Review

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Metabolic engineering of antibiotic factories: new tools for antibiotic production in actinomycetes

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Actinomycetes are excellent sources for novel bioactive compounds, which serve as potential drug candidates for antibiotics development. While industrial efforts to find and develop novel antimicrobials have been severely reduced during the past two decades, the increasing threat of multidrug-resistant pathogens and the development of new technologies to find and produce such compounds have again attracted interest in this field. Based on improvements in whole-genome sequencing, novel methods have been developed to identify the secondary metabolite biosynthetic gene clusters by genome mining, to clone them, and to express them in heterologous hosts in much higher throughput than before. These technologies now enable metabolic engineering approaches to optimize production yields and to directly manipulate the pathways to generate modified products.

Actinomycetes as sources for novel drugs

For more than 70 years, actinomycetes (order *Actinomycetales*) have been recognized as important sources for bioactive natural compounds. From the roughly 18 000 known bioactive bacterial compounds, more than 10 000 were described from bacteria of the actinomycete genus *Streptomyces* [1]. Many well-known antibiotics, such as tetracycline, erythromycin, vancomycin, and streptomycin, originate from the secondary metabolism of actinomycetes. Beyond antibiotics, other medically useful natural products that were isolated from this group of bacteria include the immunosuppressant rapamycin, the anticancer agents doxorubicin and bleomycin, the anthelmintic avermectin, and the antifungal compound nystatin.

The traditional approach to small-molecule discovery from microbial sources such as actinomycetes has generally involved cultivation of the microbes under different growth conditions, extraction of the metabolites, and analysis of the extract for bioactivity (e.g., antimicrobial activity) in a chosen assay. Once a bioactive extract is identified, a more-detailed analysis is performed, normally involving chromatography-based separation of the individual constituents, to identify the specific bioactive molecules. Very often, however, this enormous effort leads to the rediscovery of known molecules, a fact that dampened enthusiasm for natural product discovery from actinomycetes over the past two decades.

Although this general strategy is still applied today, several recent developments have renewed enthusiasm for natural product discovery from actinomycetes. Genome sequence analysis from multiple actinomycetes indicates that each bacterium can produce approximately 10-fold more secondary metabolites than has been detected during screening efforts before the availability of the genome sequence data. For this reason, actinomycetes continue to be promising sources of novel bioactive compounds [2]. In addition, the availability of new metabolic engineering strategies now provides alternative approaches to streamline and accelerate the discovery and production of bioactive natural products from microbial or metagenomic sources. Metabolic engineering is a well-established discipline that systematically engineers microbial strains for the overproduction of natural and non-natural chemical compounds that are useful to mankind [3] (Figure 1). Although similar rationales can be applied to actinomycetes, engineering actinomycetes is more difficult than engineering model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, because actinomycetes possess more diverse genomic content and biochemical machinery [4] (Figure 1). We review tools and methods recently developed for the effective metabolic engineering of actinomycetes, and discuss how these tools enable the generation of microbial cell factories for the production of antibiotics and other secondary metabolites.

Genome mining for the detection and identification of secondary metabolite biosynthetic gene clusters

The protein machinery responsible for the biosynthesis of secondary metabolites in bacteria is encoded by distinct

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Glossary

A-domain: adenylation domains of non-ribosomal peptide synthetase (NRPS) modules select, activate amino acids as amino acid adenylates, and transfer them to the peptidyl carrier protein domains of NRPS modules.
AT: acyltransferase domains encoded in modular polyketide synthase specifically select the acyl-CoA building-blocks and transfer them to acyl carrier proteins (ACPs).
ACP: acyl carrier proteins contain a phosphopantetheine prosthetic group tethering the reaction intermediates during polyketide biosynthesis.
BAC: bacterial artificial chromosomes are replicable circular DNA vectors developed from the F-plasmid. They are usually used for cloning long fragments of 150–350 Kb.
CRISPR–Cas9: clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease Cas9 is an RNA-guided endonuclease using RNA–DNA base-pairing to cleave target DNA, which is demarcated by protospacer adjacent motif (PAM) sequences. PAM sequences are short nucleotide motifs that are specifically recognized and required by Cas9 for DNA cleavage.
DH: the dehydratase domain of a PKS module is responsible for dehydrating the β-OH group of a polyketide intermediate.
ER: the enoylreductase domain of a PKS module reduces a double bond to a single bond between two adjacent extender units.
HDR: homology-directed repair is a template-dependent pathway for double-strand break (DSB) repair.
KR: the ketoreductase domain of a PKS module is responsible for reducing the β-keto of the polyketide intermediate group to a hydroxyl group.
NRPS: non-ribosomal peptide synthetases specifically activate and condense proteinogenic or non-proteinogenic amino acids in an assembly-line fashion. They are involved in the biosynthesis of many peptide antibiotics such as, for example, vancomycin.
PKS: polyketide synthases condense acyl-CoA units to form a polyketide. There are three types of PKS: type I PKSs are homologous to type I fatty acid synthases that are found for example in mammals. They act either iteratively or in an assembly-line fashion. Type II PKSs are homologous to type II fatty acid synthases as found in many bacteria. Their products usually are aromatic polyketides. Type III PKSs are homologs of plant chalcone/stilbene synthases.
Red/ET: Red/ET recombineering is a method to insert foreign DNA into chromosomes or plasmids based on short homologous sequence regions (<50 bp). The system makes use of the exonucleases/recombinases Redα/Redβ from λ phage or RecE/RecT from Rac prophage.
SMBGC: secondary metabolite biosynthetic gene clusters contain all genes required for biosynthesis, regulation, export, and very often resistance of natural products/secondary metabolites, thus all genes are encoded side-by-side. With only very few exceptions, bacterial secondary metabolite biosynthesis pathways are always organized in SMBGCs. However, SMBGCs can also be found in fungal producers.
TALEN: transcription activator-like effector nucleases are fusions of the *Fok*I cleavage domain and DNA-binding domains derived from TALE proteins. TALEs contain multiple 33–35 amino acid repeat domains, each of which recognizes a single base pair.
TE: the thioesterase domain releases the polyketide or peptide intermediate from the PKS or NRPS assembly line; in many cases the release is combined with a macrolactonization, yielding cyclic molecules.
ZFN: zinc-finger nucleases are fusions of the non-specific DNA cleavage domain from the *Fok*I restriction endonuclease with zinc-finger proteins. ZFN dimers induce targeted DNA DSBs that stimulate DNA damage response pathways.

gene clusters within their genomes, making the identification of these clusters through genome mining an important task [4]. Several major developments have advanced such genome-mining efforts, in particular the development of next-generation sequencing technologies, increased knowledge about the secondary metabolism, and novel mass spectrometry (MS) detection tools. The resulting biological information has been incorporated into several databases and software tools for the genome-wide prediction of gene clusters, the analysis of their corresponding secondary metabolite biosynthetic pathways, and the prediction of substrate specificities [5]. The currently most widely used software to identify such gene clusters is the antibiotics and secondary metabolites analysis shell (antiSMASH) [6]. antiSMASH includes rule-based as well as statistics-based algorithms to identify secondary metabolite biosynthetic gene clusters (SMBGCs; see Glossary) and offers various modules for analyzing the relevant pathways.

In addition to computational tools, genome mining is typically accompanied by proteome and/or metabolome analyses using MS for accurate linking of a target secondary metabolite and its biosynthetic gene cluster [7,8]. The biosynthesis of several classes of secondary metabolites follows a conserved biochemical logic; this is utilized to match secondary metabolite-derived analytical data with genes in the genome of the target organism [9,10]. In addition to the biochemical logic, high-quality genome annotation data for the target organism (e.g., high coverage and accurate open reading frame predictions) and the availability of sufficiently sensitive mass spectrometers determine the success of this MS-based genome mining. In this approach, the analytical data of secondary metabolites generated by MS (i.e., MS^n data) provide specific fragment patterns which contain amino-acyl or glycosyl 'tags'. Amino-acyl tags can be searched against amino acid building blocks predicted from the genome of the target organism using the conserved biochemical logic for ribosomal or non-ribosomal peptides [11–13].

Along the same lines, glycosyl tags from glycosylated natural products can be linked to their corresponding biosynthetic genes among all the glycosylation genes which are initially characterized by mining the genome of the target organism. These methods can be considered either peptidogenomics or glycogenomics, depending on the use of amino-acyl (or peptidyl) or glycosyl 'tags' obtained from the target secondary metabolites, respectively, but the general rationale of their approaches remains the same. Use of peptido- and glycogenomics led to the discovery of novel analogs of the antibiotics stendomycin from *Streptomyces hygroscopicus* ATCC 53653 [11], and arenimycin B from the marine actinobacterium *Salinispora arenicola* CNB-527, an antibiotic effective against multidrug-resistant *Staphylococcus aureus* [12], respectively. Although these methods might need to be tailored for secondary metabolites with hybrid or highly modified structures (e.g., non-ribosomal and polyketide hybrids), they can still be applicable to a variety of compounds to rapidly identify their biosynthetic genes. In another recent example, significant correlations between protein expression levels and the activities of target secondary metabolites under several different growth conditions were validated and used to link target secondary metabolites to their specific gene clusters [14]. Once positive links were identified, the respective proteins were mapped onto the predicted gene clusters of the target organism through quantitative proteomic expression data.

After the gene cluster for a target secondary metabolite has been identified, metabolic engineering can be conducted by using a microbial strain that natively harbors the specific gene cluster provided that it has adequate growth characteristics and is amenable to genetic manipulation. Otherwise, the identified gene cluster can be cloned and expressed in a heterologous host. The recent development of new cloning techniques has now greatly expedited this process.

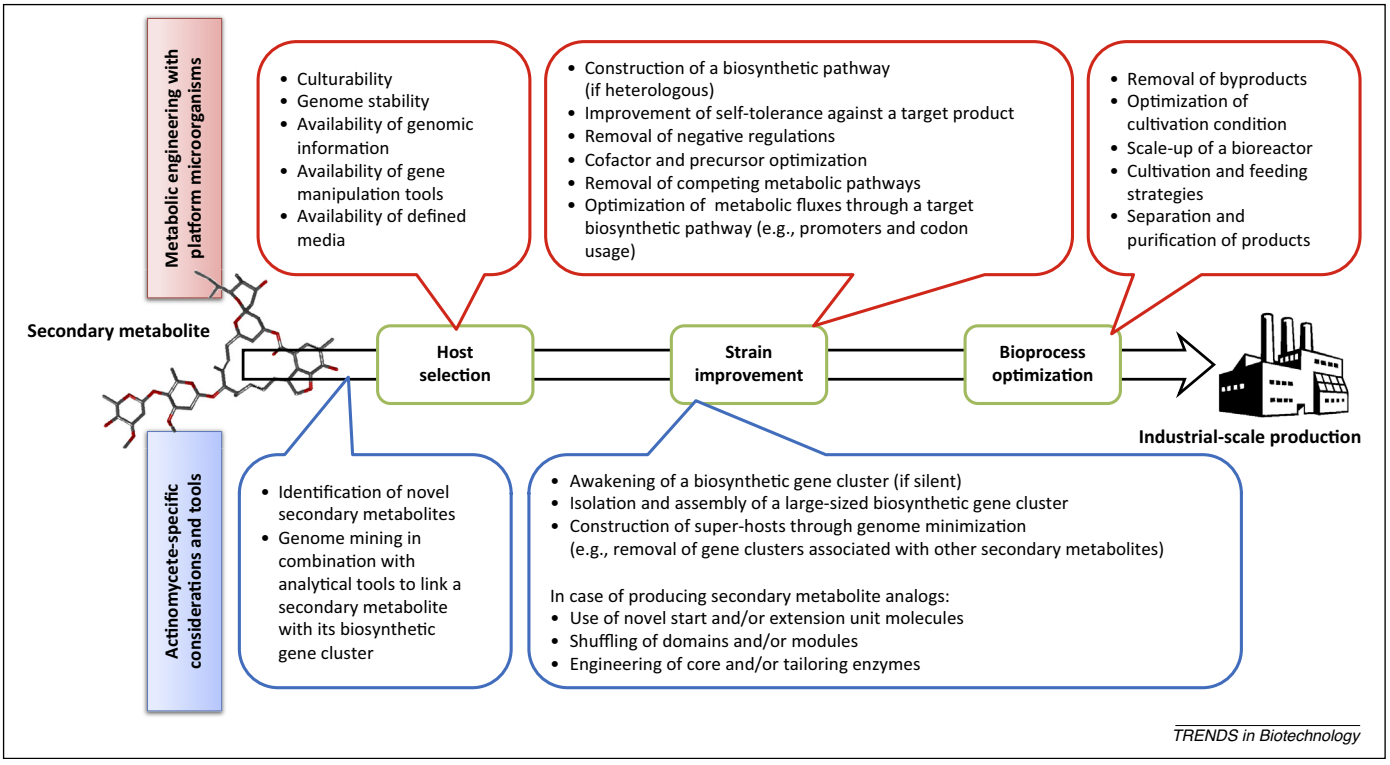

Figure 1. Representative metabolic engineering pipeline with platform microorganisms (e.g., *Escherichia coli* and *Saccharomyces cerevisiae*), shown in upper boxes, and additional considerations for actinomycetes, shown in lower boxes.

Awakening the silent genetic potential

Mining of whole genomes from actinomycetes species on average reveals the presence of 30–40 SMBGCs per strain, thus indicating a great genetic potential to synthesize secondary metabolites, including antibiotics. However, under standard laboratory conditions only a fraction of these clusters is expressed. Several methods have been developed that nonspecifically trigger the expression of such 'silent gene clusters'. It has been observed that inducing mutations in ribosomal proteins or RNA polymerase, supplementing the fermentation broth with chemicals such as rare earth elements, antibiotics, *N*-acetyl glucosamine, or particular synthetic compounds can have positive effects on the expression of SMBGCs and the yield of their resulting compounds [15]. Similar effects have been observed for co-culturing approaches in which specific antibiotics are produced upon co-cultivation of the antibiotic producer with other microorganisms [16].

In many cases, the expression of 'silent' SMBGCs was activated successfully by coexpression of homologous (i.e., from the same SMBGC) or heterologous (i.e., from a different SMBGC) pathway-specific activator genes. For example, production of glycopeptide ristomycin A could be activated in *Amycolatopsis japonicum* MG417-CF17 by coexpression of the balhimycin pathway specific activator *bbr* [17]. Alternatively, strong promoters can be inserted in front of the native regulators to trigger the activation of the silent SMBGCs, as shown for the 6-*epi*-alteramides of *S. albus* J1074 [18], or pathway-specific repressor genes can be inactivated. Finally, although the strategy of engineering pathway-specific regulation can be successfully employed in the native hosts, it is also very relevant for the heterologous expression of SMBGCs in platform strains.

Advanced DNA synthesis and cluster assembly techniques

If a cluster of interest cannot be expressed in its original host, one must necessarily express the cluster heterologously. To achieve this the cluster must be cloned into a suitable vector. There are several factors to consider when choosing a cloning strategy. These include: whether genomic DNA is readily available to serve as a template, whether one wishes to focus on a small number of clusters or scan multiple clusters in a genome, and whether to capture the cluster in its native form or to refactor it.

If template DNA is readily available, for example from genomic, environmental, or metagenomic sources, then the traditional cloning strategy has been to break down the DNA into random pieces and clone these smaller fragments into *E. coli* to store them as a library. Cosmid/fosmid library vectors are capable of carrying inserts up to 45 kb, and bacterial artificial chromosome (BAC) vectors can take inserts up to 200 kb. The library is then screened using *in situ* colony hybridization [19] or colony PCR [20] to identify colonies that contain fragments from the target cluster. In some instances, a cluster will not be cloned in its entirety into a single vector owing to its size. Cluster lengths usually range from 10 to 100 kb, and some can be even larger. When colonies containing fragments from the target cluster have been identified, the fragments can be reassembled into a single contiguous piece using restriction–ligation, λ-mediated recombination, or transformation-associated recombination (TAR) in yeast [21]. A well-constructed library should contain all the SMBGCs from a given source, allowing the analysis of multiple clusters.

More recently, TAR [22] and a related technique, linear plus linear homologous recombination (LLHR) [23], have been used as direct cloning methods to capture a target DNA sequence from a genomic DNA template more quickly and efficiently than through library construction and screening. *S. cerevisiae* or *E. coli* are transformed with genomic DNA of the producers, and the target cluster is captured into a replicable plasmid through homologous recombination catalyzed by the native recombination machinery in yeast or by a bacteriophage-derived Red/ET recombination system in engineered *E. coli* (Figure 2). The upper size limit for cluster capture is not yet known, but the 67 kb taromycin A cluster has been captured with TAR [24], and a 52 kb cluster could be directly captured with LLHR [23,25]. Once the clusters are captured, either by traditional library methods or by direct cloning, they can be further manipulated inside their *S. cerevisiae* or *E. coli* hosts using standard recombination techniques [23,25]. With these methods it is possible, for example, to introduce new promoters or regulatory elements, modify RBS sites, swap codons, or reconstruct gene modules to generate new product derivatives [26].

A target cluster can also be assembled from smaller fragments through the use of several different DNA assembly techniques. Some of these, for example Gibson assembly [27], ligation-independent cloning (SLIC) [28], TAR [22] (or DNA assembler [29]), USER fusion [30], ligase cycling reaction (LCR) [31], and successive hybridization assembling (SHA) [32], do not depend on restriction or specific recombination sites, and do not introduce scars, making them especially well suited for cluster reconstruction. Genes, pathways, and even whole bacterial genomes have been constructed from smaller fragments using these techniques (Table 1) [33].

One of the major strengths of these approaches is their use for cluster redesign. Each gene, promoter, or other element can be viewed as an independent module that can be replaced, deleted, or added to the cluster as desired. It is thus possible to replace native promoters with synthetic ones and bypass native regulation, to delete the transcriptional repressors encoded on the cluster, or to create combinatorial pathways to produce new antibiotics [34]. For *in vivo* homologous recombination-based methods, such as DNA assembler and TAR, unintended recombination events can occur between regions that have repeats or similar sequences, such as those found in the gene clusters that encode polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS). In these cases it is necessary to verify the accuracy of the constructed sequence through restriction mapping [24] or direct DNA sequencing. For *in vitro* methods in which assembly is based on homologous ends, for example Gibson assembly and SLIC, secondary structures (hairpins and stem loops) in the homologous ends can drastically lower assembly efficiency.

It should be noted that the GC content of SMBGCs from actinomycetes is usually about 65–75%, which makes the

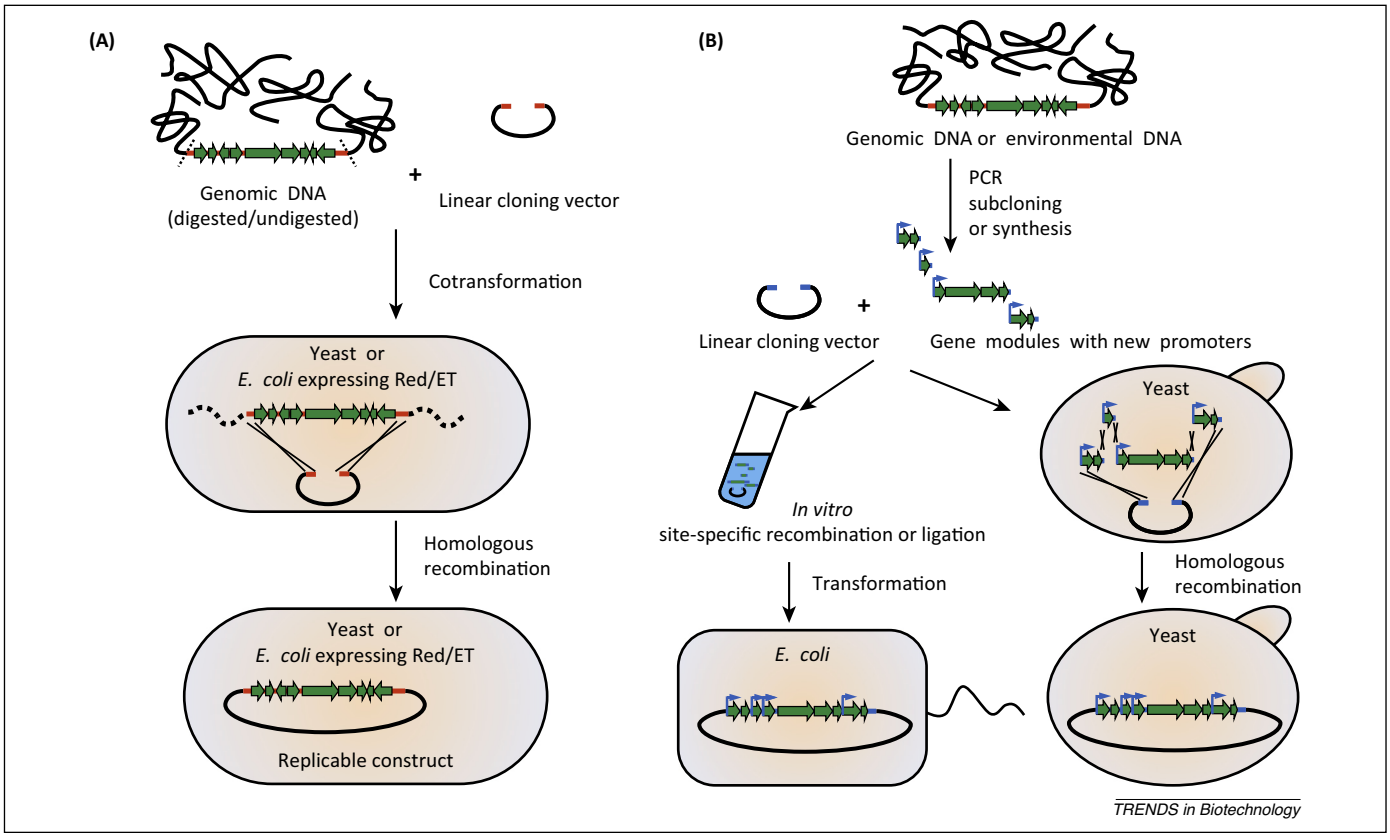

Figure 2. Direct cloning and reassembly of secondary metabolite biosynthetic gene clusters. **(A)** To capture a long cluster, a linear cloning vector flanked with homology arms is prepared and then co-transferred with the cluster-harboring genomic DNA into yeast (TAR) or engineered *Escherichia coli* (LLHR). Catalyzed by the native recombination mechanism in yeast or expressed Red/ET in *E. coli*, the homologous recombination between the cluster ends and the vector homology arms yields a circular replicable construct. **(B)** For reassembly of a cluster, genes are isolated individually by PCR, subcloning, or chemical synthesis, and are modified by the addition of homology ends, ends with restriction sites, or specific recombination sites. Strong or inducible promoters can be introduced in front of genes. Next, the gene fragments are assembled in a vector by *in vivo* homologous recombination in yeast, or by *in vitro* site-specific recombination or ligation. Assembled products can be further amplified in *E. coli*.

Table 1. Examples of isolating and reconstructing whole secondary metabolite biosynthetic gene clusters

Method	Mechanisms	Examples
Genomic library	BAC library	Cloning of the 128 kb daptomycin biosynthetic gene cluster [62]
Linear–linear homologous recombination (LLHR)	Red/ET recombination in *Escherichia coli*	Direct cloning of 10 PKS-NRPS clusters of 10–52 kb from predigested genomic DNA [23,63]
Restriction/ligation based 'conventional' cloning	PCR amplification/subcloning; cloning into suitable expression vectors	Assembly of erythromycin polyketide synthase DEBS [64] and the full erythromycin A biosynthetic pathway [65]
Transformation-associated recombination (TAR)	Homologous recombination in yeast	Direct cloning of the 67 kb taromycin A (NRPS) cluster from genomic DNA [24]
MASTER Ligation	Type II endonuclease *Msp*JI	Assembly of the 29 kb actinorhodin (PKS) cluster from four fragments [66]
DNA assembler	Homologous recombination in yeast	Assembly of the 42.6 kb spectinabilin (PKS) cluster from three fragments [67]
Site-specific recombination-based tandem assembly (SSRTA)	*In vitro* site-specific recombination by *Streptomyces* phage θBT1 integrase	Assembly of the 56 kb epothilone cluster (PKS-NRPS) from seven fragments [68]
Overlap extension PCR–yeast homologous recombination method (ExRec)	Homologous recombination in yeast	Assembly of the 45 kb xenoamicin biosynthesis gene cluster (NRPS) from six fragments [69]

handling of such DNA for sequencing, DNA synthesis, and PCR amplification more challenging than the handling of DNA with GC content closer to 50%. Consequently, assembly and cloning procedures often require additional optimization or verification steps to ensure that each cloned fragment is correct.

In the future it will also be possible to obtain entire clusters through chemical synthesis as the cost of DNA synthesis continues to decline, thereby bypassing the need for cluster cloning or assembly. At current prices, it is common to purchase shorter fragments and assemble them through the methods discussed above.

Heterologous expression and super-hosts

Actinomycetes are often chosen as hosts for the heterologous expression of SMBGCs in view of factors such as matching GC content, the availability of biosynthetic precursors from primary metabolism that support secondary metabolite biosynthesis, and the need for particular modification enzymes during secondary metabolite biosynthesis. Heterologous expression becomes necessary when the native host is difficult to culture or genetically intractable [35]. For industrial production of secondary metabolites, such as antibiotics, another important factor for choosing a heterologous host is genome stability [36] because the genomes of actinomycetes can be unstable over time, which can lead to a reduction or complete loss of secondary metabolite production [37].

Several groups have recently developed genome-minimized actinomycetes that show promise as 'super-hosts' and platform strains for heterologous expression. The common feature among these strains is the removal of unnecessary native SMBGCs. This allows the target compound synthesized by the introduced gene cluster to be detected more easily through routine analytical techniques. In one case, four endogenous SMBGCs for actinorhodin, undecylprodigiosin, coelimycin, and calcium-dependent antibiotic (CDA) were deleted from *Streptomyces coelicolor*, and point mutations were introduced into *rpoB* and *rpsL* to pleiotropically increase the level of secondary metabolite production [38]. In another case, different sets of SMBGCs were deleted from *S. coelicolor* to create a series of genome-reduced hosts, including one strain with all clusters deleted [39]. Vector-borne expression of the actinorhodin cluster in these strains, however, resulted in different yields of actinorhodin among the strains. In addition to *S. coelicolor*, the avermectin producer *Streptomyces avermitilis* has also been subjected to

Table 2. Limitations for compound production and examples of improved production yield after strain engineering[a]

Limitations	Strategies for production improvement	Target compounds with improved production yield	Refs
Regulation (feedback inhibition)	Tuning of the expression of regulatory genes	Doxorubicin	[70]
		Ristomycin	[17]
	Expression of genes involved in the synthesis of signaling molecules (γ-butyrolactones)	Actinorhodin and undecylprodigiosin	[71]
Precursor supply	Improvement of precursor availability (improved precursor titers)	Actinorhodin	[43,72]
		FK506	[73,74]
		Erythromycin	[75]
		Balhimycin	[44]
Expression and kinetics of structural enzymes	Overexpression, inactivation, and engineering of structural enzymes	Erythromycin A	[76]
Self-resistance	Overexpression of resistance genes and ribosome engineering	Actinorhodin	[77]
		Doxorubicin	[78]
Export system (often determining self-resistance)	Overexpression of export genes	Avermectin	[79]

[a]See also Table S1 in the supplementary material online.

genome reduction and probed as a potential super-host for heterologous expression. A region of more than 1.4 Mb was deleted from the 9.02 Mb linear chromosome of this organism [40,41]. The tractability of this *S. avermitilis* super-host has recently been confirmed by successful heterologous expression of more than 20 exogenous SMBGCs in this strain [40].

Despite these successes, the development of a single super-host that can ensure efficient and high-level expression of every SMBGC is unlikely. Proficient gene transfer and genetic manipulation, fermentation conditions (e.g., suitable culture medium and conditions), and an optimal trade-off of fluxes between primary and secondary metabolisms all play important roles in secondary metabolite biosynthesis, and can vary from one host to another. Additional hosts are likely to be created in the future as high-throughput genome editing tools, such as TALEN, ZFN, and CRISPR–Cas9 [42], become adapted for

Box 1. Antibiotic biosynthesis by molecular assembly lines

Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are intracellular, multifunctional enzymatic machineries that catalyze the biosynthesis of the complex core structures of many polyketides or non-ribosomally synthesized peptides. These enzymatic assembly lines have a modular organization: each biosynthetic module is responsible for adding one building-block (acyl unit in the case of PKS and amino acid in the case of NRPS) into the precursor molecule. The assembly line modules in turn consist of functional enzymatic domains that provide the required activity for polyketide or peptide biosynthesis. The domains of PKS and NRPS have diverse functions (see Table 2 in main text and Table S1 in supplementary material online) [9,10].

In the biosynthetic assembly line for the polyketide antibiotic erythromycin (Figure I), which is produced by the actinomycete *Saccharopolyspora erythraea*, the acyltransferase (AT) domain of the loading module selects a propionyl-CoA unit and loads it onto a phosphopantetheine group of its cognate acyl carrier protein (ACP). The ATs of the remaining modules 1–6 are specific for methylmalonyl-CoA building blocks, which also are loaded onto their cognate ACPs. Ketosynthase (KS) domains then catalyze the decarboxylative condensation of the different acyl units. If a module contains optional domains (e.g., KR, DH, and ER), the polyketide intermediate is further modified while still tethered to the ACP. The last module has a terminal thioesterase (TE) domain, which cleaves the precursor molecule from the last ACP and cyclizes it to the precursor 6-deoxyerythronolide B. In the final steps of erythromycin biosynthesis, this precursor molecule is modified by tailoring enzymes to the end-product erythromycin A.

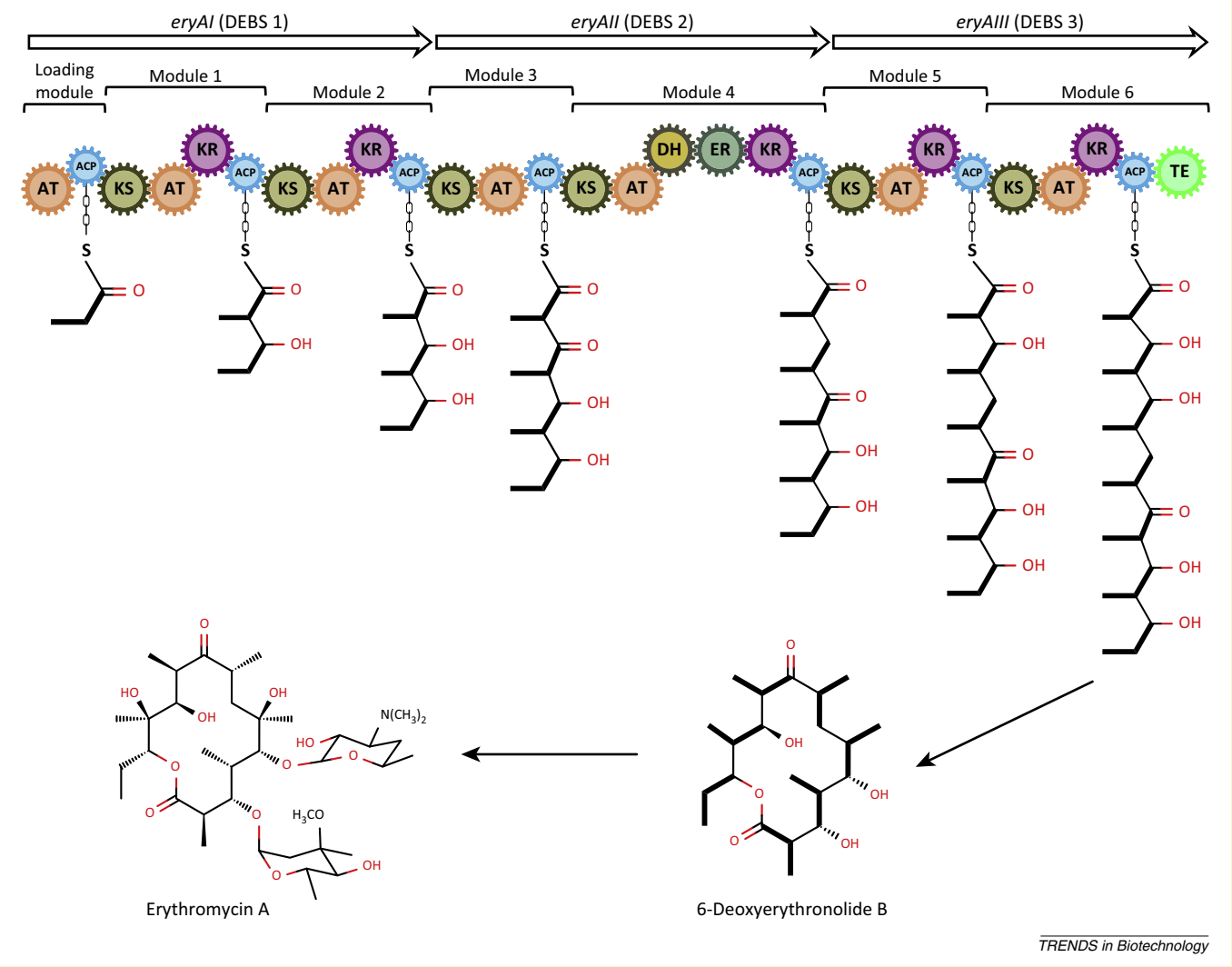

Figure I. Assembly line for the polyketide antibiotic erythromycin.

use in actinomycetes. The use of such hosts is and will be crucial to harnessing the biosynthetic potential of the many cryptic SMBGCs.

Engineering secondary metabolite biosynthetic pathways

While the techniques described above have emerged only during the past few years, genetic engineering of SMBGCs has a longstanding history in antibiotic and natural product discovery from actinomycetes. More recently, metabolic engineering strategies have been developed to increase the production of a target molecule and to create novel analogs. In cases where a molecule has been identified and is approved for clinical use, metabolic engineering becomes especially important because strain improvement and industrial fermentation are often the only routes to obtain large quantities of the molecule. Large-scale chemical synthesis frequently cannot be applied because the

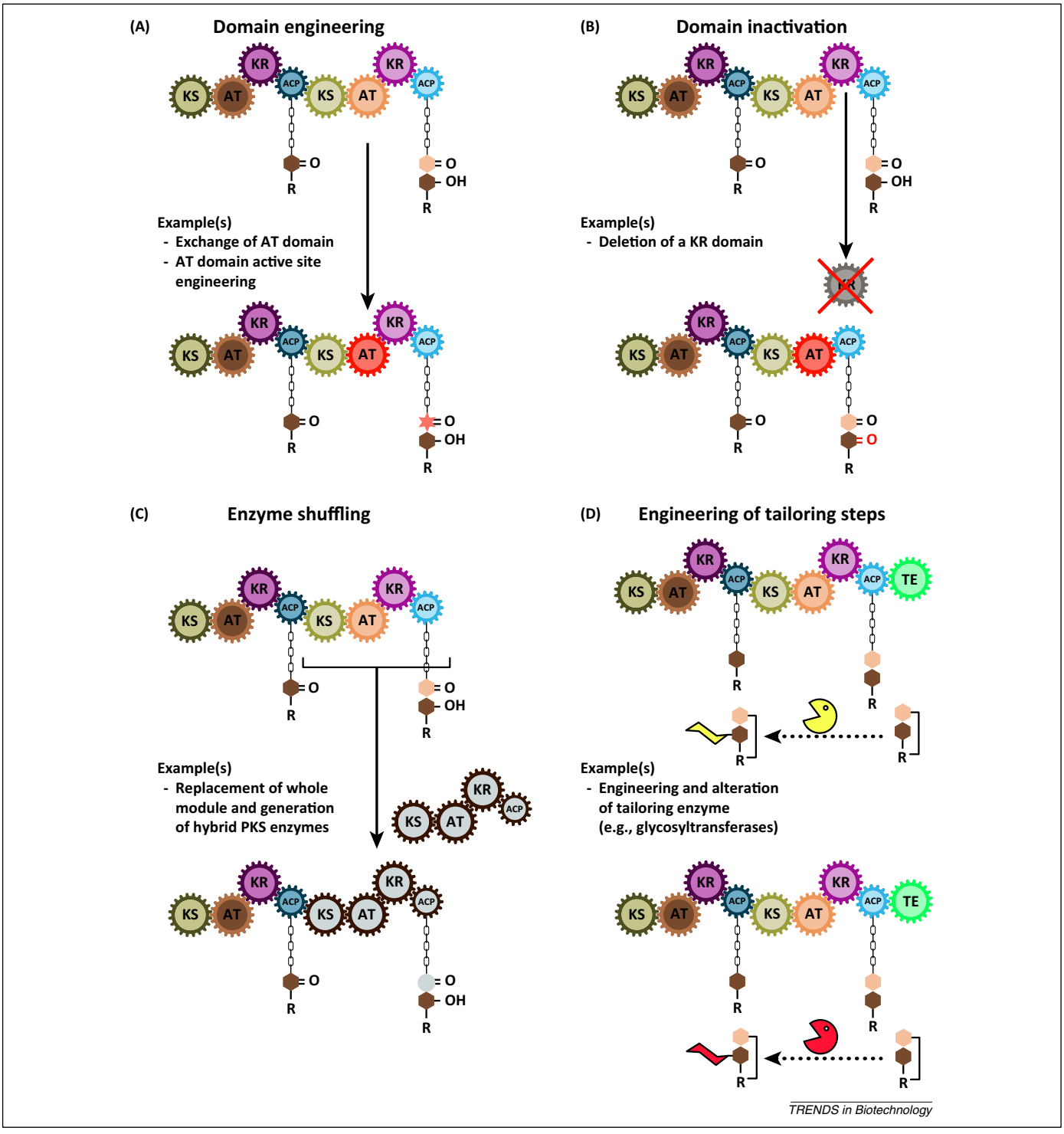

Figure 3. Strategies to engineer type I polyketide biosynthetic pathways. **(A)** Generation of novel compounds by AT domain engineering. **(B)** Interfering with β-carbon reduction by domain inactivation. **(C)** Generation of novel compounds by the replacement of whole modules. **(D)** Generation of novel compounds by engineering of tailoring modifications. Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; ER, enoylreductase; KR, ketoreductase; KS, ketosynthase; TE, thioesterase.

structures of these secondary metabolites are too complex. In such cases it is an advantage to identify factors, such as global- or cluster-specific regulation, metabolic flux, or insufficient self-resistance, which limit compound production in the native or heterologous host (Table 2; Table S1 in the supplementary material online).

Multiple approaches have been implemented to overcome these limitations and boost compound production. Several strategies, such as tuning the expression of regulatory genes, increasing the supply of precursors, and overexpressing the biosynthesis, resistance and export genes, have all led to increased compound yields (Table 2; Table S1 in the supplementary material online). For example, the overexpression of the malonyl-CoA synthesizing complex (acetyl-CoA carboxylase complex) led to a sixfold increase in actinorhodin production in *S. coelicolor* [43]. In the balhimycin producer *Amycolatopsis balhimycina*, the overexpression of two genes from the shikimate pathway, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (*dahp*) and prephenate dehydrogenase (*pdh*), increased

Table 3. Engineering strategies for secondary metabolite biosynthetic gene clusters[a]

Strategies	Examples		Refs
Engineering of PKS and NRPS assembly lines			
Combination of acyltransferase (AT) domains and AT substitution	Erythromycin and rapamycin		[47,80]
	Geldanamycin		[81]
Domain manipulations: dehydratase (DH) and ketoreductase (KR) substitution	Avermectin and rapamycin (ivermectin)		[82]
Shuffling of polyketide synthase (PKS) subunits/hybrid PKS	Mederrhodin and dihydrogranatihordin (hybrid antibiotics mederrhodins A and B)		[83,84]
	Tylosin, spiramycin, and chalcomycin		[85]
	Aureothin and luteoreticulin		[49]
Enzyme replacement by a fatty acid synthase homolog	Undecylprodiginine (replacement of the 3-ketoacyl ACP synthase III initiation enzyme by a fatty acid synthase)		[86]
PKS gene inactivation	Pactamycin		[87]
KR domain inactivation	Amphotericin B		[88]
Thioesterase fusions	Erythromycin and non-actinomycete products		[89]
Module fusion in PKSs	Tetracenomycin and R1128		[90]
Non-ribosomal peptide synthetase (NRPS) subunit alteration and module exchange combined with structural gene deletion	Daptomycin		[91,92]
NRPS-module fusions	Daptomycin		[93]
NRPS gene deletion	Streptolydigin and christolane A, B, and C (inactivation of streptolydigin NRPS genes led to the production of novel compounds)		[94]
A-domain engineering	Alteration of substrate specificity by site-directed mutagenesis	Calcium-dependent antibiotic	[48]
	Directed evolution of the A-domain	Andrimid derivatives	[95]
Engineering of other parts of the pathways			
Modification of tailoring enzymes	Modification and alteration of glycosyltransferases, halogenases, methyltransferases, discrete ketoreductase	Erythromycin A	[51,76]
		Doxorubicin	[53,96]
		Daunorubicin and derivatives	[53]
		Staurosporine and rebeccamycin	[97]
		Clorobiocin and novobiocin	[98]
		Vancomycin, teicoplanin, tylosin, indolcarbazoles, anthracyclines, and angucyclines	[99,100]
		YC-17	[101]
		Chlortetracycline and other tetracycline derivatives	[102]
Mutagenesis of tailoring enzymes	P450 epoxidase inactivation	Pimaricin	[103]
	Ketoreductase inactivation	Mithramycin and derivatives	[104]
	Hydroxylase inactivation	Tautomycin	[105]
	Inactivation of flavin-dependant monooxygenase, phosphatase, oxidoreductase	Macbecin	[106]
Precursor alteration	Gene inactivation and engineering combined with precursor feeding	Rapamycins	[107]
		Balhimycin	[108]
		Novobiocin and chlorobiocin	[109]
		Geldanamycin	[110]
	Precursor alteration by synthetic biology approaches	36-methyl-FK506	[111]

[a]See also Table S2 in the supplementary material online.

the glycopeptide production by approximately threefold [44]. Recently, a combined approach of classical random mutagenesis and metabolic engineering resulted in enhanced FK506 production from *Streptomyces* sp. RM7011 [45].

Genetic engineering has also enabled the construction of non-natural analogs of specific molecules. These studies all focus on manipulation of the gene clusters that encode the enzymatic machinery for secondary metabolite biosynthesis. Two very important classes of secondary metabolites with antibiotic activity are polyketides and non-ribosomally synthesized peptides. Most complex polyketides (e.g., erythromycin) and non-ribosomally synthesized peptides (e.g., vancomycin) are assembled by large multifunctional proteins. These PKS and NRPS enzymes resemble molecular assembly lines (Box 1) that synthesize a precursor molecule by successive incorporation of polyketide extender units or amino acids, respectively. Both enzyme classes have a highly modularized organization; each module contains several functional domains which catalyze reactions required for biosynthesis of the backbone of the core molecule (Box 1).

The modularity and assembly-line logic of PKSs and NRPSs make manipulation of these mega-enzymes an attractive strategy to create analogs. Whole genes, modules, domains, or only their specificities can be 'mixed and matched' to generate derivatives or completely new compounds. There have been many successful examples of such 'mix-and-match' or 'plug-and-play' strategies (Figure 3; Table 3; Table S2 in the supplementary material online) [46]. One specific example is the incorporation of novel building blocks into the growing backbone. Acyltransferase (AT) and adenylation (A) domains act as 'gatekeepers' that determine which precursors are incorporated into the molecule during biosynthesis. Attempts to engineer these domains to alter their substrate specificities have resulted in the isolation of novel derivatives. Successful *in vivo* AT and A domain substitutions and derivative production have been described for erythromycin [47] and CDA [48], respectively. A very recent and interesting example is the successful reprogramming of a modular (type I) aureothin PKS into a synthase producing luteoreticulin, a compound which was initially isolated from a different strain [49].

Beyond the diversification of natural products by modification of the core assembly enzymes, tailoring enzymes can also be manipulated to construct novel derivatives of specific molecules (Figure 3; Table 3; Table S2 in the supplementary material online). Tailoring enzymes act after the biosynthesis of the core backbone is finished, and they introduce a wide variety of modifications into the molecule. Enzymes such as glycosyltransferases, halogenases, methyltransferases, and hydroxylases can decorate the precursor molecule with additional chemical moieties [50]. The inactivation or reprogramming of their specificities either removes the modification step or introduces chemical groups at new positions in the compound structure. Erythromycin [51], vancomycin [52], and doxorubicin [53] are pharmacologically relevant antibiotics that have all been structurally modified through manipulation of the tailoring enzymes.

Concluding remarks and future perspectives

The current period is likely to represent the beginning of a new renaissance for the field of antibiotics and other natural products discovery. Reasons for optimism include: an enormous pool of SMBGCs from both culturable and unculturable microbes for which the associated molecules have not yet been detected; the ability to capture and assemble these clusters much more easily than in the past; the continued advances in metabolic engineering of host strains to facilitate expression of the clusters, optimization of metabolic fluxes, and detection of the molecules; and improved understanding of the chemical and biological mechanisms of secondary metabolite biosynthesis. The availability of all this knowledge now can be used to rationally design cell factories for the production of antibiotics and other natural compounds. We anticipate that the future will bring even more exciting discoveries that will greatly aid actinomycete strain engineering and heterologous, combinatorial, modular expression of natural and synthetic genes. For example, genome-wide mapping of all transcription units and transcription start-sites has now been performed for different microorganisms [54,55], and the acquisition of similar datasets for selected actinomycete strains will allow more precise engineering of the genome. In addition, new impressive insights into the biochemistry and architecture of key enzymes for antibiotic biosynthesis (e.g., complex PKSs [56,57] and NRPSs [58]), and new bioinformatic [59] and synthetic biology approaches [60,61], will enable more accurate and directed engineering of the powerful assembly lines. The next few years promise to be an exciting time for metabolic engineering of actinomycetes and new antibiotic compound discovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tibtech.2014.10.009.

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