Lawrence Berkeley National Laboratory
Recent Work

Title
Multi-chassis engineering for heterologous production of microbial natural products.

Permalink
https://escholarship.org/uc/item/9hh941xf

Authors
Ke, Jing
Yoshikuni, Yasuo

Publication Date
2020-04-01

DOI
10.1016/j.copbio.2019.09.005

Peer reviewed
Multi-chassis engineering for heterologous production of microbial natural products

Jing Ke\textsuperscript{1} and Yasuo Yoshikuni\textsuperscript{1,2,3,4,5}

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.

Addresses
\textsuperscript{1}US Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Walnut Creek, CA 94598, USA
\textsuperscript{2}Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
\textsuperscript{3}Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
\textsuperscript{4}Center for Advanced Bioenergy and Bioproducts Innovation, Urbana, IL 61801, USA
\textsuperscript{5}Global Institution for Collaborative Research and Education, Hokkaido University, Hokkaido, 060-8599, Japan

Corresponding author: Yoshikuni, Yasuo (yyoshikuni@lbl.gov)

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,
and all the discussed 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 φG31 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 conjugal 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

| 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. ambobaciens* 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 afr* ACN14a        | *S. altus* J1074, De114, 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 pltR | 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 | Iazamiride A and B | Environmental DNA | *S. altus* | 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. altus*, *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. spirioverticillatus*, 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, *S. lividans* TK24, *S. spheroides* NCIMB 11891, *S. roseochromogenes* var. oscitans DS 12.976 | λ-Red-mediated homologous recombination, phase atP-int system | While *S. coelicolor* M512 produced NPs comparable levels to their native hosts, *S. lividans* TK24 was ~5 times less productive | [71] |
| 13 | Clorobiocin   | clorobiocin BGC             | *S. coelicolor* M512, *S. lividans* TK24, *S. spheroides* NCIMB 11891, *S. roseochromogenes* var. oscitans DS 12.976 | λ-Red-mediated homologous recombination, phase atP-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 | *Pseudomonas putida* KT2440, *Agrobacterium tumefaciens* LBA4404, *E.coli* | 25 of γ-proteobacteria chalisa strains | CRAGE | This strategy enables integration of BGCs into chromosomes of diverse bacteria species | [33]** |
| 16 | Violacein     | *Pseudoalteromonas putida* KT2440, *Agrobacterium tumefaciens* LBA4404, *E.coli* | TAR-cloning and heterologous BGC expression platform | 40-fold cloning and heterologous BGC expression platform | A nonclustered LuxR-type quorum-sensing receptor from *P. luteoviolacea* 2ta16, PvlR, was identified to increase pathway transcription and violacein production | [60] |
| 17 | Metatricycloene | Uncultured environmental bacteria | Diverse *Streptomyces* species, including *S. altus* | 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 box 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* Del114, 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 isopenoid, 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 noncanonical 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, tetramycin, and lazariamide 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 (RBBSs) [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. berenices*; 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 pyrazinoines and dihydroxyazainones. 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 di-oliose [90]. S. argillaceus ΔmtmU 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.

Acknowledgements
Work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported under Contract No. DE-AC02-05CH11231. We thank Anita Walther for professional editing support.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Dayan FE, Cantrell CL, Duke SO: Natural products in crop protection. Biogeoq Med Chem 2009, 17:4022-4034.
2. Gu J, Gui Y, Chen L, Yuan G, Lu HZ, Xu X: Use of natural products as chemical library for drug discovery and network pharmacology. PLoS One 2013, 8:e62839.
3. Flüchter S, Follonier S, Schiel-Bengelsdorf B, Bengelsdorf FR, Zinn M, Dürr P: Anaerobic production of poly(3-hydroxybutyrate) and its precursor 3-hydroxybutyrate from synthesis gas by Autotrophic Clostridia. Biomacromolecules 2019 http://dx.doi.org/10.1021/acs.biomac.9b00342.
4. Mukherjee S, Sheshadri R, Varghese NJ, Elloe-Fadroski EA, Meier-Kolthoff JP, Göker M, Coates RC, Hadjithomas M, Pavlopoulos GA, Paez-Espino D et al.: 1,003 reference genomes of bacterial and archaeal isolates expand coverage of the tree of life. Nat Biotechnol 2017, 35:676-63863.
5. Medema MH, Blink K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R: antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 2011, 39:W339-346 (Web Server issue).
6. Rutledge PJ, Challis GL: Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat Rev Microbiol 2015, 13:509-523.
7. Cohen LJ, Esterhazy D, Kim SH, Lemetre C, Aguilar RR, Gordon EA, Pickard AJ, Cross JR, Emiliano AB, Han SM et al.: Commensal bacterium makes GPCR ligands that mimic human signaling molecules. Nature 2017, 549:48-53.
8. Guo CJ, Chang FY, Wyche TP: Discovery of reactive microbioderived metabolites that inhibit host proteases. Cell 2017, 168:517-526.
9. Smanski MJ, Zhou H, Claesen J, Shen B, Fischbach MA, Voigt CA: Synthetic biology to access and expand nature’s chemical diversity. Nat Rev Microbiol 2016, 14:135-149.
10. Shao Z, Rao G, Li C, Abi Z, Luo Y, Zhao H: Refactoring the silent spectinabilin gene cluster using a plug-and-play scaffold. ACS Synth Biol 2013, 2:662-669.
11. Fu J, Bian X, Hu S, Wang H, Huang F, Selibert PM, Plaza A, Xia L, Müller R, Stewart AF, Zhang Y: Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat Biotechnol 2012, 30:440-446.
12. Wenzel SC, Müller R: Recent developments towards the heterologous expression of complex bacterial natural product biosynthetic pathways. Curr Opin Biotechnol 2005, 16:594-606.
13. Bibb MJ: Regulation of secondary metabolism in Streptomyces. Curr Opin Microbiol 2005, 8:208-215.
14. Bibb MJ, Hesketh A: Analyzing the regulation of antibiotic production in Streptomyces. Methods Enzymol 2009, 458:93-116.
15. Sosio M, Giusano F, Cappellano C, Bossi E, Puglia AM, Donadio S: Artificial chromosomes for antibiotic-producing actinomycetes. Nat Biotechnol 2000, 18:343-345.
16. Martinez A, Kolvek SJ, Yip CL, Hopke J, Brown KA, MacNeil IA, Osbourne MS: Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. Appl Environ Microbiol 2004, 70:2452-2463.
17. Fuji K, Koyama T, Kai W, Kubota S, Yoshida K, Ozaki A, Aoki JY, Kawabata Y, Araki K, Tsuzaki T et al.: Construction of a high-coverage bacterial artificial chromosome library and comprehensive genetic linkage map of yellowtail Seriola quinqueraadiata. BMC Res Notes 2014, 7:200.
18. Varshney RK, Mir RR, Shatia S, Thudi M, Hu Y, Azam S, Zhang Y, Jaganathan D, You FM, Gao J et al.: Integrated physical, genetic and genome map of chickpea (Cicer arietinum L.). Funct Integr Genomics 2014, 14:59-73.

19. Miao V, Coëffet-Legal MF, Brian P, Brost R, Penn J, Whiting A, Martin S, Ford R, Parr I, Bouchard M et al.: Daptomycin biosynthesis in Streptomyces roseosporus: cloning and analysis of the gene cluster and revision of peptide stereochemistry. Microbiology 2005, 151:1507-1523.

20. Liu H, Jiang H, Halti B, Kulowski K, Muszynska E, Feng X, Summers M, Young M, Graziani E, Koehn F et al.: Rapid cloning and heterologous expression of the meridamycin biosynthetic gene cluster using a versatile Escherichia coli-Streptomyces artificial chromosome vector, pSBAC. J Nat Prod 2009, 72:389-395.

21. Nah HJ, Woo MW, Choi SS, Kim ES: Precise cloning and tandem integration of large polyketide biosynthetic gene cluster using Streptomyces artificial chromosome system. Microb Cell Fact 2015, 14:140 http://dx.doi.org/10.1186/s12934-015-0325-2.

22. Thorpe HM, Smith MC: In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc Natl Acad Sci U S A 1998, 95:5505-5510.

23. Bierman M, Logan R, O’Brien K, Seno ET, Rao RN, Schoner BE: Plasmid cloning vectors for the conjugal transfer of DNA from Escherichia coli to Streptomyces spp. Gene 1992, 116:43-49.

24. Gregory MA, Till R, Smith MCM: Integration site for Streptomyces phage φBT1 and development of site-specific integrating vectors. J Bacteriol 2003, 185:5320-5323.

25. Fogg PCM, Colloms S, Rossier S, Stark M, Smith MCM: New applications for phage integration. J Mol Biol 2014, 426:2703-2716.

26. Du D, Wang L, Tian Y, Liu H, Tan H, Niu G: Genome engineering and direct cloning of antibiotic gene clusters via phage φBT1 integrase-mediated site-specific recombination in Streptomyces. Sci Rep 2015, 5:8740.

27. Engelhardt K, Degens KF, Zotchev SB: Isolation and characterization of the gene cluster for biosynthesis of the thiopeptide antibiotic TP-1161. Appl Environ Microbiol 2010, 76:7093-7101.

28. Hickman AB, Chandler M, Dyda F: Integrating prokaryotes and eukaryotes: DNA transposases in light of structure. Curr Rev Biochem Mol Biol 2010, 45:50-56.

29. Johnson CM, Grossman AD: Integrative and conjugative elements (ICEs): what they do and how they work. Annu Rev Genet 2015, 49:577-601.

30. Lee CA, Babic A, Grossman AD: Autonomous plasmid-like replication of a conjugative transposon. Mol Microbiol 2010, 75:268-279.

31. Wozniak RAF, Waldor MK: Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol 2010, 8:552-563.

32. Brophy JAN, Triasai AJ, Adams BL, Renberg RL, Stratis- Cullum DN, Grossman AD, Voigt CA: Engineered integrative and conjugative elements for efficient and inducible DNA delivery to undomesticated bacteria. Nat Microbiol 2018, 3:1043-1053. The authors developed an approach based on ICEBs1 to overcome the problem of transferring DNA into the cell of undomesticated bacteria. The breakthrough technology makes it easier to harness undomesticated microbes and expand the range of heterologous chassis strains for NP production.

33. Wang G, Zhao Z, Ke J, Engel Y, Shi YM, Zhang Z, Bingol K, Robinson D, Wang B, Evans R et al.: Chassis-independent recombining assembled genome engineering enables rapid activation of biosynthetic gene clusters. Nat Microbiol 2019, in preparation. One of the best examples of the success of multi-chassis engineering for discovery and heterologous production of microbial natural products.

34. Balfz RH: Streptomyces and Saccharopolyspora hosts for heterologous expression of secondary metabolite gene clusters. J Ind Microbiol Biotechnol 2010, 37:759-772.

35. Gomez-Escibano JP, Bibb MJ: Engineering Streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters. Microb Biotechnol 2011, 4:207-215.

36. Zhang MM, Wang Y, Ang EL, Zhao H: Engineering microbial hosts for production of bacterial natural products. Nat Prod Rep 2016, 33:963-987.

37. Myronovskiy M, Rosenkränzer B, Nadmid S, Pujic P, Normand P, Luzhetskyy A: Generation of a cluster-free Streptomyces albus chassis strains for improved heterologous expression of secondary metabolite clusters. Metab Eng 2018, 49:316-324.

38. Komatsu M, Uchiyama T, Ōmura S, Cane DE, Ikeda H: Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism. Proc Nat Acad Sci U S A 2010, 107:2646-2651.

39. Choi KR, Cho JS, Cho U, Park D, Lee AY: Markerless gene knockout and integration to express heterologous biosynthetic gene clusters in Pseudomonas putida. Metab Eng 2018, 47:463-474.

40. Baumgart M, Unthan S, Kloß R, Radek A: Corynebacterium glutamicum chassis C1*: building and testing a novel platform host for synthetic biology and industrial biotechnology. ACS Synth Biol 2016, 7:125-144.

41. Fredens J, Wang K, de la Torre D, Funkе LFH, Robertson WE, Christova Y, Chia T, Schmied WT, Dunkelmann DL, Beranek V et al.: Total synthesis of Escherichia coli with a recoded genome. Nature 2019, 569:514-518.

42. Chin JW: Expanding and reprogramming the genetic code. Nature 2017, 550:53-60.

43. Smanski MJ, Zhou H, Claesen J, Shen B, Fischbach MA, Voigt CA: Synthetic biology to access and expand nature’s chemical diversity. Nat Rev Microbiol 2016, 14:135-149.

44. Temme K, Zhao D, Voigt CA: Refactoring the nitrogen fixation gene cluster from Klebsiella oxytoca. Proc Natl Acad Sci U S A 2012, 109:7085-7090.

45. Shao Z, Rao G, Li C, Abil Z, Luo Y, Zhao H: Refactoring the silent spectinomycin gene cluster using a plug-and-play scaffold. ACS Synth Biol 2013, 2:662-669.

46. Osswald C, Zifp G, Schmidt G, Maier J, Bernauer HS, Müller R, Wenzel SC: Modular construction of a functional artificial epothilone polyketide pathway. ACS Synth Biol 2014, 3:759-772.

47. Luo Y, Huang H, Liang J, Wang M: Activation and characterization of a cryptic polyisocyclic tetramate macro lactam biosynthetic gene cluster. Nat Commun 2013, 4:2894.

48. Lale R, Brautaset T, Valla S: Broad-host-range plasmid vectors for gene expression in bacteria. In Strain Engineering. Methods in Molecular Biology (Methods and Protocols). Edited by Williams J. Humana Press; 2011:373-343. This chapter provides methods for the use of broad-host-range plasmid vectors for expression of genes in a variety of bacteria, which provides technique support for the employment of multi-chassis hosts for heterologous NP production.

49. Montiel D, Kang HS, Chang FY, Charlop-Powers Z, Brady SF: Yeast homologous recombination-based promoter engineering for the activation of silent natural product biosynthetic gene clusters. Proc Natl Acad Sci U S A 2015, 112:8953-8958.

50. Kudla G, Murray AW, Tollery D, Plotkin JB: Coding-sequence determinants of gene expression in Escherichia coli. Science 2009, 324:255-258.

51. Cho BK, Zengler K, Qiu Y, Park YS, Knight EM, Barrett CL, Gao Y, Palsson BØ: The transcription unit architecture of the Escherichia coli genome. Nat Biotechnol 2009, 27:1043-1049.

52. Li GW, Oh E, Weissman JS: The anti–Shine-Dalgarno sequence drives translational pausing and codon choice in bacteria. Nature 2012, 484:538-541.

53. Cambry G, Guimaraes JC, Arpin A: Evaluation of 244,000 synthetic sequences reveals design principles to optimize
translational efficiency of Streptomyces species using an engineered CRISPR/Cas system. ACS Synth Biol 2015, 4:723-728.

65. Cott RE, Wang Y, Zhao H. High-efficiency multiplex genome editing of Streptomyces species using an engineered CRISPR/Cas system. ACS Synth Biol 2015, 4:1020-1028.

66. Zhang MM, Wong FT, Wang Y, Luo S, Lim YH, Heng E, Yeo WL., **Cobb RE, Enghiad B, Ang EL et al.: CRISPR-Cas9 strategy for activation of silent Streptomyces biosynthetic gene clusters. Nat Chem Biol 2017, 13:607-609.**

The authors introduced a one-step CRISPR-Cas9 knock-in strategy to activate multiple silent BGCs in Streptomyces and trigger production of unique metabolites, including a novel pentangular type II polyketide. This breakthrough strategy complements existing activation approaches and facilitates discovery of more NPs.

67. Wong W, Zhu TF. Targeted isolation and cloning of 100-kb microbial genomic sequences by Cas9-assisted targeting of chromosome segments. Nat Protoc 2016, 11:960-975.

The authors report an optimized protocol that potentially simplifies and accelerates efforts to isolate and clone large gene clusters from microorganisms by Cas9-assisted targeting of chromosome segments.

68. Wang W, Zhao X, Gabrielli T, Lou C, Ebenstein Y, Zhu TF. Cas9-assisted targeting of chromosome segments CATCH enables one-step targeted cloning of large gene clusters. Nat Commun 2015, 6:6106.http://dx.doi.org/10.1038/ncomms6106.

69. Eppelmann K, Doekel S, Marahiel MA. Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host Bacillus subtilis. J Biol Chem 2001, 276:34824-34831.

70. Eustáquio AS, Gust B, Gaim U, Li SM, Chater KF, Heide L. Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters. Appl Environ Microbiol 2005, 71:2452-2459.

71. Nah HJ, Pyeon HR, Kang CH, Choi SS, Kim ES. Cloning and heterologous expression of a large-sized natural product biosynthetic gene cluster in Streptomyces species. Front Microbiol 2017, 8:394-408.

The authors summarized a technique for producing a great amount of antibiotics NP through BGC heterologous expression systems as well as recent strategies specialized for large NP BGCs in Streptomyces heterologous hosts.

72. Zaburannyi N, Babyk M, Ostash B, Fedorenko V, Luzhetskyy A: Insights into naturally minimised Streptomyces albus J1074 genome. BMC Genomics 2014, 15:97.

73. Malcolmson SJ, Young TS, Ruby JG, Skewes-Cox P, Walsh CT: The posttranslational modification cascade to the thiopentapeptide andactinomycin biosynthesis systems: new insights and advances. Proc Natl Acad Sci U S A 2018, 115:8639-8644.

74. Laver Y, Kotlar D: Codon bias as a factor in regulating expression via translation rate in the human gene. Gene 2005, 349:127-136.

75. Nilsson G, Belasco JG, Cohen SN, van Gabain A: Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc Natl Acad Sci U S A 1987, 84:4890-4894.

76. Ongley SE, Bixion X, Neilan BA, Müller R: Recent advances in the heterologous expression of microbial natural product biosynthetic pathways. Nat Prod Rep 2013, 30:1121-1138.

77. Zhang JJ, Tang X, Zhang M, Nguyen D, Moore BS: Broad-host-range expression reveals native and host regulatory elements that influence heterologous antibiotic production in Gram-negative bacteria. mBio 2017, 8:e01291-17.

78. Berg OG, Kurland CG: Growth rate-optimized tRNA abundance and codon usage. J Mol Biol 1997, 270:544-550.

79. Gustafsson C, Govindarajan S, Minshull J: Codon bias and heterologous protein expression. Trends Biotechnol 2004, 22:346-353.

80. Lanza AM, Curran KA, Reg LG, Alper HS: A condition-specific codon optimization approach for improved heterologous gene expression in Saccharomyces cerevisiae. BMC Syst Biol 2014, 8:3-43.

81. Gould N, Hendy O, Papamichail D: Computational tools and algorithms for designing customized synthetic genomes. Front Bioeng Biotechnol 2014, 2:41.

82. Myer OD, Sumner SJ, Li S, Barnes S, Du X: One step forward for reducing false positive and negative compound identifications from mass spectrometry metabolomics data: new algorithms for constructing extracted ion chromatograms and detecting chromatographic peaks. Anal Chem 2017, 89:8696-8703.

83. Clevenger KD, Bok JW, Ye R, Miley GP, Verdian MH, Velk T, Chen C, Yang K, Robey MT, Gao P et al.: A scalable platform to identify fungal secondary metabolites and their gene clusters. Nat Chem Biol 2017, 13:895-901.

84. Wang GY, Graziani E, Waters B, Pan W, Li X, McDermott J, Meurer G, Saxena G, Andersson RJ, Davies J: Novel natural products from soil DNA libraries in a streptomycete host. J Org Lett 2000, 2:2401-2404.

85. Courtois S, Cappellano CM, Ball M, Francou FX, Normand P, Helync G, Martinez A, Kolvek SJ, Hopke J, Osborne MS et al.: Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Appl Environ Microbiol 2003, 69:49-55.

86. Martinez A, Kolvek SJ, Hopke J, Tong Yip CL, Osborne MS: Environmental DNA fragment conferring early and increased sporulation and antibiotic production in Streptomyces species. Appl Environ Microbiol 2005, 71:1638-1641.

87. Lussier FX, Chamberlain O, Côté A, Hupé JF, Denis F, Juteau P, Beaudet R, Shareck FJ: Construction and functional screening of a metagenomic library using a T7 RNA polymerase-based expression cosmid vector. J Ind Microbiol Biotechnol 2011, 38:1321-1328.

88. McMahon MD, Guan C, Handelsman J, Thomas MG: Metagenomic analysis of Streptomyces lividans reveals host-dependent functional expression. Appl Environ Microbiol 2012, 78:3622-3629.

The authors developed tools and methods for constructing and screening metagenomic libraries in S. lividans that enable discovery and characterization of bioactive clones that could not be found using E. coli as a host.

89. Itoh HA, Beauin L, Grabau JU, Brady SF: Natural product discovery through improved functional metagenomics in Streptomyces. J Am Chem Soc 2016, 138:9341-9344.

90. Fischer C, Lipata F, Rohr J: The complete gene cluster of the antitumor agent givocarcin V and its implication for the
87. Castro JF, Razmilio V, Gomez-Escribano JP, Andrews B, Asenjo JA, Bibb MJ: Identification and heterologous expression of the chaxamycin biosynthesis gene cluster from Streptomyces leeuwenhoekii. Appl Environ Microbiol 2015, 81:5820-5831.

88. Pickens LB, Tang Y, Chooi YH: Metabolic engineering for the production of natural products. Annu Rev Chem Biomol Eng 2011, 2:211-236.

89. Pérez M, Baig I, Braña AF, Salas JA: Generation of new derivatives of the antitumor antibiotic mithramycin by altering the glycosylation pattern through combinatorial biosynthesis. ChemBioChem 2008, 9:2295-2304.

90. González A, Reising LL, Lombó F, Fernández MJ, Prado L, Braña AF, Künzel E, Rohr J, Méndez C, Salas JA: The mtmVUC genes of the mithramycin gene cluster in Streptomyces argillaceus are involved in the biosynthesis of the sugar moieties. Mol Gen Genet 2001, 264:827-835.