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# Cell-free protein synthesis for nonribosomal peptide synthetic biology

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Peptide natural products have a wide range of useful applications as pesticides, veterinary agents, pharmaceuticals, and bioproducts. To discover new natural products, manipulate them for analog generation, and to harness the potential of these bioactive compounds for synthetic biology, it is necessary to develop robust methods for the expression of biosynthetic genes. Cell-free synthetic biology is emerging as an important complementary approach because it is highly desirable to express protein on a more rapid timescale and does not rely upon the genetic tractability of a strain thus improving the throughput of design-build-test-learn cycles. Additionally, generating metabolites outside the cell can overcome issues such as cellular toxicity which can hamper applications like antibiotic development. In this review, we focus on the cell-free production of peptide natural products generated by non-ribosomal peptide synthetase. Nonribsomal peptides are biosynthesized by non-ribosomal peptide synthetases which are large "mega" enzymes that provide specific challenges to heterologous expression. First, we summarize NRPSs and their corresponding peptide metabolites that are expressed in cell-free systems. With that, we discuss the requirements and challenges to express such large proteins in cell-free protein synthesis as well as host machineries that have been developed for cell-free protein synthesis that could be particularly relevant to generating non-ribosomal peptide metabolites in the future. The development of cell-free systems can then be used for prototyping to accelerate efforts towards engineered biosynthesis of these complex pathways.

KEYWORDS

**non-ribosomal peptide synthetase, modular megaenzyme, peptide natural products, synthetic biology, cell-free protein synthesis (CFPS)**

## 1 Introduction

Peptide natural products form the basis of many important molecules including agrochemicals (Cane et al., 1998; Pereira-Dias et al., 2023), bioproducts (Tao et al., 2023), and therapeutics (Corpuz et al., 2022; Girija et al., 2022). Of these applications, the most historically significant one has been as an important source for drug leads (Atanasov et al., 2015; Harvey et al., 2015). Many peptide natural products have complex chemical scaffolds (e.g., penicillin, daptomycin, and vancomycin antibiotics) (Felnagle et al., 2008; Süssmuth and Mainz, 2017). These peptide cores are biosynthesized by two major routes: non-ribosomal peptide synthetases (NRPS) and ribosomal synthesized and post-translationally modified peptide (RiPP) biosynthetic pathways. RiPPs produce peptide precursors by utilizing the ribosome and proteinogenic amino acids. A gene cluster in RiPPs biosynthetic pathways involves a precursor peptide that is then posttranslationally modified

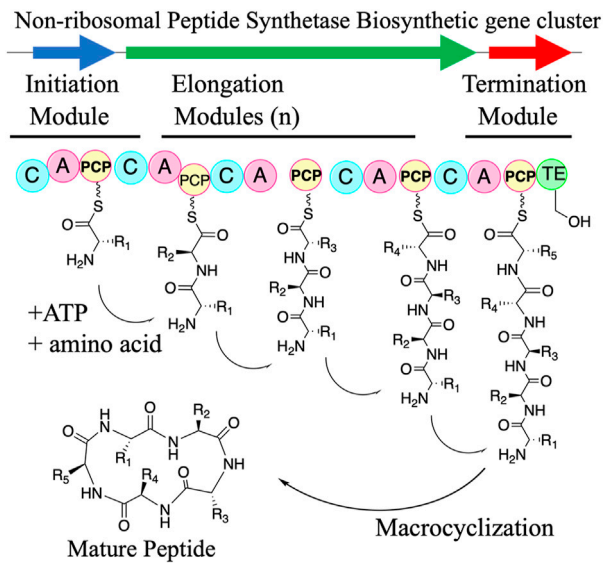


FIGURE 1
Non-ribosomal peptide synthetase (NRPS) biosynthetic logic.

and ultimately cleaved to form a small molecule. While RiPPs are an exciting class of natural products that have been the subject of recent synthetic biology efforts [reviewed elsewhere (Arnison et al., 2013; Zhang et al., 2018; Montalbán-López et al., 2021; Ongpipattanakul et al., 2022; Zhong et al., 2023)], this class of peptide natural product biosynthesis is beyond the scope of this review. In contrast to the ribosomal machinery, NRPSs are synthesized by a large multienzyme complex, which consists of many individual biosynthetic modules in an assembly-line process (Figure 1).

Briefly, NRPSs are comprised of modules, wherein each module consists of a set of discrete enzymatic domains that are responsible for the one cycle of the chain extension in a colinear fashion (Bozhüyük et al., 2019; Beck et al., 2020; Lu et al., 2023; Xu et al., 2023). A typical NRPS module contains a minimum of three core domains: the adenylation (A) domain, the condensation (C) domain, and the peptidyl carrier protein (PCP) otherwise known as a thiolation (T) domain. An A domain recognizes and activates an amino acid monomer as an adenylate followed by acyl transfer to a PCP (or a T) domain along with optional domains such as an epimerase (E) domain, cyclization (Cy) domain, or oxidase (Ox) domain. The activated amino acid is loaded by the PCP domain on a 4′-phosphopantetheine (4′-Ppant) arm which is then condensed via the C-domain. Chain release typically occurs by hydrolysis or macrocyclization via a thioesterase (TE) (Figure 1) (Felnagle et al., 2008; Miller and Gulick, 2016; Wenski et al., 2022) with other termination mechanisms also sometimes occurring such as reduction to the primary alcohol via a reductase. (Barajas et al., 2015; Heinemann et al., 2023). NRPSs must be activated by posttranslationally modifying the PCP domains with a phosphopantetheinyl transferase which provides a tether for the nascent peptide. Due to this colinear relationship between domain organization and metabolite structure, there has been extensive research focused on engineering pipeline of chimeric NRPS pathways including altering domain selectivity motifs (Koryakina et al., 2017; Drufva et al., 2020) or domain swapping (Khosla and Zawada, 1996; Kries et al., 2015; Sun et al., 2015; Zhang et al., 2015). For instance, existing antibiotic chemical scaffolds can be modified via engineered biosynthesis to generate analogs that have more targeted or improved activities and/or combat antibiotic-resistance.

In addition to bioengineering applications, strategies for heterologous expression are necessary to discover new peptide natural product scaffolds and to access adequate amounts for testing of bioactivity. The discovery of novel peptide natural products is hampered by low productivity or no productivity in a laboratory context (often called “cryptic” or “silent” biosynthetic gene clusters (Zarins-Tutt et al., 2016). Because not all of these bioactivities arise from organisms that are tractable to grow in the lab (Stewart, 2012) or produce natural products in high abundance, and because the structural complexity of peptide natural products means chemical synthesis is often non-trivial, the heterologous expression remains a valuable option for characterization and scaleup (Dias et al., 2012). For these reasons, dedicated campaigns have been pursued to generate complex natural products in more tractable heterologous hosts (Li et al., 2021). Heterologous hosts must be genetically manipulatable and generally contain both sufficient fluxes of metabolic precursors as well as an appropriate environment for protein folding and expression (Lawson et al., 2019). Challenges particularly associated with non-ribosomal peptide yields in cells include being compromised by the metabolic burden inhibiting host cell growth, the cytotoxicity of their products, misfolding, and the unavailability of necessary precursors in heterologous hosts (Li et al., 2018; Ji et al., 2022). As NRPSs are large, complex multi-domain proteins that are held together via flexible linkers (Farag et al., 2019), they are particularly prone to expression and solubility challenges and/or premature truncation, which is of particular importance when selecting appropriate transcriptional and translational machinery. These complexities result in an unwieldy number of parameters that can be varied when selecting an appropriate expression and screening strategy.

Cell-free protein synthesis (CFPS) system has emerged as a strategy to prototype expression as well as undergo biomanufacturing strategies that have limitations in cellular contexts for reasons like toxicity (Carlson et al., 2012; Hodgman and Jewett, 2012). CFPS systems are often divided into two main approaches, which are crude cell lysates/extracts supplemented with appropriate precursors and cofactor recycling systems and the *in vitro* TX-TL PURE system. The crude cell lysates are generated from cell growth and then are supplemented with cofactors and other necessary biological components for *in vitro* transcription and translation (TX-TL). Whereas TX-TL PURE system or PURExpress (Shimizu et al., 2001; Tuckey et al., 2014; Lavickova and Maerkl, 2019; Cui et al., 2022) is a system that has taken the minimal recombinant elements required for cell-free expression and is thus less complex than lysate-based systems. The lysate-based system has the advantage over the PURExpress system in that it allows for accessory genes (e.g., ones relevant to posttranslational modification) as well as chaperonins. However, it has the disadvantage that it is more complex and has the potential for more sources of background that can interfere with signal output and/or cofactor recycling (Wick et al., 2019; Wick and Carr, 2022). The lack of cellular survival objectives bypasses challenges with *in vivo* expression, and thus the protein expression process is shortened

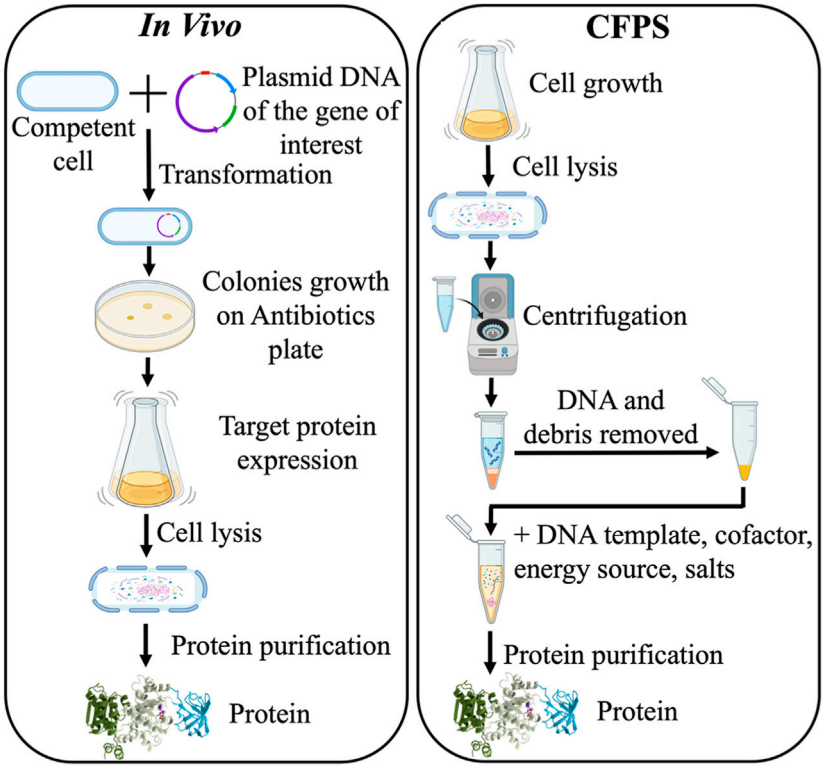


FIGURE 2
Comparison of *in vivo* recombinant DNA protein expression and cell-free protein synthesis (CFPS) methods.

significantly (from 1 to 2 weeks in living cells to 1–2 days) (Figure 2) (Finking et al., 2002; Bogart et al., 2021; Ji et al., 2022).

Developing high-throughput profiling of NRPS genes *in vitro* platforms such as cell-free expression (CFE) systems (Li et al., 2018; Bogart et al., 2021) that include both lysate-based systems and the PURExpress system can accelerate synthetic biology research. In addition to the advantages in terms of production timeframes, cell-free affords the ability to decouple limitations that exist within cell-based systems including rapidly increasing DNA concentration as well as the introduction of non-natural components like RNA polymerases, and noncanonical amino acids, all without requiring extensive cell engineering (Finking et al., 2002; Gregorio et al., 2019; Banerjee et al., 2020). Thus, this is additionally appealing for NRPS characterization and engineering because it can accelerate design-build-test-learn (DBTL) cycles. To these ends, NRPSs have recently been expressed in both lysate-based (Goering et al., 2017; Zhuang et al., 2020; Moore et al., 2021; Dinglasan et al., 2023) and PURExpress (Siebels et al., 2020a) cell-free systems. This review highlights examples of NRPS natural products that are expressed in CFE systems in synergy with different transcriptional and translational machineries. These foundational studies can be built upon to develop more sophisticated pipelines for NRPS prototyping.

## 2 Production of NRPS in CFE

While several classes of proteins have been generated using CFPS, CFPS systems are typically optimized and developed via applying tractable and detectable reporters, predominantly GFP and other fluorescent proteins like mScarlett (Martin et al., 2018; Gregorio et al., 2019; Garenne et al., 2021). NRPSs pose more demands to a cell-free system than simple fluorescent protein reporters due to their large size, complex multidomain architecture, posttranslational modification, and cofactor requirements (e.g., ATP) and thus the efforts to express NRPS enzymes and produce non-ribosomal peptide metabolites in cell-free are somewhat limited. To date, all detection of metabolite formation from an NRPS (as opposed to the expression of the NRPS protein only) in cell-free has been performed with *Escherichia coli* transcriptional and translational machinery, both in lysate-based systems as well as the PURExpress system (Table 1).

### 2.1 Cyclic dipeptide, D-Phe-L-Pro diketopiperazine (DKP)

Jewett and co-workers initially demonstrated the application of the lysate-based cell-free system to synthesize a cyclic dipeptide D-Phe-L-Pro diketopiperazine (DKP) (Prasad, 1995). DKP is a naturally occurring shunt product of early modules of the antibiotic gramicidin S biosynthetic pathway (Kurotsu et al., 1991). DKP is used as a model reporter because it previously has been used for a variety of domain and module experiment purposes including domain swapping studies (Kries et al., 2015), characterization of NRPS domain activity (Stanišić et al., 2021), and enzymatic redesign (Lilien et al., 2005). The antibiotic

TABLE 1 Examples of NRPS expression in CFPS systems.

| Year(s) | NRPS gene(s) | Non-ribosomal peptide metabolite(s) | TX/TL strategy | Metabolite Yield(s) | Protein Yield(s) | Reference(s) |
|---|---|---|---|---|---|---|
| 2017 | GrsA and GrsB1from *Brevibacillus brevis* | D-Phe-l-Pro Diketopiperazine (DKP), shunt product of Gramicidin S | *E.* coli BL21 Star (DE3) lysate | ~12 mg/L | GrsA ~106 μg/mL<br>GrsB1 ~77 μg/mL | Goering et al. (2017) |
| 2020 | GrsA from *Brevibacillus brevis* | Gramicidin S | PURExpress | Not generated | Not quantiated | Siebels et al. (2020b) |
| 2020 | Vlm1 and Vlm2 from *Streptomyces tsusimaensis* | Valinomycin | *E.* coli BL21 Star (DE3) lysate and cell free protein synthesis-metabolic engineering (CFPS-ME) | ~37 μg/L | Not quantitated | Zhuang et al. (2020) |
| 2020 | BpsA from *Streptomyces lavenduale* | Indigiodine | PURExpress | ~62 μg/mL | BpsA: ~28.7 μg/mL | Siebels et al. (2020b) |
| 2023 | BpsA from *Streptomyces lavenduale* | Indigiodine | *E.* coli BL21 Star (DE3) lysate | ~223 μg/mL | BpsA: ~95 μg/mL | Dinglasan et al. (2023) |
| 2020 | KJ12A, KJ12B, KJ12C from *Xenorhabdus* KJ12.A | RXP (rhabdopeptide-like peptides) | PURExpress | Not quantitated | Not quantitated | Siebels et al. (2020b) |
| 2020 | IndC from *Photorhabdus luminescens* | Indigiodine | PURExpress | Not generated | Not quantitated | Siebels et al. (2020b) |
| 2020 | TycB from *Bacillus brevis* | Tyrocidine | PURExpress | Not generated | Not quantitated | Siebels et al. (2020b) |
| 2023 | TycA from *Bacillus brevis* | Tyrocidine | *E.* coli BL21 Star (DE3) lysate | Not generated | Not quantitated | Dinglasan et al. (2023) |
| 2023 | Pys from *Pseudomonas entomophilia* | Pyreudione | *E.* coli BL21 Star (DE3) lysate | Not generated | Not quantitated | Dinglasan et al. (2023) |
| 2021 | TxtA and TxtB from *Streptomyces scabiei* | Thaxtomin A | *Streptomyces venezuelae* ATCC 10712 lysate | Not generated | Not quantitated | Moore et al. (2021) |
| 2021 | NH08_RS0107360 from *Streptomyces rimosus* | Uncharacterized | *Streptomyces venezuelae* ATCC 10712 lysate | Not generated | Not quantitated | Moore et al. (2021) |

gramicidin S is produced by Brevibacillus brevis via the biosynthetic pathway of two NRPS enzymes GrsA (126 kDa, one module) and GrsB (510 kDa, four modules) (43,44). Previous reports have shown that D-Phe-l-Pro diketopiperazine can be produced via GrsA and GrsB1 in isolation (Gruenewald et al., 2004). The substrates of the first two (of five) NRPS modules of the gramicidin S pathway, GrsA, and GrsB1 (the first module of GrsB, 121 kDa), go through the spontaneous intramolecular cyclization process to form DKP (Figure 3A) (Kurotsu et al., 1991; Stachelhaus et al., 1998). GrsA converts L-phenylalanine to D-phenylalanine whereas GrsB1 creates a peptide bond between D-phenylalanine and l-proline.

As an initial step to establish an *in vitro* platform for D-Phe-L-Pro DKP biosynthesis, GrsA, and GrsB1 were expressed separately in separate lysate CFE reactions (Goering et al., 2017). *E. coli* BL21 Star (DE3) lysate was applied towards 20-h batch reactions resulting in full-length, soluble GrsA and GrsB1 in yields of ~106 μg/mL and ~77 μg/mL, respectively. To ensure adequate phophopantetheinylation, the commonly used premiscuous PPTase, Sfp from *Bacillus subtilis* was used and Bodipy-CoA was applied as a fluorescent labeling reagent for high throughput screening of posttranslational modification. Subsequently, instead of expressing GrsA and GrsB1 separately, these two enzymes were co-expressed in a single-pot mixture to biosynthesize D-Phe-L-Pro DKP. This process allows *in vitro* reconstitution of the partial NRPS assembly line for *in situ* product formation. By using the synthetic DKP as a reference, the target product D-Phe-L-Pro DKP with the correct D-l stereochemistry was verified by the LC-MS/MS analysis. Previously, the yield of DKP from cell-based recombinant protein expression in *E. coli* was reported at 9 mg/L (Gruenewald et al., 2004), which is lower compared to the cell-free production system even without optimization (up to 12 mg/L). This work was the first proof of concept that an NRPS could be expressed in a cell-free system.

## 2.2 Cyclododecadepsipeptide valinomycin

Subsequent to the success of expressing the dipeptide DKP *in vitro* (Goering et al., 2017), the Jewett laboratory sought to investigate a total biosynthesis of a natural product instead of a small model shunt product (Zhuang et al., 2020). Their selected target, valinomycin, is a 36-membered cyclododecadepsipeptide has a wide range of bioactivities including insecticidal (Heisey et al., 1988), antifungal (Park et al., 2008), antiviral (Wu et al., 2004),

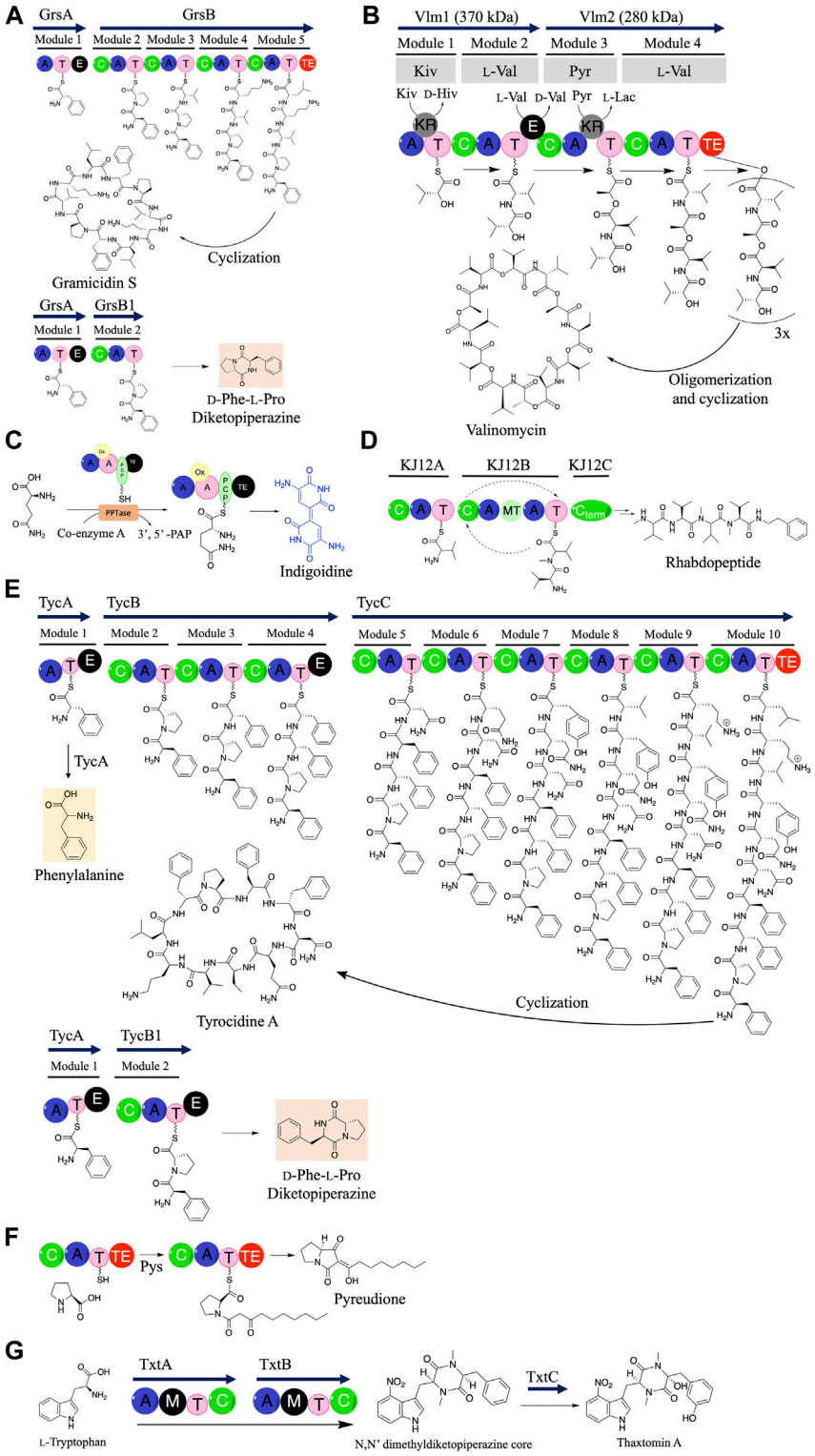


FIGURE 3
Biosynthesis of non-ribosomal peptide metabolites expressed in CFE systems. **(A)** Gramicidin NRPS. **(B)** Valinomycin NRPS. **(C)** Blue Pigment Synthetase A (BpsA). **(D)** Rhabdopeptides NRPS. **(E)** Tyrocidine A NRPS. **(F)** Pyreudione NRPS. **(G)** Thxatomin A NRPS.

anticancer efficacy (Ryoo et al., 2006), and antimicrobial (Tempelaars et al., 2011) and is naturally synthesized by various *Streptomyces* strains (Cheng, 2006; Magarvey et al., 2006; Matter et al., 2009). The biosynthetic pathway of valinomycin consists of Vlm1 (two modules, 370 kDa) and Vlm2 (two modules, 284 kDa). In the first module, the A domain activates α-ketoisovalerate (Kiv), which is subsequently reduced to D-2-hydroxyisovalerate (D-Hiv) by a ketoreductase (KR) domain. In the second module, L-Val is

activated and converted to D-Val by the A and the epimerase (E) domain. Similarly, in module 3, another KR domain reduces activated Pyruvate (Pyr) to L-Lactate; and L-Val is activated in module 4. Three tetradepsipeptide basic units of D-α-Hiv-D-Val-L-Lac-L-Val from the four modules of valinomycin synthetase oligomerized and macrolactonized by a TE domain to form valinomycin (Figure 3B) (Huguenin-Dezot et al., 2019). Valinomycin previously was expressed heterologously in *E. coli in vivo* by reconstitution of the NRPS genes (*vlm1* and *vlm2*) with the yield of 1 mg/L after a systematic bioprocess optimization (Li et al., 2015a; 2014; Jaitzig et al., 2014). As an initial step, the *E. coli* lysate-based CFPS system was used to co-express two enzymes Vlm1 (370 kDa) and Vlm2 (284 kDa) for valinomycin synthesis by adding two individual plasmid DNA templates harboring the genes vlm1 and vlm2 in CFPS reactions full-length and soluble in The expression of both proteins Vlm1 and Vlm2 was successfully achieved separately in a single-pot cell-free reaction by using the cell extracts prepared from *E. coli* BL21 Star (DE3). Sfp was once again utilized for the phosphopantetheinylation to activate to the active-holo form. To avoid the necessity for purification, the *sfp* gene was directly added with Vlm1 and Vlm2 to co-express in a one-pot reaction. The successful co-expression had a low yield of ~9.8 μg/L. To further improve the biosynthetic strategy, a type II thioesterase (TE-II) was employed to regenerate the activity of Vlm1 and Vlm2 which presumably edits via hydrolyzing stalled intermediates (Li et al., 2015b). As expected, TE-II improved valinomycin biosynthesis in a one-pot CFPS reaction of all four proteins (Vlm1, Vlm2, TE-II, and Sfp) with the final yield reaching ~37 μg/L. Taken together, this work is the first example of using an E. coli-based CFPS system to express a whole natural product gene cluster of valinomycin (>19 kb), which consists of two NRPSs (Vlm1 and Vlm2), a heterologous modification protein (Sfp), and an associated editing enzyme (TE-II). Even though this work was a significant advance in the field, the yields of valinomycin in the previous work of *in vivo* production in *E. coli* remained about three orders of magnitude higher than in CFPS reactions (Li et al., 2015b).

The co-expression of four enzymes including two large NRPSs (Vlm1 and Vlm2) together with the extended reaction duration was posited to limit resources such as energy, which was potentially responsible for the low yield of valinomycin. To overcome this constraint, cell-free metabolic engineering (CFME) was applied (Kay and Jewett, 2015; Dudley et al., 2016; Karim and Jewett, 2016), which is a platform wherein proteins are maximally expressed in cells followed by subsequent enrichment of the lysate with these proteins. Vlm1 and Vlm2 were heterologously expressed in *E. coli* BL21 (DE3), and *E. coli* BAP1, which has the *sfp* gene integrated into its genome (Pfeifer et al., 2001). The two lysates that enriched Vlm1 and Vlm2 proteins were then mixed with other cell-free components to initiate *in vitro* valinomycin biosynthesis. However, the yield of valinomycin obtained was low (~5.5 μg/L), which led to attempts to further optimize the CFME system. Hence, the effect of supplemental cofactors such as CoA, NAD, and ATP and the mass ratio of two lysates was investigated. Valinomycin formation in CFME reactions was found best at ~76.9 μg/L without cofactor supplementation and the mass ratio of 3:1 (cell lysate-Vlm1: cell lysate-Vlm2). Once again, the TE-II was applied for yield improvement of valinomycin by using a two-phase biosynthesis CFPS-CFME (CFPS-ME) system. In the first reaction phase, TEII was expressed by CFPS, which could be used to initiate the activity of Vlm1 and Vlm2 during the second CFME phase. Compared to the initial CFME yield (~5.59 μg/L), this approach improved valinomycin biosynthesis significantly with a yield of ~29.3 mg/L, which is more than 5,200 times higher. This result suggests that 1) the upstream CFPS-expressed enzyme (TEII) can efficiently activate the downstream CFME reactions (Vlm1 and Vlm2) and 2) cheap, simple precursor glucose can be converted to valinomycin substrates (Pyruvate, ketoisovalerate, and L-Val) through the glycolysis in CFPS to robustly synthesize complex natural product. Taken together, this work demonstrates the feasibility of CFPS to generate larger, more complex proteins using more complex metabolic networks within cell lysate.

## 2.3 Indigoidine and rhabdopeptides

The ability to transcribe and translate genomic DNA directly in cell-free systems was explored by Gringiner and coworkers via applying the PURExpress system directly from a commercially available kit. Two examples of NRPS products were generated to demonstrate feasibility. The first was Blue Pigment Synthetase A (BpsA) from *Streptomyces lavendulae*, which generates the pigment indigoidine (Siebels et al., 2020a). BpsA is a monomodular NRPS, consisting of an A domain that incorporates L-glutamine and undergoes spontaneous cyclization. The product is released by the oxidation (Ox) domain and thioesterase (TE) domain. BpsA activity can be directly monitored as indigoidine absorbs strongly at 590 nm (Figure 3B). The promiscuous PPTase Sfp from *B. subtilis* was added to the PURE system to synthesize the full-length activated BpsA. When the protein was phosphopantetheinylated prior to BpsA synthesis in a sequential reaction, the yield was at ~28.7 μg/mL. However, when Sfp was added while the protein was synthesized in a parallel reaction, the yield decreased to ~15.5 μg/mL. The efficiency of phosphopantetheinylation of *in vitro* synthesized BpsA was determined by labeling it with the fluorescent CoA analog (CoA-647). Subsequently, phosphopantetheinylated BpsA was used to *in vitro* biosynthesize indigoidine with the final concentration of ATP, and L-glutamine was added directly into the reaction solution at 2.65 mM and 0.95 mM respectively. The catalytic differences between BpsA expressed in cell-free *versus in vivo* in the IVPS system suggest that the folding environment may not be comparable. However, because the exact formulation of the IVPS kit is proprietary, it is difficult to generate and test hypotheses that explain this disparity. To further probe the IVPS system, the RXP-synthesizing NRPS KJ12ABC from the bacterium *Xhenorhabdus* KJ12.1 was pursued as an example of a more complex target. KJ12ABC consists of three modules, both elongation modules KJ12A (encoding a C-A-T module, 137.8 kDa) and KJ12B (encoding a C-A/MT-T module, 181.5 kDa) which prefers valine as a substrate. The KJ12C domain (62.3 kDa) encodes an unusual C-terminus standalone domain that uses phenylethylamine (PEA) for peptide chain release (Figure 3D). These three modules were expressed recombinantly in *E. coli* prior to being assayed for product formation. With the molar ratio of 10:1.5:1 (KJ12A:B:C) for the modules, three tetrapeptides synthesized were mV-V-mV-mV-PEA, V-V-V-mV-PEA, and V-mV-V-mV-PEA

(N-methylated valine (mV), valine (V), and phenylethylamine (PEA). In addition to the examples where metabolites were generated, other proteins were expressed in the PURExpress system to test the capacity to generate large proteins from genomic DNA and were successfully labeled with the fluorescent Coa-647 analog. This includes IndC (a BpsA homolog from *Photorhabdus lumincens*), GrsA, and TysB1 from tyrocidine biosynthesis as several polyketide synthases which also use phosphopantetheinylated T domains. This work set the stage for future endeavors that use the PURExpress system for genome mining applications.

## 2.4 Monomodule NRPSs: BpsA, TycA, Pys

Despite the successful proof of concept that NRPS can be successfully expressed from the Jewett and Gringiner labs, our research group hypothesized that further optimization of the CFE conditions were likely necessary to further unlock the ability to generate NRPS in cell free systems (Jewett and Swartz, 2004; Vilkhovoy et al., 2018). Moreover, CFPS tends to use GFP as the initial protein for optimization (Jewett and Swartz, 2004); however, GFP differs substantially from biosynthetically relevant proteins such as NRPSs. For instance, the A domain uses ATP to generate an aminoacyl-AMP intermediate, which could potentially compete with CFPS components (e.g., amino acids, phosphoenolpyruvate (PEP), and magnesium ions). Additionally, with a large protein beyond the size of native *E. coli* proteins, premature truncation can be a concern (Goering et al., 2017; Hurst et al., 2017; Zhuang et al., 2020). Hence, our group examined different reaction conditions to probe the limitations of NRPS CFE (Dinglasan et al., 2023). Reporters were utilized, as many of the methods to evaluate full-length protein translation are low throughput (e.g., SDS-PAGE gels or proteomic evaluation via mass spectrometry) (Goering et al., 2017; Zhuang et al., 2020) This work applied a tetracysteine (TC) peptide tag expressed at the C-terminus of the expressed protein to investigate factors that limit full-length NRPS translation, and consequentially, metabolite production in an *E. coli* lysate-based cell-free system (Dinglasan et al., 2023) via an exogenously added organoarsenic dye that binds to the TC tag to form a fluorescent complex, which was previously established in the PURExpress cell-free system (Wick et al., 2019).

As an initial step, BpsA-TC was expressed in *E. coli* BAP1 (a BL21 (DE3) derivative with a chromosomally integrated copy of *sfp* (Pfeifer et al., 2001)) lysates to validate that the TC tag does not abolish the catalytic function of NRPSs expressed in cell-free systems. The presence of the blue pigment indigoidine in BAP1 lysate reactions confirmed indicates full-length expression and catalytically functional BpsA-TC protein. Variant concentrations of the precursor l-glutamine, CoA, and plasmid DNA were tested, and to a maximum yield of ~900 μM indigoidine, which is roughly 3.6 fold higher than the yield reported in the PURE cell-free system (250 μM) (Siebels et al., 2020b). Next, a protocol for TC tag detection in lysate-based CFE reactions was established by using the organoarsenic FlAsH reagent. Detecting BpsA-TC in lysates using FlAsH-based opens possibilities to investigate and optimize parameters that could constrain TX/TL processes of NRPS proteins including catalytically active A domains that consume stoichiometric ATP, precursor availability (e.g., amino acids), and require cofactors like $Mg^{2+}$. To disentangle size from catalytic activity, a point mutant with an inactivated A domain was generated. Next, to probe the demands on the ATP pool, various concentrations of PEP and ATP were tested for BpsA-TC, BpsA-TC with an inactive A domain (BpsA E315A), and superfolder GFP (sfGFP). The results verified that the catalytically active A domain contributes to the additional catabolism of ATP, which causes higher energy substrate requirements for functional NRPS TX/TL compared to proteins of the same size.

In addition, magnesium ion concentrations were investigated because of their important role in almost all stages of TX/TL such as regulating the ionic strength of the lysate reaction environment and stabilizing nucleic acids and ribosomes. Once again, a difference in the expression trend was observed between the wild-type enzyme, BpsA E315A, and sfGFP implying that the active A domain impacts NRPS CFE $Mg^{2+}$ requirements. Another important parameter for NRPS protein synthesis concentration of amino acids which are both the monomers of translated proteins as well as the substrate of NRPSs. A range of different amino acids concentrations were tested using the optimal concentrations of PEP (77 mM) and $Mg^{2+}$ (18 mM) reaction mix for BpsA, BpsA E315A, and sfGFP. Interestingly, there are no significant differences in expression levels across testing conditions except a substantial decline at 8 mM for the wild type, which indicates the role of the A domain in disrupting this cofactor flux. To further investigate, 2 mM amino acid with increasing concentration of l-Gln was tested against BpsA-TC and BpsA-TC_E315A. The result showed that additional l-Gln in the reaction impairs the expression of both proteins. Altogether, optimal BpsA expression conditions were different compared to standard CFE conditions which were developed for small proteins like sfGFP.

After optimizing full-length NRPS expression by testing different CFE conditions with a C-terminus TC tag and the FlAsH dye, BpsA-TC, and indigoidine concentrations were identified to compare between reactions made with standard GFP optimized (containing 33 mM PEP and 12 mM $Mg^{2+}$) and BpsA optimized (containing 77 mM PEP and 18 mM $Mg^{2+}$) reaction mixes. Both BpsA-TC and indigoidine concentrations show improvement using the optimized mix condition. In addition, other monomodular NRPSs with similar architectures to BpsA also benefit from the total set of reaction conditions optimized. Two NRPSs were selected for testing proof of concept: pyreudione synthetase, Pys (142 kDa), which adenylates l-proline in the synthesis of amoebicidal pyreudiones (Figure 3E) and TycA (124 kDa), which incorporates and isomerizes an L-phenylalanine residue into the cyclic antibiotic decapeptide tyrocidine (Figure 3F). The improvement of expression of these two enzymes were even more dramatic than observed improvement in BpsA expression. Together, this work reveals the power of applying reporters for high throughput assaying of full-length NRPS expression and demonstrates the necessity of optimizing on systems beyond GFP.

# 3 Prospects for applying different transcriptional/translational machineries

CFPS systems have predominantly focused on *E. coli*, however, there are challenges with large and complex proteins like NRPSs that

result in *E. coli* not always being the most appropriate host machinery such as poor solubility, codon usage bias mismatch, and lack of appropriate posttranslational modifications. Therefore, there has been extensive research investigating alternative CFPS host organisms transcriptional/translational machineries, many of which may be more suitable for specific NRPSs. Expressing NRPS enzymes in the cell-free system has centered around *E. coli*-based systems (Goering et al., 2017; Siebels et al., 2020b; Zhuang et al., 2020; Dinglasan et al., 2023). To date, the only alternate cell-free system for NRPS expression has been *Streptomyces venezuelae* (Moore et al., 2021). Other transcriptional/translational machineries have the potential to greatly contribute to expanding the toolkit to generate NRPSs from a variety of metabolically gifted organisms which are discussed below.

## 3.1 Gram-positive hosts

### 3.1.1 *Streptomyces* spp.

*Streptomyces* spp. and other closely related Actinobacteria are known for their gifted secondary metabolism and are the source of numerous clinically relevant NRPS-derived natural products including daptomycin (Debono et al., 1988; Miao et al., 2005; Gu et al., 2007), bleomycin (Shen et al., 2002), and vancomycin (van Wageningen et al., 1998; van Wageningen et al., 1998; Choroba et al., 2000; Choroba et al., 2000; Recktenwald et al., 2002). *Streptomyces* sp. harbor genomes known for their high guanine and cystosine (G + C) content (ranging between 67% and 72%) (Sharma, 1999; Kieser et al., 2000; Drufva et al., 2022) and utilize a myriad of complex genetic regulatory mechanisms (Zhou et al., 2020) which can cause translational issues in tractable hosts such as *E. coli* (Hwang et al., 2021; Sword et al., 2023). This combined with relatively cumbersome genetics, typically requiring intergenic conjugation (Mazodier et al., 1989; Flett et al., 1997) or protoplast preparation (Xu et al., 2020; Kieser et al.), has motivated the development of *Streptomyces*-based CFPS for rapid prototyping (Moore et al., 2023). Currently, the *Streptomyces*-based CFPS system is the most prevalent non-*E. coli* CFPS platform developed with bacterial natural product biosynthetic machinery in mind (Moore et al., 2021; 2017; Li et al., 2017; Toh et al., 2021; Xu et al., 2022b; 2022a; Moore et al., 2023). In the 1980s, initial efforts were made to develop a *Streptomyces* CFPS platform as a tool for biological investigation (Thompson et al., 1984). With the significant rise in fundamental understanding of *Streptomyces* biology over the past half century, Jewett and others sought to develop a high-yielding *Streptomyces* CFPS protocol in 2017 (Li et al., 2017). An optimized *Streptomyces lividans* system using the reporter-enhanced green fluorescent protein (eGFP) was generated (Li et al., 2017), with an initial yield of ~45 μg/mL in batch reactions using the cell-free optimized vector pJL1-eGFP (Li et al., 2017). Additionally, other commonly used *Streptomyces* strains were tested to this CFPS system, which includes *S. lividans* B-122, *S. lividans* 66, *S. coelicolor* ISP-5233, and *S. coelicolor* M1152 to produce eGFP. Results showed that the *S. lividans* strains produced more eGFP than the *S. coelicolor* strains, and *S. lividans* B-1227 has a faster protein synthesis rate compared to the *S. lividans* 66. Importantly, they validated the effectiveness of using a *Streptomyces*-based CFPS system by expressing high G-C content genes originating from *Streptomyces*; these included *tbrP* (72% GC), *tbrQ* (78% GC), and *tbrN* (75% GC) which are tailoring genes in the biosynthesis of the non-ribosomal petide tambromycin (Goering et al., 2016) and the type II thioesterase (TEII, 64% GC) gene that is from the valinomycin BGC of *S. tsusimaensis*. (Li et al., 2015b). As expected, the results showed the solubility of these proteins using the *Streptomyces*-based CFPS system is higher compared to *E. coli*-based platforms despite having a lower titer (Li et al., 2015b). This implied the benefit of using a *Streptomyces*-based CFPS for NRPS cell-free expression but also the necessity for further optimization. By optimizing translation-related factors and creating a toolkit of promoter and ribosomal binding site libraries achieving the highest to-date reading of eGFP value of 515.7 ± 25.3 μg/mL (Xu et al., 2020; 2022b).

Another second prominent effort developed by Freemont, Moore, and others *Streptomyces*-based CFPS platform utilizes a *S. venezuelae* transcription-translation (TX-TL) system (Moore et al., 2017), achieving a yield of up to ~1.3 μM sfGFP by optimizing the cell-extract preparation process (Moore et al., 2017; Xu et al., 2020). Subsequently, other parameters of this system were screened for the improvement of protein synthesis, which includes a promoter, minimal energy solution, ATP source, and RNase inhibitor (Moore et al., 2021). Notably, the strongest promoter observed was SP44, with 2.2 times more active than *kasOp**, the initial promoter (Moore et al., 2021). This system was then used to test for the expression of high G-C content genes, which includes the oxytetracycline enzymes (OxyA, -B, -C, -D, -J, -K, -N, and -T) from *Streptomyces rimosus*, the thaxtomin A biosynthesis from *Streptomyces scabiei* (TxtA and TxtB), and an uncharacterized NRPS (NH08_RS0107360) from *S. rimosus*. Most of these enzymes were successfully expressed, which proves the robustness of expressing high G-C genes using this platform. *Streptomyces*-based CFPS systems were developed with the hope of overcoming the challenges that were faced when expressing native *Streptomyces* genes in *E. coli* CFPS, which included solubility issues, lack of post-translational modifications, codon bias, and complexity (Moore et al., 2021; Ji et al., 2022). Evidently, *Streptomyces*-based CFPS systems have proven to be more suitable as CFPS hosts for NRPSs than *E. coli*, (Li et al., 2017), especially considering many NRPSs with high GC-content genes, are derived from *Streptomyces* (Ji et al., 2022). Even though there is limited research expressing NRPS proteins using *Streptomyces*-based CFPS systems, further development, and optimization will undoubtedly accelerate natural products research.

### 3.1.2 *Bacillus* spp.

*Bacillus subtilis* is a robust and efficient heterologous host that is generally used industrially for heterologous expression (Yang et al., 2021; Ejaz et al., 2022). A number of canonical NRPS pathways come from *B. subtilis* and other closely related Firmicutes including lipopeptides like surfactin (Sen, 2010), polymyxins (Komura and Kurahashi, 1985; Choi et al., 2009; Kim et al., 2015), bacillaene (Butcher et al., 2007), and plipastatin (Gao et al., 2018). Early reports of efforts to develop *B. subtilis* CFPS faced challenges due to the requirement for exogenous mRNA, protease inhibitors, DNAse treatments, or less efficient energy systems that discouraged the development of this platform (Kelwick et al., 2016). The more recent development of the *B. subtilis* cell-free transcription-translation

platform was overcoming these shortfalls by adapting optimization techniques from the *E. coli* cell-free system (Kelwick et al., 2016). Initial efforts focused on *B. subtilis* 168, a strain that is highly characterized and heavily applied as a laboratory host which achieved a low yield of 0.011 μM GFPmut3b (a variant of GFP that is more fluorescent than wild type (Cormack et al., 1996) but not under patent protection like superfolder GFP) (Kelwick et al., 2016). It was hypothesized that *B. subtilis* 168 was not quite suitable for a cell-free transcription-translation reaction due to the presence of endogenously expressed proteases and a resultant degradation of translated proteins (Kelwick et al., 2016). Next, efforts were applied to a protease-deficient strain *B. subtilis*, WB800N which was found to result in ~72-fold improvement in protein yield (up to 0.8 μM GFPmut3b (Kelwick et al., 2016). Despite improved expression, overall yields remain lower than those observed in *E. coli* cell-free transcription-translation systems (Kelwick et al., 2016). A similar *B. subtilis* 168 cell-free system was successfully used to diagnose the bottleneck of the N-acetylneuraminic acid (NeuAc) biosynthesis pathway in *B. subtilis in vivo* by finding the limiting factors through *in vitro* analysis (Tian et al., 2020). Subsequently, an attempt was made to compose a standardized approach to develop a cell-free platform for poorly characterized non-model organisms termed native cell-free (NCF) transcription–translation with *Bacillus* spp. (Moore et al., 2018). The method was applied to *B. megaterium* DSM319, a far less characterized *Bacillus* spp. (Moore et al., 2018). The *Bacillus megaterium* NCF system, however, is further proof of concept that NCF can be applied to non-model organisms (Moore et al., 2018).

## 3.2 Other gram-negative hosts

### 3.2.1 Vibrio natriegens

Fast-growing organisms are of special interest as one of the main benefits of CFPS is acceleration of the timeframe of the DBTL cycle. *Vibrio natriegens*, a Gram-negative marine bacterium, is known for having the fastest growth rate of any non-pathogenic organism with a reported doubling time of less than 10 min (Lee et al., 2016; Weinstock et al., 2016; Fernández-Llamosas et al., 2017). Genetic engineering tools for *Vibrio natriegens* have been widely developed and manipulated for heterologous recombinant protein expression. (Weinstock et al., 2016; Hoffart et al., 2017). *Vibrio natrigens* can grow rapidly under a range of media conditions (including minimal media supplemented with various carbon sources) and under both aerobic and anaerobic conditions meaning it is an extremely versatile host (Weinstock et al., 2016; Hoffart et al., 2017). The extraordinary growth rate of *V. natriegens* is typically attributed to its high number of ribosomes per cell. (Aiyar et al., 2002). Furthermore, *V. natriegens* also contains more rRNA operons compared to *E. coli* (Des Soye et al., 2018).

In recent years several groups have focused on optimizing the *V. natriegens* as a CFPS platform (Des Soye et al., 2018; Failmezger et al., 2018; Wiegand et al., 2018; Zhu et al., 2020). Of these studies, the reporter protein GFP has been applied and the highest yield achieved was 1.6 ± 0.05 g/L using the *Vnat* cell-free platform, which is competitive with existing *E. coli* CFPS platforms (Des Soye et al., 2018). Noticeably, the optimal incubation reaction temperature for maximum protein yield was found at 26°C and 30°C with extracts incubated at 30 °C produced 3 folds more protein compared to those at 37°C. The result supports the theory that the rapid growth of *V. natriegens* at higher temperatures is because of the larger number of ribosomes and not more efficient ribosomes (Wiegand et al., 2018). Additionally, the kinetic analysis showed that *V. natriegens* extracts sustained elevated protein expression rates over 60 min as compared to *E. coli* extracts sustained for 20 min (Wiegand et al., 2018). *Vibrio natriegens* CFPS demonstrated competitive yields with *E. coli* CFPS and observed higher sfGFP yields (~9.3 μM) than *Bacillus subtillis* (0.8 μM) and *S. venezuelae* (1.3 μM) (Wiegand et al., 2018). Noticeably, the *V. natriegens* CFPS was viable with linear templates, as opposed to plasmids which also accelerates prototyping (Zhu et al., 2020). Although *V. natriegens* CFPS platforms have not been applied to NRPS production, the robustness of *V. natriegens'* extracts in the CFPS platform means that it may be a viable alternative to *E. coli*, especially for NRPS from Proteobacteria.

### 3.2.2 *Pseudomonas* spp.

*Pseudomonas putida* is a Gram-negative bacterium, that often serves as a laboratory workhorse organism for its well-characterized genome, robust culture conditions, and catabolism of a broad range of carbon sources (Loeschcke and Thies, 2015). With a complete genome sequenced, numerous genetic tools have been developed to engineer *P. putida* (Wang et al., 2018). There are also multiple biosynthetic gene clusters including NRPSs harbored within *Pseudomonas* spp. (Ackerley et al., 2003; Gao et al., 2014; González et al., 2017) and *P. putida* has been shown to be a robust host for heterologous expression of biosynthetic gene clusters (Domröse et al., 2017; Cook et al., 2021). An initial effort was made for the *Pseudomonas* organism for CFPS using the lysate crude extract from *Pseudomonas fluorescens* in 2004 (Nakashima and Tamura, 2004). However, it faced challenges with the complicated cell extract preparation process, which led to low protein yield. Recently, an optimized CFPS method has been developed by the Jewett laboratory using the lysate crude extract of *P. putida* (Wang et al., 2018). Adopting the protocol used for the *E. coli* CFPS system (Jewett and Swartz, 2004; Li et al., 2016), cell extract from *P. putida* was harvested, giving a low yield for sfGFP. Thus, this system was then optimized by manipulating reaction conditions such as lysate concentration, plasmid concentration, reaction temperature, and $Mg^{2+}$ concentration. The yield achieved was ~200 μg/mL in 4h batch reactions, which is more than ten times the un-optimized yields. Even though the current yields are significantly lower than that of the *E. coli*-based CFPS system (>1,000 μg/mL), they are ten times higher than the *S. cerevisiae*-based CFPS system (<20 μg/mL) (Schoborg et al., 2016; Wang et al., 2018). While *Streptomyces*-based CFPS platforms have been established to express high GC genes, *P. putida* also has high GC (~60%) content. The process of harvesting crude cell extract from *P. putida* is less complex than for *Streptomyces* organisms as it does not have a clumpy mycelial physiology. (Loeschcke and Thies, 2015), which makes it a great potential for an alternative CFPS strategy with *Streptomyces*-based CFPS platforms to express GC-rich genes (Loeschcke and Thies, 2015). Developing *P. putida*-based CFPS platforms contributes to a future application for CFPS screening platforms and expands the protein synthesis toolkit for synthetic biology.

### 3.2.3 Cyanobacteria

Cyanobacteria are Gram-negative oxygenic photosynthetic prokaryotes (Laxmi et al., 2023) that are capable of using solar energy to synthesize organic carbons from atmospheric carbon dioxide, which makes them an ideal target for biofuel and biochemical research (Choi et al., 2020; Choi et al., 2021). Their genomes have been known for containing a plethora of NRPS, and other natural product genes, hence, they have been a popular target for genome mining (Ehrenreich et al., 2005; Barrios-Llerena et al., 2007; Choi et al., 2021; Laxmi et al., 2023). However, as heterologous hosts, they present challenges since they can be polyploid and are slow to appropriately segregate heterologous genes (Choi et al., 2021). For these reasons, developing a cell-free system using Cyanobacteria crude extract could accelerate DBTL cycles in a fashion analogously to other hosts that have slow genetic manipulation timelines like *Streptomyces sp*. Recently, a CRISPR/Cas12a-based assay coupled with a cell-free system was developed as a tool for prototyping promoters using model cyanobacteria *Synechocystis* sp. PCC 6803, which has the potential to be applicable to other host organisms (Choi et al., 2021). The study created a promoter library and was able to use the assay to confirm a positive correlation between *in vivo* and *in vivo* performance in cyanobacteria *Synechocystis* (Choi et al., 2021). This method can be useful for NRPS and other natural product gene expression in cyanobacteria by significantly shortening the workflow time.

## 3.3 Eukaryotic CFE systems

### 3.3.1 Saccharomyces cerevisiae

The most prominent eukaryotic expression system origin is *Saccharomyces cerivisae* (Yin et al., 2007). Importantly, there are many fungal NRPS pathways (Domröse et al., 2017; Zhang et al., 2023; 2022) that could be accelerated using *S. cerevisiae* cell-free systems. While cell-free systems for plant (Madin et al., 2000; Murota et al., 2011; Buntru et al., 2022; 2014; Buntru et al., 2021) and animal (Ezure et al., 2010; Makrydaki et al., 2021) based expression systems exist, these are out of the scope of this review, which focuses on peptide natural products from microorganisms. Notably, *Saccharomyces cerevisiae* is a great candidate to harness the benefit of both microbial-based and eukaryotic systems due to its fast growth characteristics (Salari and Salari, 2017). Furthermore, its capability of growing aerobically and anaerobically has contributed to its industrial success in food, brewing, biotechnology, and drug discovery (Parapouli et al., 2020). *Saccharomyces cerevisiae* has been extensively engineered as a heterologous host for the expression of numerous NRPSs *in vivo* such as β-lactam benzylpenicillin, beauvericin, bassianolide, indigoidine synthetase (BpsA), tyrocidine synthetase (TycA), and surfactin synthetase (SrfAC) (Tippelt and Nett, 2021). The ability of *S. cerevisiae* to heterologously express NRPSs *in vivo* suggests that it might be a great candidate for expressing NRPSs using *S. cerevisiae* CFPS systems. The most recent *S. cerevisiae* CFPS-based TX/TL studies achieved yields of 4.33 ± 0.37 μg/mL luciferase GFP (Hodgman and Jewett, 2014) and 17.0 ± 3.8 μg/mL sfGFP in a 10 h semi-continuous reaction (Schoborg et al., 2014). Although *S. cerevisiae* CFPS-based system has been successful when expressing reporters, attempts to express NRPS enzymes have not been performed yet. Optimizing conditions to express multidomain enzymes using *S. cerevisiae* CFPS-based platforms will open more doors to get access to "difficult-to-synthesize" proteins. Very recently, other fungal systems have been explored including *Neurospora crassa* and *Aspergillus niger* which was able to produce peroxygenases with activities reaching up to 105 U $L^{-1}$ (Schramm et al., 2022). Due to the success of *Aspergillus* sp. as heterologous hosts in recent years (Chiang et al., 2013; Anyaogu and Mortensen, 2015; Lin et al., 2023), these systems could have broad utility for NRPS expression.

### 3.3.2 Leishmania tarentolae

Another prominent CFPS system derived from eukaryotes developed by Alexandrov and coworkers harnesses the non-pathogenic single-celled flagellate *Leishmania tarentolae* (Mureev et al., 2009; Kovtun et al., 2011; Johnston and Alexandrov, 2014; Gagoski et al., 2015), which is inexpensive in bioreactors and has been successful in expressing a range of complex eukaryotic proteins (Mureev et al., 2009; Kovtun et al., 2010; Gagoski et al., 2017; Wu et al., 2022; Moradi et al., 2023). *Leishmania tarentolae* can be fermented on a large scale and its genetic modification process is not complicated (Kovtun et al., 2010). Additionally, many proto- and metazoan proteins were expressed by using *L. tarentolae* lysate (Kovtun et al., 2010). In particular, this system expressed proteins in *L. tarentolae* extract, and utilized Species-Independent Translation Initiation Sequence (SITS) allowing for a divergent range of applications (Kovtun et al., 2011). While the *L. tarentolae* system has not been applied to proteins from microbial secondary metabolism, NRPSs have been identified in some protozoan species, especially dinoflagellates such as *Symbodinium tricnidorium*, *Cladocopium* sp. (Beedessee et al., 2019) and *Ostreopsis ovata* (Yamazaki, 2016). The *Leishmania* system has been shown to be remarkably tolerant to complexity and has been shown to produce up to 300 ug/mL of recombinant protein in 2 h (Mureev et al., 2009). Furthermore, this system is suitable for high throughput screening as a result of more than 50 pure proteins obtained from the coding sequence manipulation process without cloning (Kovtun et al., 2011). This is suggestive its promise as a general host for NRPS discovery, but particularly for NRPS from understudied origins, as dinoflagellate biosynthetic gene clusters are less well characterized than bacterial or fungal ones.

# 4 Conclusion and future perspectives

With current technologies in genome sequencing and genome mining, a vast number of novel natural products have been discovered. Cell-free synthetic biology is a powerful platform that offers rapid, high-throughput, cost-effective, and robust approaches to access and harness these valuable natural products as well as generate analogues and apply the biosynthetic steps for bioproduct generation. CFPS accelerates the DBTL cycle the metabolic engineering. In this review, we focus on non-ribosomal peptide natural products that are expressed using cell-free strategies, different lysate host organisms used in CFPS (Table 1), and the potential of using CFPS to accelerate engineering non-ribosomal peptides and their analogs. The knowledge gap in using a universal platform with high yield, cost-effectiveness, inability, and scalable

CFPS systems to synthesize any complex proteins such as NRPS is still a bottleneck.

With the increasing tools available to control, modify, and optimize conditions for CFPS systems with various lysate hosts, the promise of CFPS as a fruitful platform for bioengineering efforts remains high. For instance, the unique characteristics of the flexible linkers holding NRPS domains and modules together draw significant interest in studying these NRPS parts such as module and domain swapping, module and domain deletions, and insertions (Kries et al., 2015; Winn et al., 2016; Beck et al., 2020). Different approaches have been developed to engineer the A domain (Kries et al., 2015); however, expressing mutants often leads to low production or no production at all (Winn et al., 2016). Binding domains or modules are substantially longer than subdomains, hence, subdomain swapping could greatly contribute to NRPS engineering based on gene synthesis and bioinformatics research. The subdomain swapping strategy was successfully done on the A domain of the GrsA biosynthesis of gramicidin S synthetase (Kries et al., 2015). Following, the production of the novel pyoverdine derivatives proved to be transferable to different NRPS systems at high yields by exchanging the A domain (Calcott et al., 2020; Messenger et al., 2023). Another noticeable example of domain swapping is the exchanging of the T domain of the NRPS IndC with synthetic and natural T domains shows improvement in indigoidine production (Beer et al., 2014). These are a few examples of domain engineering strategies, illuminating a promising approach to generating novel molecules as well as increasing natural product production.

Taking advantage of the flexibility of cell-free systems, exogenous elements can be rapidly added to the reaction directly and manipulated, allowing the synthesis of potential peptide natural products. In recent years, many efforts have investigated non-ribosomal peptide precursors derived from amino acids (Kurusu et al., 1987; Kajimura and Kaneda, 1996; Milne et al., 2006; Han et al., 2012; Thirlway et al., 2012). CFPS affords the ability to alter host machinery, regulatory elements and rapidly engineer chimeric NRPS pathways as well as express new NRPS machineries that come from intractable organisms. In a synergistic fashion, it also affords the incorporation of unnatural building blocks to increase molecular diversity as well as overcome cellular toxicity issues that arise from heterologous expression of bioactive metabolites. While CFPS remains an emergent technology for the production of secondary metabolites, advances in lysate systems with transcriptional and translational machineries that are more appropriate to NRPS expression and cofactor balance specific to this class of natural products will afford CFPS to be applied to NRPS enzymes of increasing complexity.

## Author contributions

TS: Conceptualization, Writing–original draft, Writing–review and editing. GA: Writing–original draft, Writing–review and editing. CB: Conceptualization, Funding acquisition, Resources, Supervision, Writing–original draft, Writing–review and editing.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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