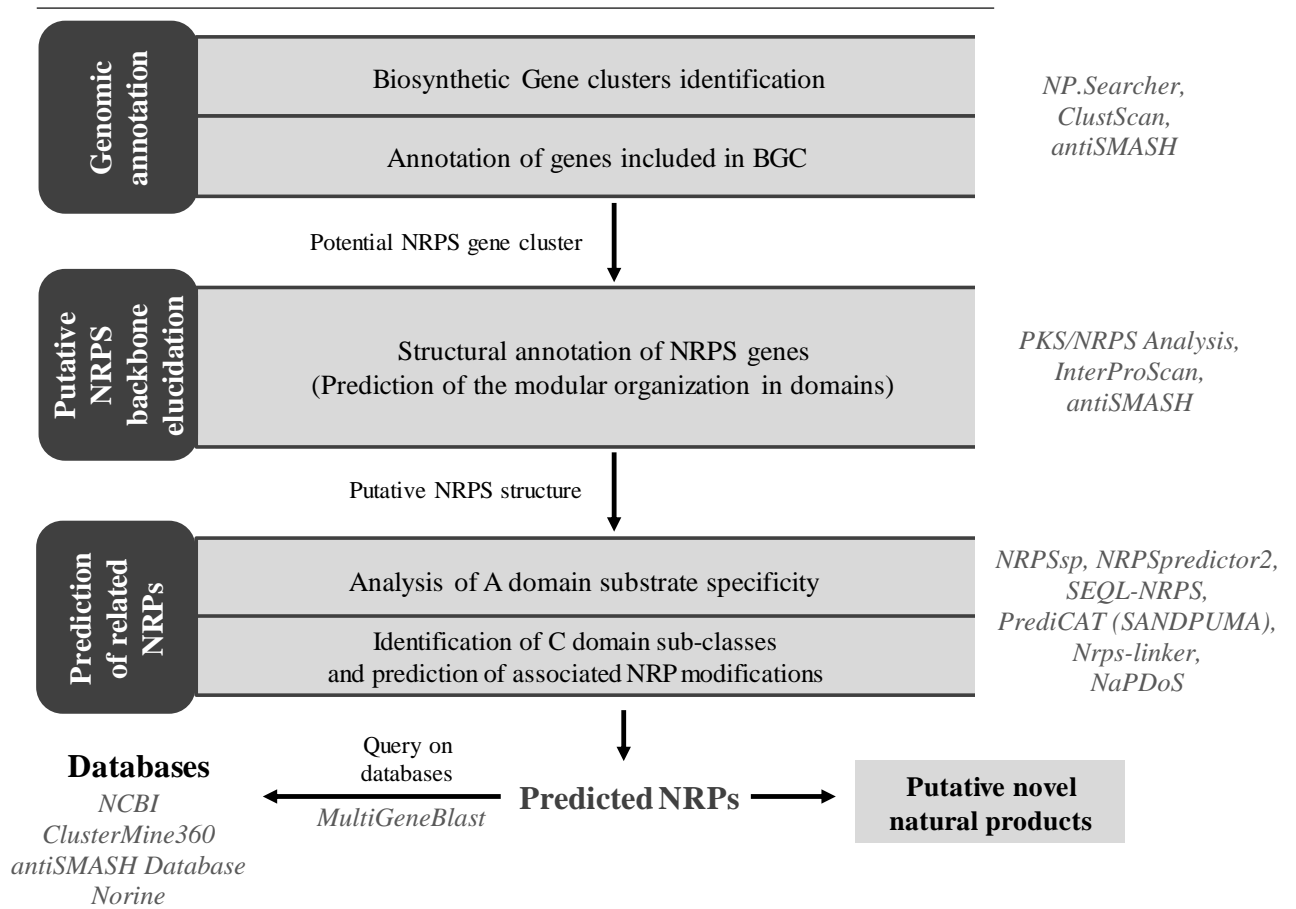
1997 **Figure 5:** Overview of the limiting steps of NRP heterologous production and of the main solutions
1998 to overcome them and consider upgrading production rates.

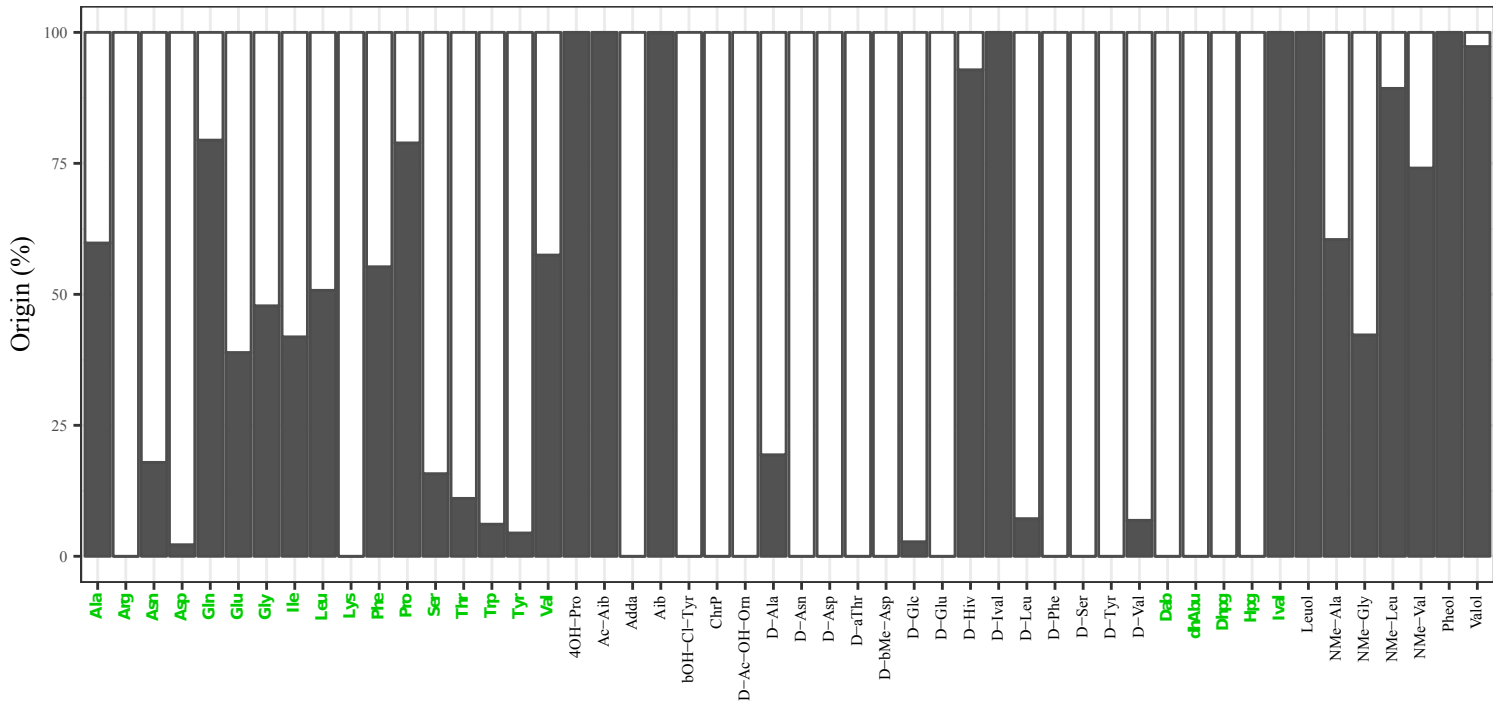
1999 **Figure 6: A.** Presentation of the gene cluster involved in the biosynthesis of penicillin in *Penicillium*
2000 *chrysogenum*. **B.** Schematic representation of the penicillin G biosynthetic pathway in *P.*
2001 *chrysogenum*. ACV is assembled by the ACVS (encoded by the *pcbAB* gene), before to be modified
2002 into isopenicillin N by IPNS (encoded by *pcbC* gene), subsequently converted into penicillin G
2003 through combined activities of IAT and PCL (respectively encoded by *penDE* and *pclA* gene). *PclA**
2004 gene is not part of the penicillin biosynthetic cluster.



Second Generation Sequencing, Third Generation Sequencing, Hybrid methods
Genomic data

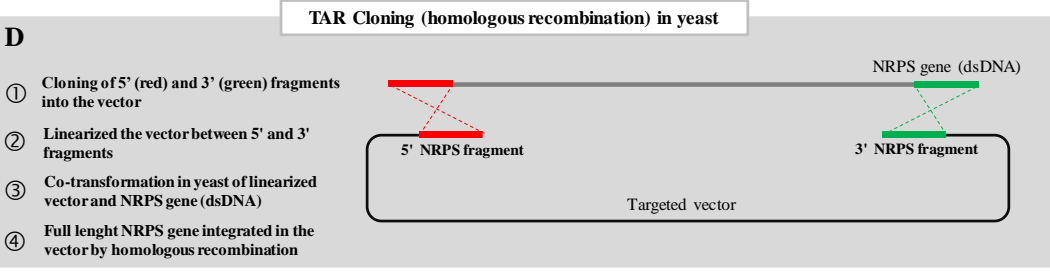
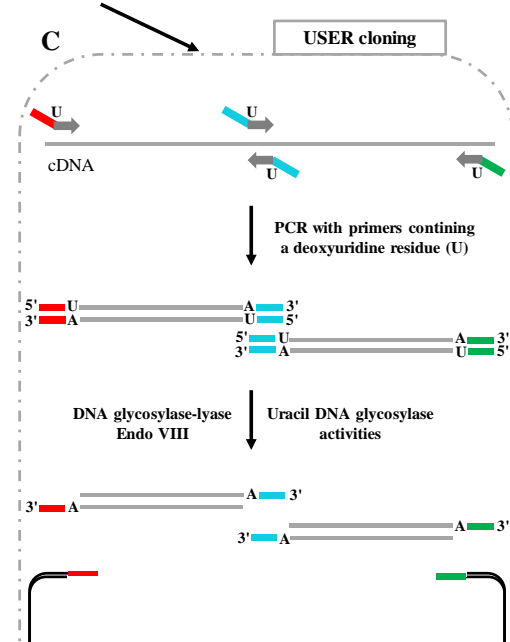
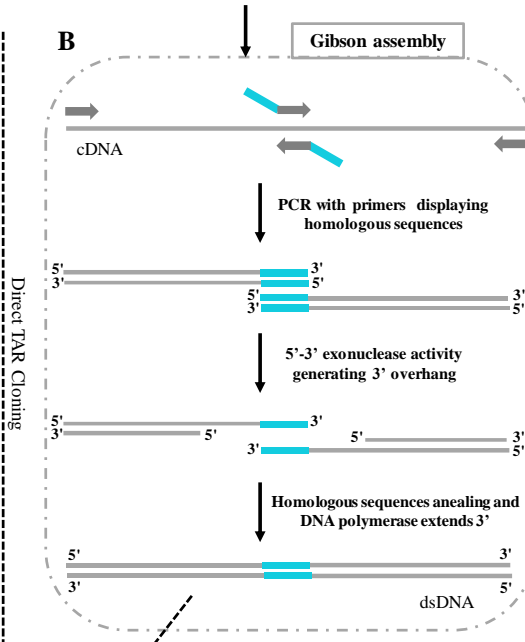
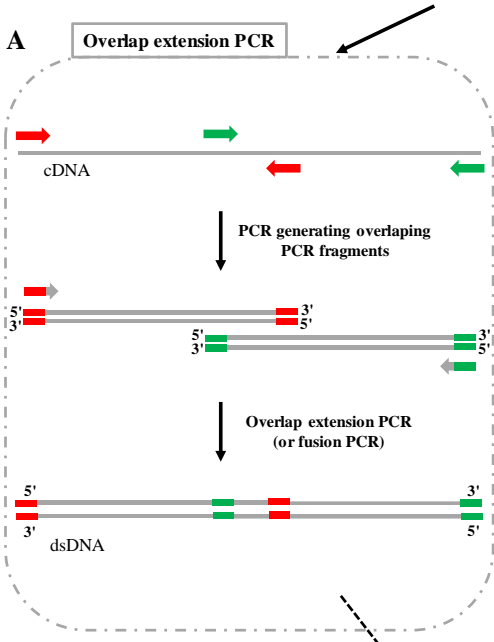
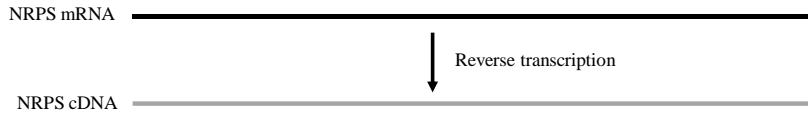
SPADes & Canu





Proteinogenic amino acids

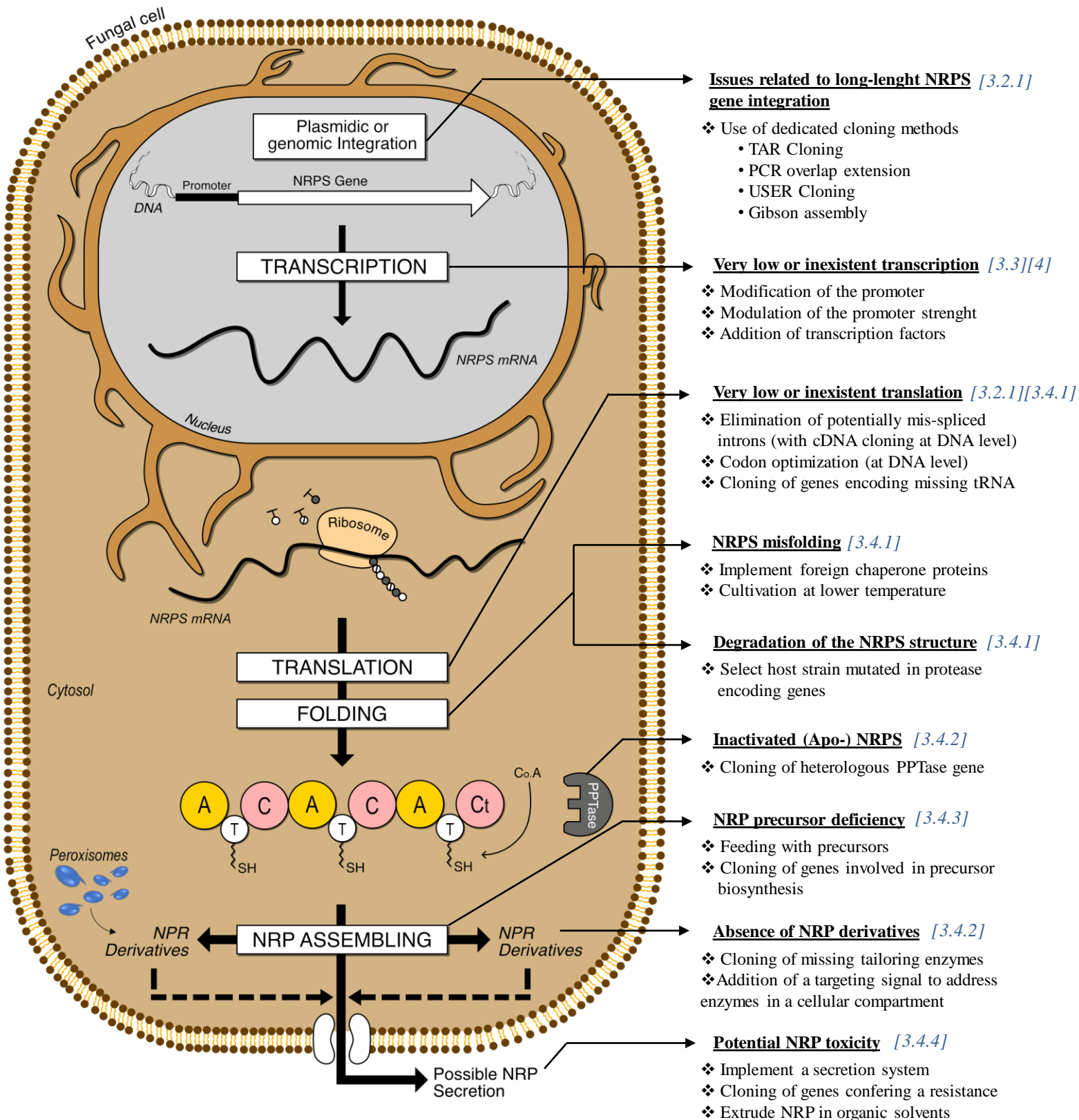
Exotic and D amino acids

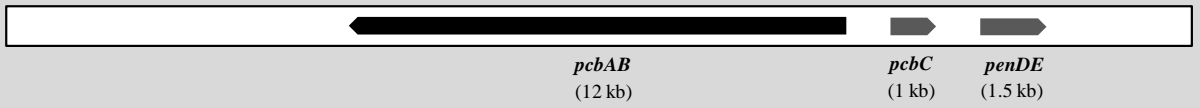


Limiting steps of NRP heterologous production

[Corresponding text section]

❖ Main solutions to overcome them



A*P. chrysogenum*
DNA**B**