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Multi-chassis engineering for heterologous production of microbial natural products

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Microbial genomes encode numerous biosynthetic gene clusters (BGCs) that may produce natural products with diverse applications in medicine, agriculture, the environment, and materials science. With the advent of genome sequencing and bioinformatics, heterologous expression of BGCs is of increasing interest in bioactive natural product (NP) discovery. However, this approach has had limited success because expression of BGCs relies heavily on the physiology of just a few commonly available host chassis. Expanding and diversifying the chassis portfolio for heterologous BGC expression may greatly increase the chances for successful NP production. In this review, we first discuss genetic and genome engineering technologies used to clone, modify, and transform BGCs into multiple strains and to engineer chassis strains. We then highlight studies that employed the multi-chassis approach successfully to optimize NP production, discover previously uncharacterized NPs, and better understand BGC function.

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Introduction

Microorganisms provide a variety of bioactive natural products (NPs) used in medicine and agriculture (e.g. antibiotics and pesticides), as well as in protection of the environment and development of materials [1–3]. In bacteria, NP biosynthesis pathways are encoded by biosynthetic gene clusters (BGCs), which are often found in a single large, contiguous genomic region. With the advent of genome sequencing [4], bioinformatics programs [5], and genetic/genome engineering technologies, BGCs can now be transferred from native strains to various heterologous hosts more easily and successfully. However, about 90% of these BGCs are inactive or only partially activated under standard fermentation conditions, and regulatory triggers required for full activation of the cryptic genes are typically unknown [6]. Therefore, many of the microorganisms' chemical innovations are untapped.

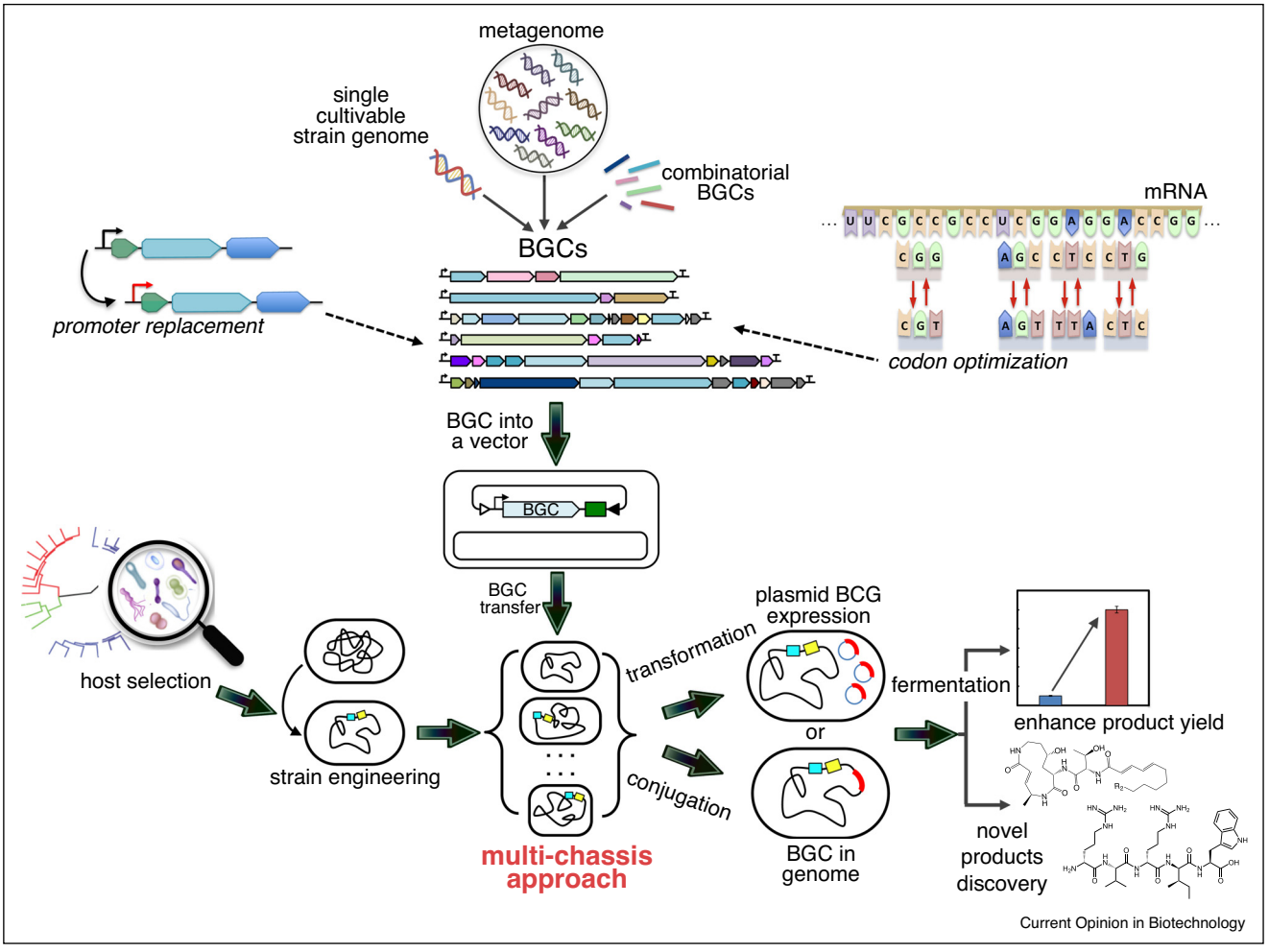
For functional expression of NPs, synthetic biology approaches such as codon optimization, refactoring, and/or DNA synthesis can theoretically be used to uncouple BGCs from their native regulatory constraints [7–10]. However, this approach requires carefully adapting the BGC to a species currently available for heterologous expression, such as *Escherichia coli*, *Bacillus subtilis*, or *Streptomyces lividans*. This approach also requires that all transferred genes be functionally expressed and all translated products fold properly and undergo appropriate post-translational modifications. The chassis strain itself must be able to supply all substrates and co-factors and tolerate all intermediates and products. Because of these complex requirements, BGC expression often fails to produce the corresponding product [11,12].

Clearly, the host organism is critical in heterologous production of NPs. However, research has primarily focused on a few model chassis strains as hosts. To demonstrate that exploring a wider range of chassis strains is worthwhile, this review describes engineering of non-conventional strains that has facilitated discovery of novel NPs by producing higher-than-native product yield. This review also highlights new tools and methodologies used to engineer diverse microbial strains to confirm the potential of the multi-chassis approach for heterologous production of NPs (Figure 1).

Genetic/genome engineering tools and techniques for heterologous NP production using multiple chassis

NP production is often regulated at multiple levels [13,14] that are difficult to determine. To facilitate robust expression of BGCs in diverse hosts, broadly applicable genetic parts, tools, and methodologies are important for functional BGC expression. In this section, we review those particularly important for multi-chassis approaches,

Figure 1



The general workflow of the multi-chassis engineering for heterologous expression of microbial natural products.

and all the disccused NPs and related approaches are summarized in Table 1.

Genome engineering techniques that enable the multi-chassis approach

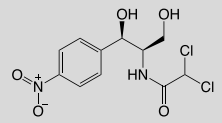
Several shuttle plasmids based on bacterial artificial chromosomes (BACs) have been developed [15–18], including some *E. coli-Streptomyces* shuttle BACs [19–21]. These shuttle BACs often contain an attP-int system from the *Streptomyces* phage [22], which catalyzes site-specific recombination of the phage attachment site (*attP*) with the bacterial attachment site (*attB*), forming two hybrid sites (*attL* and *attR*) [23,24]. Both ϕC31 and ϕBT1 attP-int loci have been well exploited to construct versatile vectors. These systems can integrate large BGCs of over 100 kb into the bacteria chromosome [23–27].

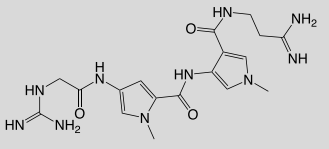
Use of integrative and conjugative elements (ICEs) is a similar approach. These modular mobile elements are integrated into chromosomes of firmicute species, mediated by the attP-int system [28,29]. ICEs undergo autonomous rolling-circle replication and are conjugally transferred between cell populations [30,31]. Brophy et al. recently developed a transposon mini-ICE from *B. subtilis* (ICEBs1) [32••]. This mini-ICEBs1 element achieved efficient transfer of large DNA constructs (~100 kb) into 35 firmicute strains isolated from humans and soil. While these attP-int systems are powerful tools to domesticate previously undomesticated strains, their host ranges are usually limited to a small group of bacteria. Integration efficiency also decreases exponentially as DNA construct size increases [32••].

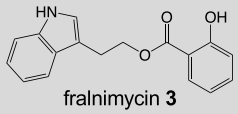
We recently developed 'chassis-independent recombinase-assisted genome engineering' (CRAGE), which can integrate large, complex BGCs into genomes of undomesticated strains with high accuracy and efficiency. A landing pad (LP) containing mutually exclusive *lox* sites is first

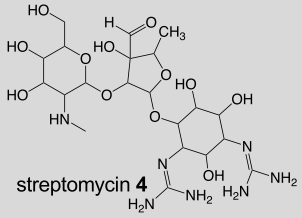
Table 1

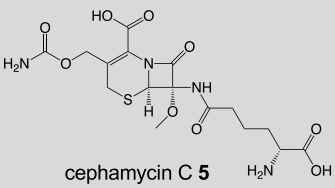
Summary of the heterologously produced natural products discussed in this paper

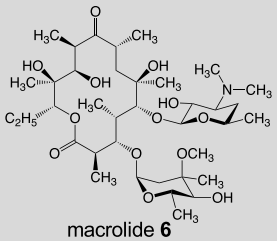

chloramphenicol **1**

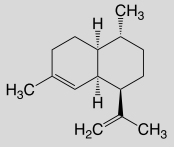

congocidine **2**

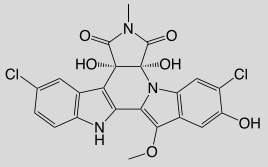

fralnimycin **3**

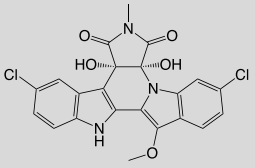

streptomycin **4**

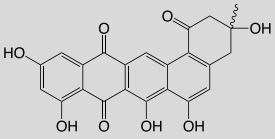

cephamycin C **5**

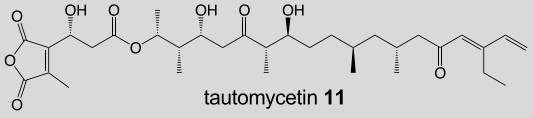

macrolide **6**

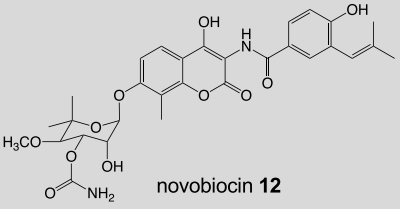

amorpha-4,11-diene **7**

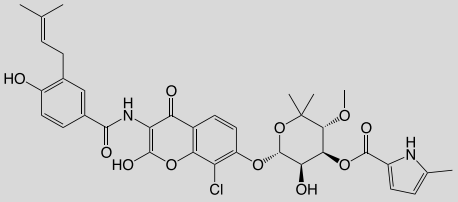

lazarimide A **8**

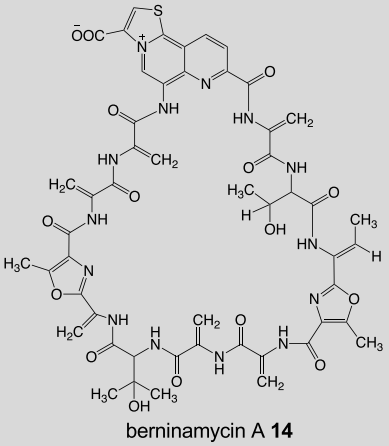

lazarimide B **9**

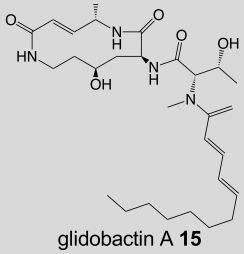

pentangular type II polyketide **10**

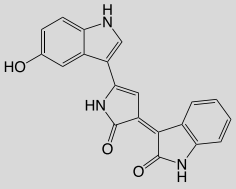

tautomycetin **11**

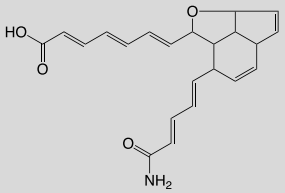

novobiocin **12**


clorobiocin **13**


berninamycin A **14**


glidobactin A **15**


violacein **16**


metatricycloene **17**

No	NP	BGC source	Heterologous host	Strategy	Comments	Ref.
1	Chloramphenicol	*S. venezuelae* ATCC 10712	*S. coelicolor* M145, M1146, M1152, M1154	Deletion of four BGCs competing for a common precursor, *rpoB* & *rpsL* mutations.	Point mutations to transcription (*rpoB*) and translation (*rpsl*) machinery in the host with quadruple BGC deletion increased productivity by 40-fold	[35]

Table 1 (*Continued*)

No	NP	BGC source	Heterologous host	Strategy	Comments	Ref.
2	Congocidine	*S. ambofaciens* ATCC23877	*S. coelicolor* M145, M1146, M1152, M1154	Deletion of four BGCs competing for a common precursor, *rpoB* & *rpsL* mutations	Point mutations to transcription (*rpoB*) and translation (*rpsl*) machinery in the host with quadruple BGC deletion increased productivity by 40-fold	[35]
3	Fralnimycin	*Frankia alni* ACN14a	*S. albus* J1074, Del14, M1152, M1154	Deletion of 15 BGCs	This approach improved compound detection limit	[37]
4	Streptomycin	*S. griseus* IFO 13350	*S. avermitilis*, *S. avermitilis* SUKA17	Reduced genome with deletion of 1.4 Mb non-essential genes.	Productivity higher than that of the native strain	[38]
5	Cephamycin C	*S. clavuligerus* ATCC 27064	*S. avermitilis*, *S. avermitilis* SUKA17	Reduced genome with deletion of 1.4 Mb non-essential genes. Expression of an extra copy of *ccaR*	Productivity higher than that of the native strain	[38]
6	Macrolide	*S. platensis* Mer-11107, 75 kb	*S. avermitilis, S. avermitilis SUKA17*	Reduced genome with deletion of 1.4 Mb non-essential genes. Expression of an extra copy of the regulatory gene *pldR*	PKS production at the industrial production level	[38]
7	Amorpha-4, 11-diene	*Artemisia annua*	*S. avermitilis, S. avermitilis* SUKA17	Reduced genome with deletion of 1.4 Mb non-essential genes. A synthetic gene optimized for *Streptomyces* codon usage	S. avermitilis could efficiently produce a plant terpenoid	[38]
8, **9**	lazarimide A and B	Environmental DNA	*S. albus*	Replaced native BGC promoter with bi-directional promoter-RBS cassettes	This approach can streamline the discovery of NPs from silent BGCs	[49]
10	Pentangular type II polyketide	*S. roseosporus* NRRL15998	*S. albus*, *S. lividans*, *S. roseosporus*, *S. venezuelae*, *S. viridochromogenes*	One-step CRISPR-Cas9 knock-in strategy to activate silent BGCs in five *Streptomyces* species	This polyketide was produced only in *S. viridochromogenes*; potentially a scalable approach facilitating novel NP discovery	[67••]
11	Tautomycetin	*S. spiroverticillatus*, 80 kb	*S. coelicolor* M145, *S. lividans* TK21, *Streptomyces* sp. CK4412, *S. coelicolor* TMC003	pSBAC was used along with cluster tandem repeat integration	*S. coelicolor* M145 produced >4-fold higher yield than that of the native strain	[21]
12	Novobiocin	novobiocin BGC	*S. coelicolor* M512,			[71]
13	Clorobiocin	clorobiocin BGC	*S. lividans* TK24, *S. spheroides* NCIMB 11891, *S. roseochromogenes var. oscitans* DS 12.976	λ-Red-mediated homologous recombination, phase attP-int system	While *S. coelicolor* M512 produced NPs comparable levels to their native hosts, *S. lividans* TK24 was ~5 times less productive	[71]
14	Berninamycin A	*S. bernensis* UC 5144	*S. lividans* TK24, *S. venezuelae* ATCC 10712	pSET152+bern plasmid, conjugative transfer	*S. lividans* produced this NP 2.4-fold greater than in the native species	[74]
15	Glidobactin A	*Photorhabdus luminescens* subsp. laumondii TTO1	25 of γ-proteobacteria chassis strains	CRAGE	This strategy enables integration of BGCs into chromosomes of diverse bacteria species	[33••]
16	Violacein	*Pseudoalteromonas luteoviolacea* 2ta16	*Pseudomonas putida* KT2440, *Agrobacterium tumefaciens* LBA4404, *E.coli*	TAR-cloning and heterologous BGC expression platform	A nonclustered LuxR-type quorum-sensing receptor from *P. luteoviolacea* 2ta16, PviR, was identified to increase pathway transcription and violacein production	[60]
17	Metatricycloene	Uncultured environmental bacteria	Diverse *Streptomyces* species, including *S. albus*	Optimized methods for constructing high quality metagenomic libraries in the best *Streptomyces* host	Multi-chassis approach improved the efficiency of novel NP discovery	[85]

integrated into chromosomes of recipient strains, mediated through a transposase. The BGCs, also flanked by mutually exclusive *lox* sites, are subsequently transferred into recipient strains via conjugation. With the activity of a Cre recombinase, we demonstrated integration of BGCs ranging from 10 to 48 kb with high efficiency [33••]. The utility of CRAGE, however, is currently limited to species within a few bacterial phyla including proteobacteria and actinobacteria. We are currently modifying the system to further extend the utility of CRAGE.

Genome-reduced strains used to improve heterologous NP production

Engineering chassis strains by deleting endogenous pathways competing for the same substrates can improve NP production and simplify NP identification [34]. For example, eliminating a few BGCs from *S. coelicolor* significantly enhanced production of **1** and **2** [35,36]. Myronovskyi *et al.* [37] expanded this approach and eliminated 15 BGCs from *S. albus* Del14, which led to enhanced production and discovery of compound **3**, which was previously uncharacterized.

Several studies have shown that strains with minimized genomes are useful NP production hosts. By deleting 1.4 Mb of nonessential *S. avermitilis* genes, Ikeda *et al.* [38] facilitated efficient production of **4**, **5**, **6**, and **7**. Choi *et al.* [39] used a similar approach with *Pseudomonas putida* to produce four proof-of-concept bioproducts (a protein, a polyketide, an isoprenoid, and an amino acid derivative). Baumgart *et al.* [40] created a series of genome-reduced *Corynebacterium glutamicum* strains. The final strain, with 412 genes (13.4% of the genome) deleted, showed wild-type-like growth behavior and tolerance of multiple stresses, such as oxygen limitation.

Notably, a variant of *E. coli* with a 4 Mb synthetic genome was created recently through high-fidelity convergent total synthesis [41]. Design included genome-wide replacement of synonymous codons (two sense codons and a stop codon). This synthetic *E. coli* with fewer synonymous sense codons may be easier to manipulate as a heterologous chassis for *in vivo* biosynthesis of non-canonical biopolymers [42].

Pathway refactoring and codon optimization based on established heterologous expression systems

Pathway refactoring refers to rewriting the genetic parts with defined function in a certain BGC for finer control [43,44]. BGCs can undergo modifications from simple promoter replacement to extensive swapping of genetic parts to optimize expression levels and increase production titers [44–47].

Replacing native BGC promoters with well-characterized promoters can streamline NP discovery from transcriptionally silent/cryptic BGCs. Diverse promoters with different expression characteristics are now available for this purpose [48•]. Montiel *et al.* [49] integrated bi-directional promoter-RBS cassettes into several BGCs, which activated rebeccamycin, tetarimycin, and lazarimide BGCs in *S. albus*. Promoter replacement also led to discovery of potent antiproliferative agents, **8** and **9** [49].

The efficiency and accuracy of codon usage is different for different processes and characteristics such as mRNA folding [50], gene expression [51,52], translatability [53], and co-translational folding and protein levels [50,54,55], as well as for fitness through RNA toxicity [56]. Rare codons potentially slow down the translation rate [57,58]. Translation is sometimes more efficient when genes are expressed in genetically distant strains [59,60]. Alternatively, translation efficiency can significantly improve when codons are optimized for the host organism [61,62]. Decreasing cost for de novo DNA synthesis makes codon-optimization an increasingly amenable strategy for increased heterologous NP production [63,64].

Pathway refactoring and codon optimization are therefore effective approaches, especially when BGC expression is largely controlled at the transcriptional and/or translational levels.

CRISPR-Cas9-mediated genome editing

CRISPR-Cas9 has rapidly become a promising genome-editing tool. Through sgRNA guidance, CRISPR-Cas9 cuts target double-strand DNA precisely 3 bp upstream of a specific protospacer adjacent motif (PAM). Subsequent repair of the break allows modification of the target locus. CRISPR-assisted targeted deletions can efficiently eliminate endogenous BGCs in hosts [65,66]. Additionally, CRISPR technology has enabled expression of multiple BGCs of different classes in five *Streptomyces* species and triggered production of unique metabolites, including a novel pentangular type II polyketide **10** in *Streptomyces viridochromogenes* [67••]. Furthermore, Jiang and Zhu used CRISPR-Cas9 in vitro and developed a new cloning method, Cas9-Assisted targeting of chromosome segments (CATCH) [68••]. From intact bacterial chromosomes, CATCH allows single-step targeted cloning of near-arbitrary genomic sequences of up to 100 kb [69].

Applying the multi-chassis approach — adapting a BGC to available chassis strains

Ideally, an expression host suite would include several species, perhaps including industrial strains that could produce NPs at very high levels [34]. Although uncertainty remains because of insufficient systematic analysis, in general NP researchers conclude that chassis strains closely related to a native BGC strain can efficiently activate the functions of the transferred BGCs. Regulatory elements that silence the BGC might not be conserved in these chassis strains, but other factors important

for BGC expression, such as promoters [12] and ribosome binding sites (RBSs) [70], may be similar to those of native strains. Although it is hard to predict specific host compatibility from phylogeny, parallel expression of NP constructs in multiple chassis strains provides a way to screen and optimize NP production levels and thereby facilitate discovery of novel NPs.

The multi-chassis approach helps optimize and enhance the titer of NPs produced heterologously

A versatile *E. coli-Streptomyces* shuttle BAC plasmid such as pSBAC was used along with cluster tandem integration to express an 80-kb tautomycetin (TMC) BGC from *S. spiroverticillatus* in several *Streptomyces* strains, including *Streptomyces coelicolor* M145 and *S. lividans* TK21 [21]. A tandem repeat of the TMC BGC was integrated into *S. coelicolor*. This strain produced 13.31 mg/L of **11**, a >4-fold higher yield than that of the native strain. Eustáquio *et al.* also demonstrated varied NP expression levels in different heterologous hosts. When the entire novobiocin and clorobiocin BGC was transformed into *S. coelicolor* M512 and *S. lividans* TK24, *S. coelicolor* M512 derivatives produced antibiotics in yields (31 mg/L **12** and 26 mg/L **13**, respectively) comparable to those of natural producer strains (35 mg/L **12** by *S. spheroides* and 25 mg/L **13** by *S. roseochromogenes*), whereas *S. lividans* TK24 derivatives were at least five times less productive [71].

Nah et al. listed about 90 actinomycetes NP BGCs that have been successfully expressed in various *Streptomyces* hosts [72••]. *S. coelicolor* and *S. lividans* were two competent strains for heterologous expression, thanks to their well-characterized genetic and biochemical properties. About 12% of the BGCs were expressed in another heterologous host, *S. albus*, which has fast growth and an efficient genetic system [73]. Compared with the original NP-producing strains, expression levels were higher for approximately 14% of the NPs and lower for 12% when they were expressed in the *Streptomyces* hosts. Production yield of the BGC of **14** in *S. lividans* was 2.4-fold greater than that in the native species of *S. bernensis*; there was no production in *S. venezuelae* [74]. In brief, species within the *Streptomyces* genus exhibit varied NP production yields from heterologous BGC expression, which further demonstrates the significance of the heterologous host screening and selection process for successful NP BGC expression.

A previous study by authors of this review demonstrated the efficacy of the multi-chassis approach [33••]. The same BGC was introduced in parallel into a phylogenetically diverse portfolio of 25 γ-proteobacteria strains using CRAGE [33••]. This approach substantially increased successful BGC expression. For example, the BGC for **15** (*plu1877–plu1881*) was inactive in its native strain even after homologous expression, suggesting strict regulation. In contrast, most of the strains harboring this BGC produced at least one of the three NPs previously identified. Interestingly, the production ratios of the three metabolites shifted dramatically (~10 000-fold) among the chassis strains. This result indicates that product specificity of BGCs can also change significantly, depending on the background physiology of each strain. Notably, the heterologous production titer of **15** reached as high as 177 mg/L, which is 700-fold higher than the production titer previously reported for the same BGC expressed heterologously in *E. coli* [75]. This suggests greater enhancement of NP production when BGCs are expressed in species more closely related to the native strain than in distantly related species.

However, some exceptions exist. Zhang et al. expressed the BGC of **16** from *Pseudoalteromonas luteoviolacea* 2ta16 and revealed robust production in two proteobacterial chassis strains, *P. putida* KT2440 and *Agrobacterium tumefaciens* LBA4404, but achieved very little production in various laboratory strains of *E. coli*, despite their closer phylogenetic relationship to the native strain than *A. tumefaciens*' relationship to the native strain [60]. This result suggests optimal heterologous NP producers exist among distantly related strains that can be identified only empirically.

The multi-chassis approach facilitates discovery of novel metabolites

Discovery of NPs through activation of putative BGCs often relies on suitable host strains providing yields sufficient for NP identification, purification, and NMR characterization [76]. In our previous study [33••], among nine heterologously expressed BGCs, eight previously uncharacterized NPs were identified from four BGCs. Notably, none of these newly detected NPs were detected in *E. coli*. An additional advantage of the multi-chassis strategy was also demonstrated. Untargeted searches for novel metabolites by LC–MS tend to yield high rates of false positives [77,78]. However, when the same pathway is expressed simultaneously in multiple hosts, we can use independent occurrence of the same feature under different physiological conditions to narrow down the search space considerably. This concept is analogous to FAC-MS scoring [79].

Cultivable microbes have historically been used to discover bioactive NPs. However, they make up as little as 1% of environmental microbiomes. Functional metagenomics screening has revealed the presence of large numbers of unexplored BGCs, suggesting the biosynthetic potential of the uncultured majority. A prerequisite for identifying NPs is successful expression of BGCs from metagenome libraries. Some attempts at metagenomic screening in *Streptomyces* using a single host have been proven insufficient [80–83,84•]. On the other hand, Brady *et al.* successfully used diverse *Streptomyces* species as chassis to improve metagenomic screening [85]. *S. albus*, in particular, exhibited the highest

propensity for heterologous expression of BGCs, leading to the discovery of **17**.

Guo *et al.* found a widely distributed family of NRPS gene clusters in human gut microbiomes [8]. By heterologously expressing a subset of these clusters in *E. coli* or *B. subtilis*, they showed the BGCs encoded biosynthesis of pyrazinones and dihydropyrazinones. Guo *et al.* subsequently demonstrated that the active form of these molecules is the peptide aldehyde, which bears potent protease inhibitory activity and selectively targets a subset of cathepsins in human cell proteomes [8]. Their findings show that an approach combining bioinformatics, synthetic biology, and heterologous BGC expression can rapidly expand knowledge of the metabolic potential of the microbiota while avoiding the challenges of cultivating fastidious commensals.

Rational design of novel NPs via combinatorial assembly of BGC modules [86,87] is another important area to which the multi-chassis approach can contribute, because the regulatory complexity of combinatorial BGCs requires appropriate expression hosts [88]. For example, through expression of combinatorial BGCs in two different hosts, Pérez *et al.* generated seven new mithramycin derivatives with antitumor activity against different tumor cell lines [89]. The expressing hosts were the wild type *S. argillaceus* and its mutant M7U1, with an inactivated *mtmU* gene coding a 4-ketoreductase involved in the biosynthesis of D-oliose [90]. *S. argillaceus* Δ*mtmU* produced twice as many compounds as did the wild type. Similar results may be readily obtained if the multi-chassis approach is employed for this BGC.

Concluding remarks and future perspectives

NPs and their derivatives continue to provide high value in medicine, agriculture, environmental protection, and materials development. The multi-chassis approach for heterologous BGC expression can facilitate improvement of NP production, discovery of NPs from diverse natural resources, and even rational design and engineering of bioactive 'unnatural' NPs. Continued technological and conceptual advances in strain engineering as well as genetic and genome editing techniques will open up opportunities to fully realize the multi-chassis approach and to explore and harness the immensely diverse chemical repertoire of nature.'

Conflict of interest statement

We filed a patent application for the method described in this manuscript.

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