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Systems biology and biotechnology of *Streptomyces* species for the production of secondary metabolites

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ABSTRACT

Streptomyces species continue to attract attention as a source of novel medicinal compounds. Despite a long history of studies on these microorganisms, they still have many biochemical mysteries to be elucidated. Investigations of novel secondary metabolites and their biosynthetic gene clusters have been more systematized with high-throughput techniques through inspections of correlations among components of the primary and secondary metabolisms at the genome scale. Moreover, up-to-date information on the genome of *Streptomyces* species with emphasis on their secondary metabolism has been collected in the form of databases and knowledgebases, providing predictive information and enabling one to explore experimentally unrecognized biological spaces of secondary metabolism. Herein, we review recent trends in the systems biology and biotechnology of *Streptomyces* species.

Contents

Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; COBRA, constraint-based reconstruction and analysis; DoBISCUIT, Database Of BIoSynthesis clusters CUrated and InTegrated; MRSA, methicillin-resistant *Staphylococcus aureus*; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier protein; PKS, polyketide synthase; PrISM, Proteomic Investigation of Secondary Metabolism; SBSPKS, Structure Based Sequence Analysis of Polyketide Synthases.

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1. Introduction

Streptomyces species, belonging to the *Actinobacteria* family, are aerobic and Gram-positive soil bacteria that show filamentous growth from a single spore. As their filaments grow through tip extension and branching, they ultimately form a network of branched filaments called a substrate mycelium (Dyson, 2011). Upon environmental stresses, for example nutrient limitation, and on the solid cultivation condition, streptomycetes move from the vegetative phase (i.e., substrate mycelium) to a reproductive sporulation phase in the form of aerial multinucleated mycelium. On the other hand, they have a linear chromosome, approximately 8 Mb to 10 Mb depending on the specific species, with high GC content and several plasmids in a linear or circular form. One of the unique features of the genome in the *Streptomyces* species is the presence of biosynthetic gene clusters that encode enzymes contributing to the production of secondary metabolites with a variety of chemotypes, including polyketides, lactams, nonribosomal peptides, and terpenes (Fig. 1) (Nett et al., 2009). Many of the secondary metabolites are produced during the shifting phase from the substrate mycelium to sporulation, accompanied by morphological differentiation (e.g., formation of the aerial multinucleated mycelium) (Dyson, 2011; Flardh and Buttner, 2009).

Streptomyces species have been an important source of medicines, especially antibiotics (Fig. 1). From the late 1940s to the 1960s, also known as the golden age of antibiotics discovery, many antibiotics were isolated from various *Streptomyces* species and entered clinical use (Berdy, 2005). Although the portion of recently discovered antibiotics isolated from *Streptomyces* species has declined to about 20–30%, recent genome sequencing data indicate that this genus possesses the ability to produce many more bioactive secondary metabolites than had been previously appreciated, many of which have not yet been elucidated. The medical uses of these secondary metabolites are not just confined to antibiotics (e.g., daptomycin), but also include immunosuppressants (e.g., rapamycin), antifungals (e.g., amphotericin B), anticancers (e.g., doxorubicin), and antiparasitics (e.g., ivermectin) (Newman and Cragg, 2007). A major attention for the *Streptomyces* species comes from a current urgent need to discover novel antibacterial compounds because of the rapid rise in antibiotic-resistant microbial pathogens, for example methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative pathogens, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Bush et al., 2011; Fischbach and Walsh, 2009).

Consequently, the systematic investigation of *Streptomyces* species is becoming more important given that they are already a proven source of medically-useful compounds with diverse structures and that they have the potential to produce even more secondary metabolites than what has been isolated from them to date (Baltz, 2008, 2011; Craney et al., 2013). Importantly, vast amount of information collected on the *Streptomyces* species makes them better amenable to the application of high-throughput techniques (e.g., combination of genome mining and mass spectrometry) and gene manipulations to maximize their potential of producing potent antibiotics, compared to other antibiotics-producing microorganisms (Nett et al., 2009). The *Streptomyces* species also stand competitive in comparison with plant cells as extracting and

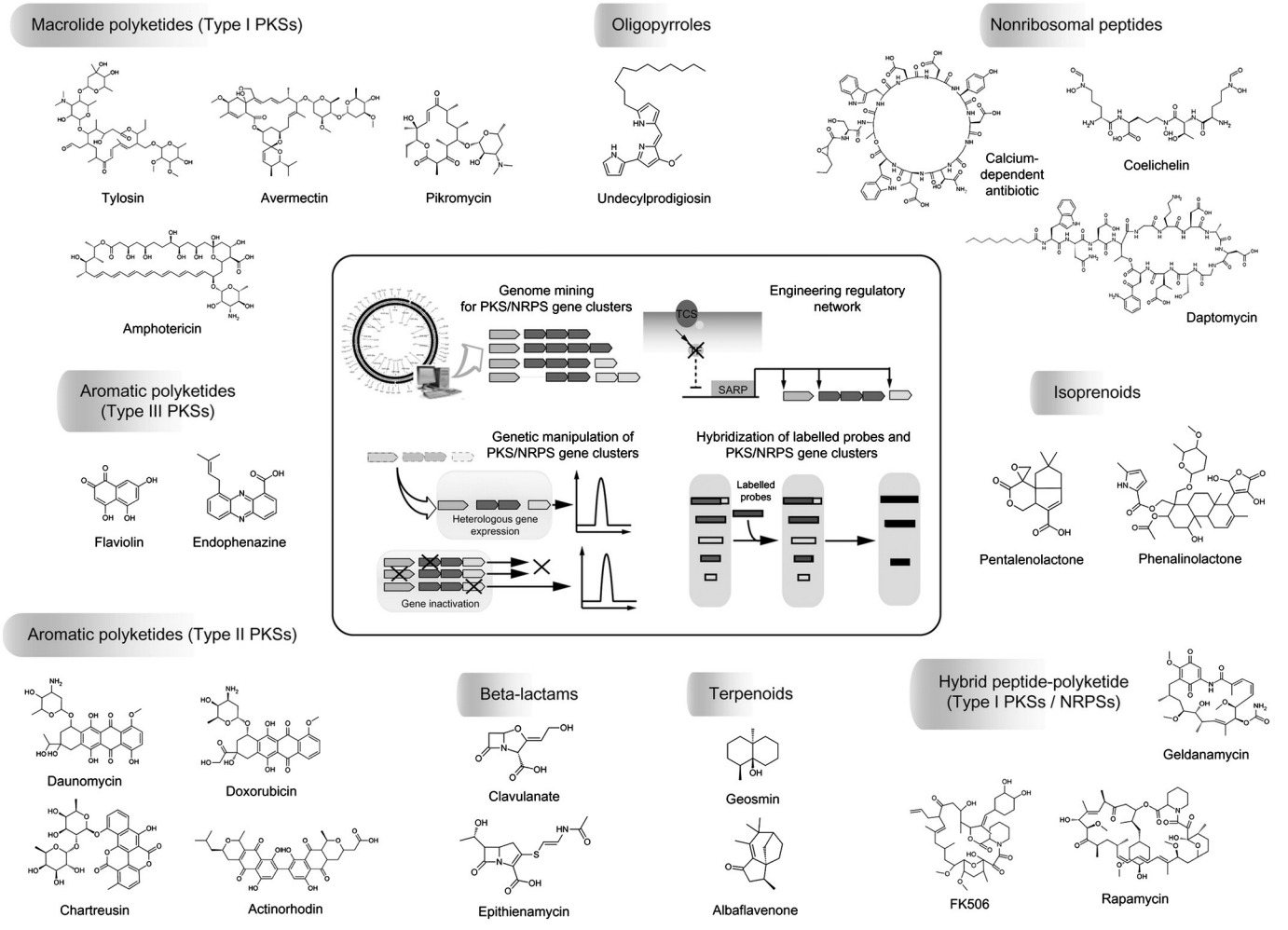

Fig. 1. Representative secondary metabolites produced from *Streptomyces* species with molecular and computational tools available shown in the middle. Parentheses next to each secondary metabolite type indicate the types of associated biosynthetic enzymes. Abbreviations: SARP, *Streptomyces* antibiotic regulatory protein; TCS, two-component system.

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characterizing bioactive compounds from plants are extremely difficult in addition to their long cultivation time (Xu, 2011). However, this is not to mean that all the other approaches, including chemical synthesis and identification of new antibiotic targets, are not necessary; they have their own strengths complementary to the engineering of *Streptomyces* species, and should also be actively pursued to enrich options for combating antibiotic-resistant pathogens (Bush et al., 2011).

Here, we review the biochemical analyses and metabolic engineering of *Streptomyces* species with emphasis on the uses of various systems biology tools, namely omics techniques, databases, knowledgebases, and constraint-based flux analysis, in order to boost the production of secondary metabolites. Because of the extensive scope of the literature on the *Streptomyces* species, detailed reviews relevant to discussions in each section are also provided.

2. Brief introduction to secondary metabolism of *Streptomyces* species

We first briefly introduce secondary metabolism of *Streptomyces* species in order to clearly grasp biological characteristics of these microorganisms, and justify the rationale of applying systems biological tools for their biochemical analyses and metabolic engineering. In this section, although polyketides and nonribosomal peptides are focused herein, reviews for other types of antibiotics are also provided.

2.1. Biosynthesis of secondary metabolites

Two large classes of secondary metabolites produced by *Streptomyces* species are polyketides and nonribosomal peptides, which have a wide variety of structural and physiological functions. Polyketide biosynthesis shares common mechanisms with fatty acids biosynthesis, in that each repetitive elongation step involves a Claisen condensation that incorporates the extender unit into the growing chain. However, polyketide biosynthesis differs from fatty acids biosynthesis because the former can utilize more diverse acyl-CoA units, for example malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA, and the β-carbon in the newly-incorporated extender unit can have different oxidation states. Furthermore, structural modification of secondary metabolites, including glycosylation, amination, and methylation, which takes place after the polyketide backbone has been synthesized, is another unique biochemical feature of secondary metabolite biosynthesis.

Polyketide synthases (PKSs), the enzyme complexes that produce secondary metabolites, can be categorized into three types depending on their structures and functions. Two (types I and II) are found in microorganisms, and type III PKSs are also found in *Streptomyces* species, but are concentrated mostly in plants (Yu et al., 2012). Type I PKSs (modular PKSs) are multifunctional and multimodular proteins producing mostly macrolides. They consist several modules, each of which is composed of domains with specific functions. Each module always carries three core domains: acyltransferase (AT), acyl carrier protein (ACP), and ketosynthase. These domains are responsible for elongating the polyketide backbone by incorporating a wide range of extender units (e.g., malonyl-CoA). Some modules also contain accessory domains, for example β-ketoreductase, dehydratase, and enoylreductase, that catalyze the reduction of the β-carbon in the extender unit in an NADPH-dependent manner. These accessory domains are present in some modules, but not others. When the polyketides reach the final module of the PKS, polyketide elongation is terminated by hydrolysis in the thioesterase domain.

Depending on the chromosomal site of the AT domain, the modular type I PKSs are further categorized into *cis*- and *trans*-AT type I PKSs. The latter corresponds to the type I PKSs without the AT domains, and in this case, the AT domain exists as a discrete enzyme apart from the PKS gene cluster. The *trans*-AT type I PKS contains a short fragment of the AT domain, which functions as a docking domain without catalytic activity and helps in connecting the discrete AT and other AT-less modules within the PKS (Musiol and Weber, 2012). Moreover, it should be noted that the type I PKSs also exist in an iterative mode in addition to the modular mode, but are found commonly in fungi, and rarely in the *Streptomyces* species. In contrast to the modular type I PKSs, the iterative type I PKSs have a single module with multiple domains that repeatedly catalyze reactions of chain elongation and β-carbon reduction (Moss et al., 2004).

In contrast to the type I PKSs, type II PKSs are a complex of individual monofunctional proteins, producing aromatic polyketides (e.g., tetracyclines and actinorhodin). Type II PKSs elongate polyketides through repetitive condensation reactions catalyzed by heterodimer-ketosynthase and ACP, and appear to have more substrate specificity by almost always utilizing acetyl-CoA and malonyl-CoA as starter and extender units, respectively. After the condensation procedure, the aromatic polyketides can be reduced by ketoreductase and post-translationally modified by aromatase, cyclase, and glycosylase.

Meanwhile, in contrast to the type I and II PKS systems, type III PKSs, homodimer of ketoreductase enzyme, does not use ACP for the carbon chain elongation and acts directly on the extender units in the form of acyl-CoA to catalyze iterative condensations of the extender units (Yu et al., 2012).

Nonribosomal peptide synthetases (NRPS) are also multimodular and multifunctional enzymes, similar to type I PKSs, and biosynthesize antibiotics that contain one or more D-amino acids. One example is the β-lactam class of antibiotics (Schwarzer et al., 2003). Each module in an NRPS usually consists of adenylation, peptidyl carrier protein (PCP or thiolation), and condensation domains. Epimerization domains are also commonly found in many NRPS modules; they convert the PCP-tethered aminoacyl substrate from the L to the D form. Epimerization is the most common route to obtain the D form of an amino acid during NRPS biosynthesis. Collectively, these domains function to elongate peptidyl-S-PCP with incoming amino acids, and transfer it to the next module. As in the type I and II PKSs, the elongation procedure is terminated by the thioesterase domain in the last module. More detailed descriptions of the biosynthetic mechanisms associated with PKSs and NRPSs are available elsewhere (Fischbach and Walsh, 2006; Rix et al., 2002; Walsh and Fischbach, 2010).

In many cases, hybrid secondary metabolites are also produced from various combinations: hybrid compounds of polyketides and peptides produced from the modular type I PKSs connected with the modules of NRPSs (Hertweck, 2009), and hybrid polyketides from combinations of the type I and II PKSs, the type I and type III PKSs, and fatty acid synthase and PKSs (Moore and Hertweck, 2002). Furthermore, these PKSs use and structurally modify dozens of starter and extender units as building precursors, enabling unique elongations, such as β-branching mechanisms (e.g., isoprenoid-like β-alkylation and Michael-type β-branching) (Bretschneider et al., 2013; Calderone, 2008), and additional modifications after the elongation step, for instance, O-heterocyclization (Hertweck, 2009). It should be noted that there also exist antibiotics other than polyketides and nonribosomal peptides, including peptide antibiotics (e.g., lantibiotics) (Claesen and Bibb, 2010; Martin, 1998; Voller et al., 2012; Zhang et al., 2011), and terpenoids (Gebhardt et al., 2011; Zhu et al., 2011), which are equally important, and many of their biosynthetic mechanisms are still awaiting to be elucidated.

2.2. Relationship between primary and secondary metabolisms

Primary metabolism significantly influences secondary metabolism by providing the precursor metabolites and reducing equivalents. Primary metabolites serve as building precursors for secondary metabolites, including acetyl-CoA, glucose-6-phosphate, glyceraldehyde-3-phosphate, α-ketoglutarate, and oxaloacetate, mostly generated from central carbon metabolism (Rokem et al., 2007). Also, metabolites outside central carbon metabolism contribute to the biosynthesis of the extender units, for instance L-valine for both methylmalonyl-CoA and

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ethylmalonyl-CoA, propionyl-CoA for methylmalonyl-CoA, and L-methionine for chloroethylmalonyl-CoA. In addition to the acyl-CoA form, there also exist extender units bound with ACP, such as methoxymalonyl-ACP and hydroxylmalonyl-ACP, in which case, 1,3 bisphospho-D-glycerate serves as a substrate for the elongation of polyketide chains (Chan et al., 2009). Because these metabolites are essential components of biomass, secondary metabolite biosynthesis inevitably competes with biomass formation; for example, acetyl-CoA and NADPH units used for polyketide biosynthesis cannot be used for fatty acid biosynthesis. The metabolic shift from primary to secondary metabolisms actually causes decreases in cellular growth and greater fluxes through the pentose phosphate pathway for more generation of NADPH (Borodina et al., 2008).

These tight connections between primary and secondary metabolisms suggest that primary metabolism should be systematically analyzed for the optimal operation of secondary metabolism. Relevant supportive studies include investigating the relationship between carbon fluxes towards biomass formation and antibiotic production by changing carbon and nitrogen sources (Wentzel et al., 2012) or varying initial seeding volumes of cells (Cheng et al., 2013) in cultivation media. For both studies, omics techniques revealed that reactions related to amino acid pathway (e.g., glutamate) helped in concentrating fluxes towards the biosynthesis of various precursors necessary for secondary metabolites. Genetic studies also manifested the importance of primary metabolism. Gene knockout of *fhaAB* encoding forkhead-associated proteins reduced TCA cycle activity, leading to the enhanced fluxes towards acetyl-CoA precursor and subsequently the actinorhodin biosynthesis (Jones et al., 2011). In the same line, knockout of SCO2951 and SCO5261 genes both encoding malic dehydrogenases indicates that these enzymes play a crucial role in balancing the biosynthesis of actinorhodin and fatty acids (Rodriguez et al., 2012). In addition to the bioprocess and genetic engineering means, chemical perturbation using small molecules from the Canadian Compound Collection also clearly revealed competitive relationships between primary and secondary metabolisms (Craney et al., 2012). The study shows that partial inhibition of fatty acid biosynthesis using a small molecule called ARC2, a tricosan-like molecule, enhanced the actinorhodin production by enriching the acetyl-CoA pool. This study demonstrates that reducing the building precursors fed into primary metabolism leads to the increased antibiotic production. Above studies suggest the issue of optimally allocating metabolic resources between primary and secondary metabolisms, wherein primary metabolism should be engineered in a way that enhances the pool of building blocks, while minimizing the impact to cellular growth (Olano et al., 2008).

2.3. Regulation and signal transduction of secondary metabolism

Regulation and signal transduction stimulated from environmental cues also play an important role in the control of secondary metabolite production in *Streptomyces* species. The biosynthesis of many secondary metabolites is often initiated when the cell senses a stressful condition of nutrient depletion (e.g., glucose, nitrogen, and phosphate) in the extracellular environment, or specific small signaling molecules, such as γ-butyrolactones (Liu et al., 2013; Rokem et al., 2007; van Wezel and McDowall, 2011). In this metabolic switching process, a variety of global transcriptional regulators, including AdpA (Ohnishi et al., 1999) and DasR (Rigali et al., 2008), and two-component systems, including AbsA1/A2 (Sheeler et al., 2005), AfsKRS (Umeyama et al., 2002) and PhoP/R (Sola-Landa et al., 2003), play roles that deliver these extracellular signals (e.g., nutritional stresses) to genes related to the biosynthesis of secondary metabolites. Because a wider range of genes are involved in the activation of secondary metabolism, high-throughput techniques have been particularly useful in elucidating hidden genome-wide interactions among genes (Table 1). A relevant study is the time-series transcriptome analysis of *Streptomyces coelicolor* throughout its cultivation to reveal dynamic changes in genome-wide gene expression patterns associated with signaling cascades and regulations during the metabolic shift from the primary to secondary metabolism (Nieselt et al., 2010). Regulatory and signaling networks of *Streptomyces* species with emphasis on secondary metabolite production have been extensively reviewed elsewhere (Chen et al., 2010; Dyson, 2011; Williamson et al., 2006).

It should be noted that the regulatory and signaling networks are interwoven into a complex network in *Streptomyces* species, as in other microorganisms, and their connecting points should be taken into account for more effective systems metabolic engineering. For example, *redZ*, the response regulator of a two-component system, is reported to specifically cross-regulate genes associated with the production of actinorhodin and calcium-dependent proteins (Huang et al., 2001). For the PhoP/R two-component system, PhoP directly binds to both global and pathway-specific transcription factors, including *afsS*, *atrA*, *cdaR*, and *scbR-scbA* genes associated with secondary metabolite production (Allenby et al., 2012). In metabolic engineering, such regulations usually hamper the overproduction of secondary metabolites, and hence often are the target of removal via genetic manipulations (Lee et al., 2012; Shao et al., in press). Effective tools and algorithms along with multiple omics data sets are further needed to accurately elucidate hidden relationships among transcription factors and signaling cues regulating the secondary metabolite production (Castro-Melchor et al., 2010).

3. Databases to knowledgebases for understanding and engineering secondary metabolism of *Streptomyces* species

More than 100 genome sequencing projects for the *Streptomyces* species have been completed or are in progress according to the Genomes Online Database (Pagani et al., 2012) as of August, 2013. Such genome sequences and annotation data have become important ingredients for genome mining that reveal novel gene clusters associated with biosynthesis of polyketides and nonribosomal peptides (Jenke-Kodama and Dittmann, 2009; Nett et al., 2009). For example, the genome mining of *S. avermitilis* showed that at least 20 gene clusters are putatively associated with secondary metabolite biosynthesis (Ikeda et al., 2003). This trend clearly highlights the importance of databases that enable user-defined access to the systematically organized genomic information with focus on the biosynthesis mechanisms of polyketides and nonribosomal peptides (Table 2).

Several databases exist that provide genomic information of *Streptomyces* species with emphasis on gene clusters involved in secondary metabolism. The first such database is Structure Based Sequence Analysis of Polyketide Synthases (SBSPKS), which contains 167 validated biosynthetic gene clusters with around 4400 catalytic domains for polyketides and nonribosomal peptides (Anand et al., 2010). A recently developed database, Database Of BIoSynthesis clusters CURated and InTegrated (DoBISCUIT), provides manually curated genome data with literature on more than 190 biosynthetic gene clusters for polyketides and nonribosomal peptides (Ichikawa et al., 2013). This database provides genome annotation data and relevant citations for not only secondary metabolites, but also those beyond secondary metabolism, including transporters, post-translational modifications, transcriptional regulations, and tolerance for self-protection against secondary metabolites. Resources also exist for identifying and annotating various types of secondary metabolic gene clusters. For instance, antiSMASH identifies gene clusters by annotating an input genome sequence, and categorizes them according to known secondary metabolite classes (Blin et al., 2013; Medema et al., 2011). PKMiner is a domain classifier predicting novel biosynthetic gene clusters of type II PKSs and aromatic polyketide chemotypes based on their annotated aromatase and cyclase domains (Kim and Yi, 2012). These resources demonstrate that significant amounts of genomic information on *Streptomyces* species have been accumulated, and more biochemical knowledge is actively being extracted through genome mining.

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Table 1
Applications of omics techniques to secondary metabolism of *Streptomyces* species. Cistrome refers to a set of genes that have sites for DNA binding factors, and are often analyzed with ChIP-on-chip assay. Abbreviations are: A-factor, 2-isocapryloyl-3R-hydroxymethyl-c-butyrolactone; CDA, calcium-dependent antibiotic; ChIP-on-chip assay, chromatin immunoprecipitation-on-chip assay; CSR, cluster-situated regulatory; iTRAQ, isobaric tag for relative and absolute quantitation; MI, early compartmentalized mycelium; MII, late multinucleated mycelium; and SILAC, stable isotope labeling by/with amino acids in cell culture.

Types of omics data	Host strains	Insights	Refs
Transcriptome	*Streptomyces coelicolor*	The cross-regulation by CSR genes, including *cdaR*, *actII-ORP4*, *redD* and *redZ*, controls the biosynthesis CDA, actinorhodin and undecylprodigiosin. CSRs show different levels of gene expressions along time series under *afsS* activation.	Huang et al. (2005)
Transcriptome	*Streptomyces coelicolor* A3(2)	Knockout of *afsS* gene affects the control of gene expression levels for 117 genes, many of which are associated with actinorhodin biosynthesis and response systems in stationary phase under depletions of phosphate, nitrogen, and sulfate.	Lian et al. (2008)
Transcriptome	*Streptomyces griseus*	Regulation study of A-factor with *S. griseus* Δ*afsA* mutant shows that A-factor likely induces expression of 152 genes, 74 of which are directly bound by A-factor. These genes are probably involved in morphogenesis and biosynthesis of secondary metabolites, including streptomycin and two putative NRPSs. Competitive electrophoretic mobility shift assay showed that AdpA induced by A-factor directly binds to 37 putative promoter regions, which upregulated 74 genes.	Hara et al. (2009)
Transcriptome	*Streptomyces avermitilis*	Analysis of *S. avermitilis* Δ*aveI* mutant reveals that *aveI* gene influences activation of biosynthesis gene clusters for avermectin and oligomycin, and genes associated with their precursor metabolites, such as those encoding crotonyl-CoA reductase. Genes for protein and lipid biosynthesis required for biomass formation were downregulated under *aveI* deletion condition.	Chen et al. (2009)
Transcriptome	*Streptomyces coelicolor*	Transcriptional regulatory network is constructed, which contains information on functional interactions between cistrons and transcriptional regulatory factors for various pathways, including those in secondary metabolism, thereby providing an overall architecture of the regulatory patterns in *S. coelicolor*.	Castro-Melchor et al. (2010)
Transcriptome	*Streptomyces coelicolor*	Switching from exponential growth to stationary phase causes upregulation of *phoP*/*R* and *redD* genes associated with secondary metabolism and downregulation of *glnk* and *pyrR* genes linked to nitrogen metabolism.	Nieselt et al. (2010)
Transcriptome and cistrome	*Streptomyces coelicolor*	In *S. griseus* Δ*adpA* mutant, AdpA, a global transcriptional regulator, transcriptionally activates 342 genes, and represses 224 genes, in cooperation with other regulatory proteins.	Higo et al. (2012)
Transcriptome and cistrome	*Streptomyces coelicolor*	*phoP* gene under phosphate depletion activates genes for phosphate uptake system and cell wall biosynthesis, while suppressing genes for nitrogen assimilation, oxidative phosphorylation, nucleotide biosynthesis, glycogen catabolism, and transcriptional regulators for production of antibiotics (e.g., actinorhodin) and morphological development.	Allenby et al. (2012)
Transcriptome and cistrome	*Streptomyces coelicolor*	Crp, a cyclic AMP receptor protein, influences secondary metabolism by binding to 8 secondary metabolite biosynthesis gene clusters and directly activates 6 gene clusters, including actinorhodin, undecylprodigiosin, calcium-dependent antibiotic, yellow-pigmented secondary metabolites, NRPSs, and albaflavenone.	Gao et al. (2012)
Transcriptome	*Streptomyces coelicolor*	Major genes involved in secondary metabolites (e.g., actinorhodin, undecylprodigiosin, CDA, yellow pigmented secondary metabolites, and geosmin) are highly associated with the MII phase.	Yague et al. (2013)
Transcriptome and proteome	*Streptomyces coelicolor*	Transcriptomic and proteomic analyses of *S. coelicolor bldA* gene knockout mutant identified increased ppGpp concentration and upregulated 147 BldA-controlled genes that are involved in gene clusters for actinorhodin, prodiginine, CDA, germicidin, coelichelin, and desferrioxamines.	Hesketh et al. (2007)
Transcriptome and proteome	*Streptomyces griseus*	Acidic pH perturbation was shown to strongly upregulate genes for sigma factors, including *sigH* (heat shock), *sigR* (oxidative stress), *sigB* (osmotic shock), and *hrdD* (pH shock).	Kim et al. (2008)
Transcriptome and proteome	*Streptomyces griseus*	Comparison of secreted proteins from wild-type and Δ*adpA* identifies 24 putative catabolic enzymes, including proteases and lipases, which seem to be associated with degradation of biomass components leaked from stationary phase.	Akanuma et al. (2009)
Transcriptome and proteome	*Streptomyces coelicolor*	Two-component system-associated SCO5785 activates biosynthetic genes of actinorhodin and undecylprodigiosin as well as sporulation.	Rozas et al. (2012)
Proteome from iTRAQ-labeling with LC–MS/MS	*Streptomyces coelicolor*	Genes for primary metabolism, including those associated with ribosomal proteins, TCA cycle, energy production, and lipid metabolism, were activated during MI phase, while genes involved in biosynthesis pathways of actinorhodin, type II polyketides, epimerase, and β-lactamase were upregulated during MII phase.	Manteca et al. (2010a)
Proteome from iTRAQ labeling/ LC-MS/MS	*Streptomyces coelicolor*	Biosynthesis of actinorhodin and ansamycins appears to be greater at MII phase than MI phase. Enzymes related to hydrophobic coating and final sporulation are more activated at late MII than early MII phase.	Manteca et al. (2010b)
Proteome with SILAC with iTRAQ reagents	*Streptomyces coelicolor*	Dynamic analysis of turnover rates from mRNA transcription to protein degradation during metabolic shift phase estimated degradation rates of 115 intracellular proteins.	Jayapal et al. (2010)
Fluxome using ^{13}C-metabolic flux analysis	*Streptomyces noursei*	TCA cycle and pentose phosphate pathway carried decreased and greater fluxes, respectively, as biomass formation decreased and nystatin production increased.	Jonsbu et al. (2001)
Fluxome using ^{13}C-metabolic flux analysis	*Streptomyces tenebrarius*	Fluxes through Embden–Meyerhof–Parnass pathway and pentose phosphate pathways are increased, whereas those in Entner–Doudoroff pathway are decreased during metabolic shift from growth phase to antibiotic tobramycin production phase.	Borodina et al. (2005b)
Fluxome using ^{13}C-metabolic flux analysis	*Streptomyces coelicolor*	Gene knockout of *pfkA2* gene causes fewer ^{13}C-labeled carbons in pyruvate, which means that NADPH production is increased by higher fluxes through pentose phosphate pathway.	Borodina et al. (2008)
Metabolome using LC–ESI–MS/ MS with silicon oil density centrifugation	*Streptomyces venezuelae*	Antibiotic precursor productions (e.g., acyl-CoA pools) increased when the cellular growth reached stationary phase and the supplementation of acyl-CoA into the growth medium increased polyketide production.	Park et al. (2007)

Combined with the knowledge of biochemistry of secondary metabolism in *Streptomyces* species, databases are evolving towards knowledgebases that provide more detailed predictive information that can be used in systems metabolic engineering. The carbon backbone of secondary metabolites can now be predicted using functionally characterized domains in putative biosynthetic gene clusters and their substrate specificity, especially for the modular Type I PKS systems. These tools are used to functionally classify and characterize each domain through homology-based and pattern recognition approaches (e.g., BLAST, HMMer3, and Glimmer); they extract useful information for the substrate specificity of AT domains in PKSs and adenylation domains in NRPSs, respectively, and predict carbon backbone structures. This feature has been incorporated into several databases and genome annotation tools, including SBSPKS, MapsiDB, and ClustScan for PKSs and antiSMASH and NRPSpredictor2 for NRPSs. In particular, NRPSpredictor2 database predicts substrate specificity of proteins encoded by NRPS gene clusters through support vector machine applied to physicochemical properties, such as polarity and hydrophobicity, of

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Table 2
Databases and knowledgebases specific to *Streptomyces* species. Abbreviations are: PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; and NIS, NRPS-independent siderophore.

Classification	Database name	URL	Comments	Refs
Genome annotation/mining database	StrepDB	http://strepdb.streptomyces.org.uk	Genome annotation server for six *Streptomyces* species	–
	antiSMASH	http://antismash.secondarymetabolites.org	Identification, annotation, and analysis of microbial and fungal PKS/NRPS genes responsible for biosynthesis of secondary metabolites	Blin et al. (2013)
	MapsiDB	http://gate.smallsoft.co.kr:8008/pks	Genome mining of type I (modular and iterative) PKS gene clusters	Tae et al. (2009)
	ClusterMine360	http://clustermine360.ca	Annotation of microbial PKS/NRPS gene clusters linked to antiSMASH	Conway and Boddy (2013)
	ClustScan	http://csdb.bioserv.pbf.hr/csdb/	Genome mining of PKS, NRPS, NIS synthase and hybrid synthase/synthetase gene clusters in actinobacteria	Starcevic et al. (2008)
	DoBISCUIT	http://www.bio.nite.go.jp/pks	Annotation of gene clusters necessary for biosynthesis of secondary metabolites based on literatures	Ichikawa et al. (2013)
	NaPDoS	http://napdos.ucsd.edu	Prediction of PKS/NRPS gene clusters and their potential secondary metabolites based on phylogenetic analysis	Ziemert et al. (2012)
	NP.searcher	http://dna.sherman.lsi.umich.edu	*In silico* prediction of structure of secondary metabolites by using genome mining based on annotated PKS/NRPS gene	Li et al. (2009)
	NRPSpredictor2	http://nrps.informatik.uni-tuebingen.de	Prediction of substrate specificity for adenylation domains of NRPSs	Rottig et al. (2011)
	NRPSsp	http://www.nrpssp.com	Prediction of substrate specificity for NRPS modules in bacteria and fungi	Prieto et al. (2012)
	PKMiner	http://pks.kaist.ac.kr/pkminer	Genome mining of type II PKS gene clusters	Kim and Yi (2012)
	SBSPKS	http://www.nii.ac.in/~pksdb/sbspks/master.html	Structural and sequence analyses with linker sequences between modules for PKS gene clusters	Anand et al. (2010)
Secondary metabolites database	NORINE	http://bioinfo.lifl.fr/norine/	List of 1122 nonribosomal peptides produced by NRPS gene clusters	Caboche et al. (2008)
	Novel Antibiotics Data Base	http://www.antibiotics.or.jp/journal/database/database-top.htm	List of 5430 novel antibiotics reported in the Journal of Antibiotics from 1947 to 2003	–
	StreptomeDB	http://www.pharmaceutical-bioinformatics.de/streptomedb/	List of 2444 natural products isolated from *Streptomyces* species, sorted with text mining and manual curation	Lucas et al. (2013)

the active site region in the NRPS adenylation domain (Rottig et al., 2011).

Despite better insights on secondary metabolism obtainable through the current databases and knowledgebases, they still have limitations to overcome. For instance, the homology-based and pattern recognition approaches preclude the accurate prediction of substrate specificity of domains for unusual substrates, for example, amino acids in the NRPS gene clusters and substrates other than malonyl-CoA and methylmalonyl-CoA in the PKS gene clusters. This is due to a relative lack of structural information on active sites of adenylation and AT domains binding unusual substrates (Yadav et al., 2003). Furthermore, predicting precise chemical structures of putative polyketides and nonribosomal peptides beyond their carbon backbones is limited by the lack of information on biochemical mechanisms associated with glycosylation, methylation, halogenation, and cyclization. Although NP.searcher also considers post-translational modifications to predict chemical structure for a novel secondary metabolite, their prediction accuracy needs to be further improved (Li et al., 2009). Despite these challenges, genome mining in combination with various biochemical and high-throughput techniques has been carried out, for instance Proteomic Investigation of Secondary Metabolism (PrISM) (Bumpus et al., 2009; Chen et al., 2012) discussed below, which will allow elucidation of more putative biosynthetic gene clusters and their resulting secondary metabolites.

4. Constraint-based flux analysis of *Streptomyces* species metabolism

As mentioned above, a deep understanding of primary metabolism is very important to studies of secondary metabolism as primary metabolism provides necessary precursors and cofactors for the production of secondary metabolites. One way to systematically analyze both primary and secondary metabolisms is constraint-based reconstruction and analysis (COBRA) of metabolic networks (Orth et al., 2010). In contrast to signaling and transcriptional regulatory networks, metabolism is more amenable to modeling at large scale because metabolic reactions are relatively well defined in terms of biochemistry, and various assumptions, such as pseudo-steady state, are applicable. In particular, after the release of genomic data for *Streptomyces* species (Bentley et al., 2002; Ikeda et al., 2003), genome-scale metabolic models specific to this genus began to be actively reconstructed (Borodina et al., 2005a).

Constraint-based flux analyses, an approach frequently employed to simulate metabolic network models, have been used to design strategies of systems metabolic engineering for different *Streptomyces* species (Table 3). The identification of genes to manipulate in targeted producing strains (Huang et al., 2013) and analysis of intracellular flux distributions in a network model in combination with omics data (Alam et al., 2010; Borodina et al., 2008), both at a genome level, are not novel areas of the COBRA approach, and have also been applied to *Streptomyces* species. Also, the analysis of the metabolic network models with omics data enables not only their validations, for instance with ^{13}C-based flux data (Borodina et al., 2008), but also the identification of previously unknown regulatory mechanisms within secondary metabolism (Alam et al., 2010). In the latter case, the comparison of transcriptome data with predicted flux data enables closer investigations of genes and reactions in secondary metabolism showing inconsistent mutual activities caused by regulations that cannot be observed from constraint-based flux analysis alone (Alam et al., 2010).

The generation of genome-scale metabolic models for *Streptomyces* species can now be accomplished in a high-throughput manner, for instance by using Model SEED (Henry et al., 2010). This capability presents advantages when choosing a heterologous production host as it enables comparative analyses of several production hosts in a short period of time (Zakrzewski et al., 2012). For example, with the Model SEED, comparative flux analyses of genome-scale metabolic models for 38 actinobacteria with respect to their biomass and secondary metabolite production were performed (Zakrzewski et al., 2012). In this

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Table 3
Constraint-based reconstruction and analysis approaches to the study of *Streptomyces* species. CDA herein stands for calcium-dependent antibiotics.

Host organism	Metabolic model	Simulation methods	Insights	Refs
Streptomyces coelicolor	Metabolic model having over 200 reactions	Maximizing biomass formation rate at limited consumption rates of carbon, nitrogen, sulfur, phosphate, and potassium	Limited nitrogen uptake leads to the highest production rate of actinorhodin biosynthesis and the lowest maintenance energy requirement.	Naeimpoor and Mavituna (2000)
Streptomyces coelicolor	Genome-scale metabolic model having over 400 reactions	Maximizing CDA production rate under limited glucose consumption rate and constrained biomass formation rate	Fluxes through nitrogen assimilation, pentose phosphate pathway, and reactions associated with prephenate and α-ketoglutarate are positively correlated with CDA production rate.	Kim et al. (2004)
		Single gene knockout simulations to improve CDA production rate	CDA production rate is increased by inactivation of anthranilate synthase and glutamate synthase.	
Streptomyces coelicolor A3(2)	Genome-scale metabolic model based on 711 genes, having 971 reactions and 500 metabolites	Maximizing antibiotic production rate under the minimal glucose medium with a constrained specific growth rate	*S. coelicolor* more effectively produces polyketides because of its efficient production of methylmalonyl-CoA, butanoyl-CoA, and propionyl-CoA, compared to *S. cerevisiae*.	Borodina et al. (2005a)
Streptomyces peucetius var. *caesius* N47	Genome-scale metabolic model having 624 reactions and 515 metabolites	Flux balance analysis with changes in temperature, pH, and airflow in a bioreactor	The increase of pH from 6.5 to 7.5 appears to be the most influential factor among the three examined perturbations for the metabolic shift from the cellular growth to the ε-rhodomycinone production phase.	Kiviharju et al. (2007)
Streptomyces coelicolor A3(2)	Genome-scale metabolic model having unique 700 reactions	Constraint-based flux analysis of *pfkA* gene knockout mutant using minimization of metabolic adjustment	Decrease in the Embden–Meyerhof–Parnass pathway fluxes by deletion of *pfkA2* gene enhances fluxes through pentose phosphate pathway and production rates of secondary metabolites, including actinorodin and CDA.	Borodina et al. (2008)
Streptomyces clavuligerus ATCC 27064	Genome-scale metabolic model based on 864 genes, having 1492 reactions and 1173 metabolites	Single gene knockout simulation for reactions associated with pSLC4 megaplasmid in *S. clavuligerus* ATCC 27064 under minimal medium condition	pSCL4 megaplasmid in *S. clavuligerus* does not contain genes essential for the primary metabolism.	Medema et al. (2010)
Streptomyces coelicolor	Genome-scale metabolic model having 1015 reactions and 643 metabolites	Maximizing biomass formation rate under defined minimal medium during growth and antibiotic production phases	Comparisons of previously reported transcriptome data (Nieselt et al., 2010) and the predicted metabolic flux values from this study show high consistency during metabolic switch from 20 h to 60 h for the following pathways: arginine, cysteine, glutamate, glutamine, glycine, fatty acid, histidine, homoserine, isoleucine, leucine, lysine, and methionine, as well as actinorhodin and undecylprodigiosin. Metabolic pathways dedicated to biomass formation are downregulated, while antibiotic biosynthesizing pathways are upregulated when carbon, nitrogen, and phosphate sources are depleted.	Alam et al. (2010)
Streptomyces lividans and *Streptomyces coelicolor*	Information not available for *S. lividans* metabolic model	Maximizing biomass formation rate under limited palmitate as a sole carbon source	*S. lividans* without glyoxylate shunt did not grow in the medium with limited palmitate as a sole carbon source, indicating that *S. lividans* may operate alternative reactions for glyoxylate bypass pathway.	Lewis et al. (2010)
Streptomyces lividans TK24	Genome-scale metabolic model having 702 reactions and 602 metabolites	Constrain-based flux analyses with a unique combination of four objective functions under complex medium: 1) minimization of the total sum of absolute fluxes, 2) the quadratic sum of fluxes, 3) the number of non-zero reactions, and 4) maximization of ATP yield per flux unit	Constraint-based flux analyses (left) revealed that maximizing ATP yields per flux unit best predicts intracellular fluxes under complex medium. The predicted intracellular flux distributions showed that NADPH is largely produced by TCA cycle and NAD(P)-transhydrogenase, not by pentose phosphate pathway under complex medium. Adding glutamate and aspartate further activated malic enzyme and phosphoenolpyruvate carboxykinase, which increased specific growth rate under complex medium.	D'Huys et al. (2012)
Streptomyces roseosprorus	A metabolic model having 138 reactions and 109 metabolites	Comparison of flux distributions under two optimizations: maximization of daptomycin biosynthesis rate and specific growth rate	Comparison of flux ratios from two different objectives predicted *zwf2*, *dptI* and *dptJ* as overexpression targets to enhance daptomycin production, which was experimentally validated.	Huang et al. (2012)
38 actinobacterial species	All genome-scale metabolic models for 38 strains generated by Model SEED, but model statistics not available	Maximizing production rates of 15 antibiotics for each 38 actinobacterial strain under defined minimal medium	Microorganisms that have a high number of gene clusters for secondary metabolites do not necessarily show a strong correlation with production rates of the examined antibiotics.	Zakrzewski et al. (2012)
		Trade-offs between biomass formation and secondary metabolites production	The degree of metabolite precursors shared by pathways for biomass formation and secondary metabolite production differs for each organism, and it may be possible that a certain antibiotic be produced without a decrease in the biomass formation.	

study, biosynthesis pathways for several natural products were inserted into each model. Simulations provided insights on the selection of the most suitable strains for the production of particular secondary metabolites and their corresponding metabolic status. In particular, overproduction capability for secondary metabolites is not always positively correlated with the versatility of biosynthesis metabolism. As genomes of more than a hundred *Streptomyces* species will become available in the near future, high-throughput generation and investigation of metabolic models of these species will be more important in systems metabolic engineering.

Also, simulation of genome-scale metabolic network models has provided insights on bioprocess engineering aspects, especially the effects of using complex media, which is particularly important for the condition-controlled cultivation of *Streptomyces* species; simulations under complex media have rarely been systematically investigated before. In a relevant study, in contrast to traditional constraint-based metabolic simulations under defined media with single carbon sources, *in silico* simulations were performed in order to identify metabolic flux distributions in *S. lividans* under complex media (D'Huys et al., 2012). Through random sampling and a unique combination of objective functions (e.g., minimization of the total sum of absolute fluxes, the quadratic sum of fluxes, the number of non-zero reactions, and maximization of ATP yield per flux unit) under complex media, it was shown that a nutrient-enriched medium does not necessarily lead to optimal biomass production. Although this model is aimed at recombinant protein production rather than secondary metabolites, this approach gives novel insights on the importance of bioprocess engineering that governs environmental conditions.

Finally, despite improved accuracy and consistency of the metabolic network models, the current COBRA approaches have inherent limitations for the analysis of secondary metabolism because they are only applicable to predicting fluxes in primary metabolism during mid-exponential growth phase. Therefore, it is necessary to identify novel objective functions that properly describe the metabolic shift from exponential growth phase to stationary phase producing secondary metabolites in addition to integrating the metabolic network models with omics data (Khannapho et al., 2008). Approaches based on ordinary differential equations and kinetic parameters for regulatory networks are one of the options that could supplement constraint-based flux analysis (Mehra et al., 2008). For instance, a bistable switch ScbA-ScbR network regulating secondary metabolite production in *S. coelicolor* was developed with 17 kinetic equations and 21 parameters. This dynamic model discovered that ScbA functions with ScbR through positive feedback as consistent with experimental data. By identifying accurate kinetic parameters for enzymes in regulation cascades, such as AfsA and A-factor, the optimal production of secondary metabolites should be accurately simulated. Furthermore, future challenges will be to integrate the COBRA approach with different types of modeling approaches, thereby more precisely addressing complex biological systems (Lewis et al., 2012).

5. Systems metabolic engineering of *Streptomyces* species

5.1. Enhanced production of secondary metabolites

Rational metabolic engineering has played a role in strain improvement for the overproduction of various secondary metabolites using *Streptomyces* species, as in the production of many primary metabolites (Li and Townsend, 2006; Madduri et al., 1998; Palaniappan et al., 2003). In the past, *Streptomyces* species had successfully been engineered based on intuition for the biosynthesis mechanisms of secondary metabolites. Representative engineering approaches (Pickens et al., 2011) include: improving precursor and cofactor pools (Ryu et al., 2006), removing competing pathways (Komatsu et al., 2010), overexpressing transcriptional regulators positively affecting antibiotic production (Chen et al., 2008), improving enzyme conversion rates for bottleneck reactions (Jiang et al., 2013), and overexpressing genes conferring tolerance for the toxicity of produced antibiotics (Malla et al., 2010). However, as with producing primary metabolites, it is likely that additional unrecognized gene targets still exist that can lead to enhanced production of target secondary metabolites. This rational metabolic engineering based on intuition and already known information can be aided with systems biological concepts and tools that provide counterintuitive targets for gene manipulations and bioprocess engineering for the maximal production of secondary metabolites of interest (Lee et al., 2012).

The high-throughput techniques and their resulting omics data (Table 1 and 2) provide genome-wide correlations between individual genes and a target secondary metabolite, thereby producing a list of additional gene targets for prioritization at both transcriptional regulatory and metabolic levels. A relevant approach is comparative transcriptome analysis, which was successfully employed to enhance the production of actinorhodin in *S. coelicolor* (Fig. 2A) (Kim et al., 2011). In this study, gene expression patterns for a double knockout mutant strain (Δ*wblA* and ΔSCO1712) and the wild type strain were investigated using microarray data. The two genes that were deleted are both downregulators of actinorhodin biosynthesis. A comparison of the two transcriptome data sets revealed that 14 genes were consistently expressed even in the absence of the two downregulators. Among these genes, disruption of the *pfkA2* gene (SCO5426) encoding one of three 6-phosphofructokinase isoenzymes in *S. coelicolor* was reported to enhance the production of actinorhodin according to a previous ^{13}C-based flux analysis (Borodina et al., 2008). Blockage of glycolysis by inactivating the *pfkA2* gene redirected fluxes towards the pentose phosphate pathway, which increased the NADPH production and enriched an acetyl-CoA pool, both necessary for the production of actinorhodin. Cultivation experiments of the triple gene knockout mutant (Δ*wblA*, ΔSCO1712, and ΔSCO5426) showed that the titer of its actinorhodin production was 1.3 and 1.7 times greater than that of the double knockout mutant and the single gene knockout mutant (Δ*wblA*), respectively (Kim et al., 2011). This study is one example of the application of transcriptome data in systems metabolic engineering. Many related methods, for instance the simultaneous acquisition and analysis of several omics data sets and ^{13}C-based flux analysis, will provide more complete pictures to identify novel gene targets and insights.

As with the transcriptome analysis, constraint-based flux analysis is also a powerful tool that can contribute to systems metabolic engineering of *Streptomyces* species. Constraint-based flux analysis and subsequent genetic manipulations were successfully applied jointly to boost production of FK506 (Tacrolimus), an immunosuppressant used during allogeneic organ transplant operations, from *Streptomyces tsukubaensis* (Fig. 2B) (Huang et al., 2013). First, in order to select gene targets to increase FK506 production, a genome-scale metabolic model of *S. tsukubaensis* was reconstructed and subjected to a simulation method called minimization of metabolic adjustment (Segre et al., 2002). For the prediction of overexpression targets, the maximal specific growth rate was fixed, and non-zero fluxes were increased and selected as targets if they contributed to FK506 production. From these simulations, two gene knockout targets (*gdhA*, *ppc*) and four gene amplification targets (*dahp*, *gdhA*, *accA2*, and *zwf2*) were selected. The mutant that produced FK506 in the largest amount was one in which *gdhA* was deleted and *dahp*, *accA2*, and *zwf2* were overexpressed, resulting in a 2.8-fold increase compared to the wild-type. Although this study is a successful example of using constraint-based flux analysis, this *in silico* approach should be used with caution as it is not ideal for the simulation of secondary metabolism. In such cases, complementary approaches, including both omics data (Kim et al., 2011) and precise kinetics (Lee et al., 2008), should be considered for integrative analysis.

Systems metabolic engineering also considers the effects of variables associated with both up- and down-stream processes because they confer additional effects on microbial metabolism. Recent relevant examples include effects of varying initial seeding volumes (Fig. 2C) (Cheng et al., 2013) and components in the medium (Wentzel et al., 2012) for cell

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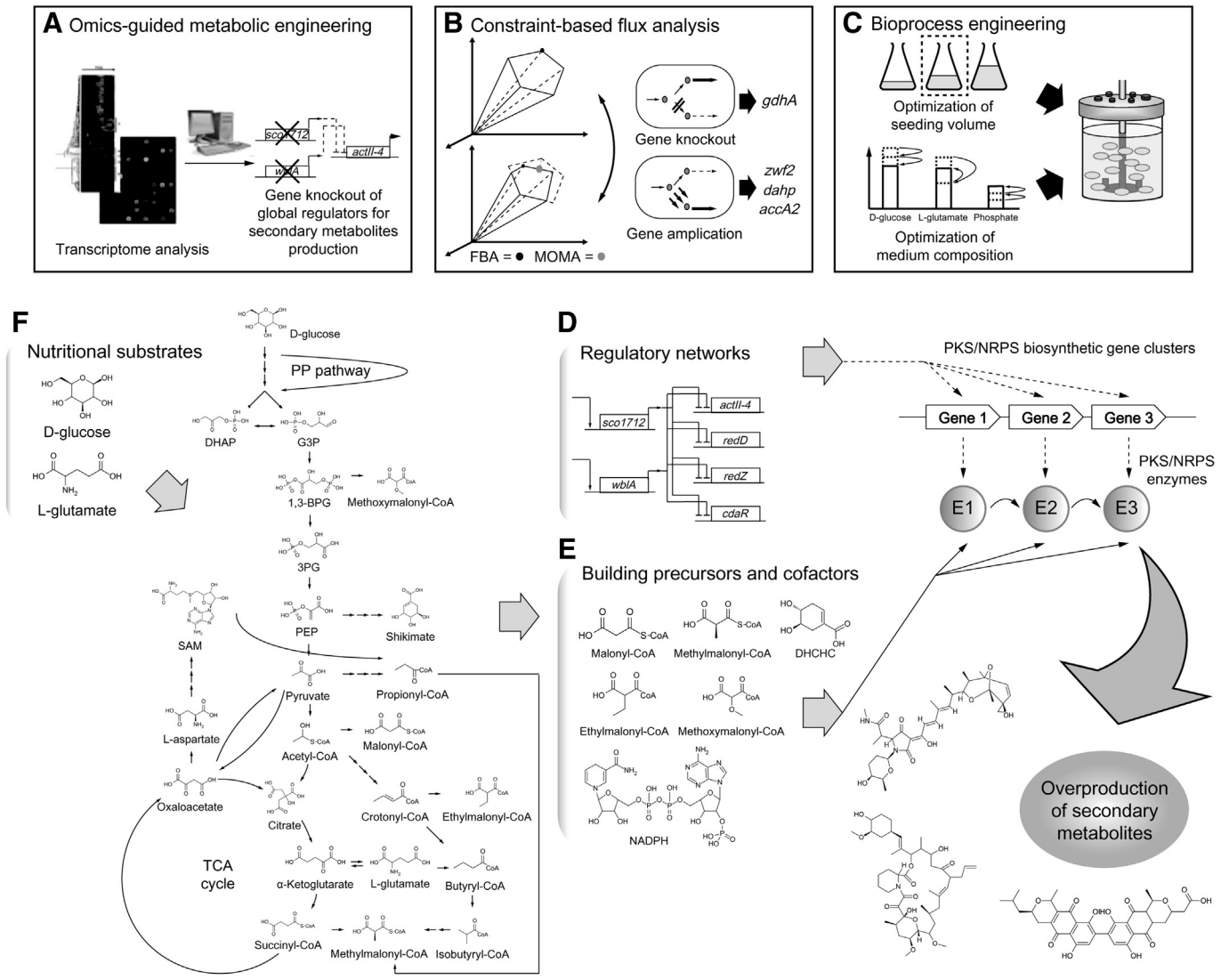

Fig. 2. Metabolic engineering aided with systems biological tools for the enhanced production of secondary metabolites. Production titer of secondary metabolites can be improved by knocking out global regulators associated with secondary metabolite biosynthesis based on transcriptome analysis (A and D), inactivating and/or amplifying genes predicted using constraint-based flux analysis (B and E), and varying initial seeding volumes and medium compositions (C and F). In constraint-based flux analysis, MOMA enables realistic prediction of suboptimal intracellular fluxes in gene knockout mutants (Segre et al., 2002), while FBA provides optimal flux values simply by maximizing the specific growth rate under the given condition. Abbreviations of metabolites are: 1,3-BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; DHCHC, 3,4-dihydroxycyclohexanecarboxylate; DHAP, dihydroxyacetone phosphate; FBA, flux balance analysis; G3P, glyceraldehyde-3-phosphate; MOMA, minimization of metabolic adjustment; PEP, phosphoenolpyruvate; PP, pentose phosphate; and SAM, S-adenosyl-L-methionine.

cultivation and secondary metabolite production. Initial seeding volume, represented by pitching ratio, was shown to affect the production titer of streptolydigin from *Streptomyces lydicus* (Cheng et al., 2013). Streptolydigin production was greater at 30% pitching ratio (v/v), compared to 1% or 10% ratio. Proteome and metabolome analyses showed that the pitching ratio influenced the activity of several amino acid pathways, especially glutamate and proline, both precursors of streptolydigin. Because glutamate and proline are actively drawn towards streptolydigin biosynthesis, they needed to be provided exogenously, which can increase the yield of streptolydigin production. In the same manner, an optimal minimal medium was designed to produce actinorhodin derivatives and undecylprodigiosin from *S. coelicolor*, which is critical in the overall cost of bioprocesses (Wentzel et al., 2012). A primary consideration was to ensure that the defined medium produces sufficient biomass concentration and maximal titer of the target secondary metabolite production. Due to this, the effects of feeding D-glucose and L-glutamate were investigated in depth under various fermentation conditions and also using ^{13}C-labeled metabolite analysis, revealing that L-glutamate appears to be a preferred C and N source; L-glutamate is more efficient in supplying acetyl-CoA, a precursor of actinorhodin and undecylprodigiosin (Wentzel et al., 2012). These cultivation conditions should be systematically taken into account and properly controlled for consistent systems biology studies since such environmental variables can obscure the effects of gene manipulations conducted during strain development.

5.2. Production of novel secondary metabolites

Identifying novel secondary metabolites and their biosynthetic mechanisms is another challenge of *Streptomyces* species in addition to overproduction of already known compounds. Using *Streptomyces* species, such novel compounds have typically been sought through combinatorial biosynthesis approaches (Wong and Khosla, 2012), including rearrangement of biosynthetic modules (Menzella et al., 2005), improving substrate promiscuity of biosynthetic enzymes (Koryakina et al., 2013; Sundermann et al., 2013), and diversified glycosylation systems

(Han et al., 2011). However, our objective here is to identify novel compounds that can actually impact the medical problems instead of simply creating a compound library. To address this, we discuss representative systems biology approaches that suggest context-specific solutions, largely by conducting omics data analysis (Bumpus et al., 2009; Chen et al., 2012; Kaysser et al., 2011; Li and Walsh, 2010), adaptive laboratory evolution (Charusanti et al., 2012), and synthetic biological approaches (Baltz, 2011; Shao et al., in press).

Conventional systems biological approaches have taken advantage of the secondary metabolite producers' genomic information that is easily obtainable. The underlying approach is to search for homologues of secondary metabolite-producing genes in the target microorganism of interest (Kaysser et al., 2011; Li and Walsh, 2010). This sequence similarity approach often comes with the rationale of conserved biochemical reactions (i.e., modules and domains of PKSs and NRPSs discussed above) that biosynthesize polyketides and nonribosomal peptides, and molecular biology involving gene knockout mutations and heterologous cloning to examine proper product formation. Using the conserved biochemistry of polyketides and nonribosomal peptides, and their chemical structures, it is possible to anticipate their potential biosynthetic pathways, which then facilitates identification of their gene clusters in another microorganism's genome. Ideally, identification of novel gene clusters can subsequently lead to systems metabolic engineering for the enhanced production of secondary metabolites (Gottelt et al., 2010). However, this homology-based approach raises a concern that it might not be suitable for screening novel compounds aiming at specific targets, such as pathogens or cancers, due to the following reasons: 1) certain secondary metabolites are produced only under special circumstances (e.g., unusual medium composition or co-cultivation system) other than typical lab conditions; and 2) relationships between the compound of interest and their biosynthetic genes should be known a priori.

Systems biology offers several approaches to overcome the aforementioned limitations of the homology-based approach. Following are representative studies driven towards identification and production of novel secondary metabolites; other approaches with similar fundamentals are also discussed in recent reviews (Baltz, 2011; Chiang et al., 2011; Frasch et al., in press). The first category of the systems biological approaches is the combination of different high-throughput techniques, for example mass spectrometry combined with genome mining as in the case of PrISM (Bumpus et al., 2009; Chen et al., 2012). This combined systematic approach identifies novel secondary metabolites and associates them with previously unknown but relevant gene clusters (Fig. 3A). The strength of this proteomic approach is its ability to detect expressed genes and their products that are often not observable under lab conditions. For example, PrISM subjects NRPSs and PKSs isolated using SDS-PAGE to LC–MS/MS analysis, and the resulting raw LC–MS/MS data are searched against the protein database NCBInr to identify corresponding gene clusters. For the peptides with low to no homologies, *de novo* peptide sequencing was conducted, and searched against a custom database. In either case, the target peptides were reverse translated into nucleotides for PCR amplifications, so that each amplified peptide can be mapped to conserved domains of NRPSs and PKSs, and the corresponding gene clusters can subsequently be identified. DNA sequence for novel NRPSs and PKSs and LC–MS analysis of the cell supernatant can be used to deduce the structure of unknown secondary metabolites. This platform was applied to 26 actinomycetes with unsequenced genomes, and led to the identification of ten NRPSs/PKSs gene clusters from six strains (Chen et al., 2012). This could be a valuable tool for *in situ* discovery of novel gene clusters and their products under specific conditions of interest. An orthogonal active site identification system is another similar proteome-level identification of PKSs and NRPSs, but in this approach, probes specific to the active sites of ACP and thioesterase were used to enrich and subject them to multidimensional LC–MS (Meier et al., 2009). Another combined approach of mass spectrometry and genome mining, called natural product peptidogenomics, was also successfully used to identify chemical structures of target peptides and their respective biosynthetic gene clusters using peptide samples (Kersten et al., 2011). As with the PrISM, combined analysis of peptide sequence tags using mass spectrometry and subsequent computational searches elucidated structures of stendomycin I and its analogs along with their gene sequence clusters as a demonstration. Ideally, these combined approaches can be followed by systems metabolic engineering for the overproduction of novel secondary metabolites.

In addition to the use of high-throughput techniques, microbial adaptive evolution can be a very powerful approach for enabling *Streptomyces* species to produce novel metabolites. This potential was demonstrated with a so-called competition-based adaptive laboratory evolution platform (Fig. 3B) (Charusanti et al., 2012). In this platform, *Streptomyces clavuligerus* was inoculated onto agar media, and subsequently challenged with the pathogen MRSA strain N315. The core idea of this platform is that repeated co-cultivation would stimulate *S. clavuligerus* to produce antibiotics against the competing pathogen. Replicates of *S. clavuligerus* were isolated from the co-cultivation system and re-challenged with the MRSA. This cycle of MRSA challenge and separation of *S. clavuligerus* was repeated until the zone of inhibition, an indicator of the growth inhibition of MRSA on the agar plates, became stable and larger than any previously observed ones. Although a final strain appeared to produce holomycin, a known compound, according to subsequent chemical analyses, it is possible to produce novel compounds because other replicates of *S. clavuligerus* did not produce holomycin. Competition against other pathogens can also be an intriguing option for the production of pathogen-specific antimicrobials. Finally, as genome sequencing of the final evolved strains pinpoints specific mutations that took place throughout the course of adaptive laboratory evolution, rational construction of the evolved strain should also be possible (Atsumi et al., 2010; Utrilla et al., 2012).

Finally, now with strong molecular tools, synthetic biological strategies can be deployed to identify and produce novel secondary metabolites from silent gene clusters that are not expressed under the typical lab settings (Baltz, 2011; Shao et al., in press). Recently, a plug-and-play scaffold was demonstrated to produce spectinabilin using its silent gene cluster in a heterologous host. This scaffold has promoter and helper modules, each of which ensures the transcription of the silent gene clusters and DNA replication, respectively, in a heterologous production host under the desired experimental condition. Hence, simply inserting the target silent gene cluster into the scaffold would enable its expression free of complex repressive regulations. The key is to select an ideal heterologous host and a set of strong promoters for the proper expression of the silent gene cluster. Although novel compounds were not mentioned in Shao et al. (in press), this synthetic biological tool is expected to contribute to the efficient identification of novel compounds with the additional aid of mass spectrometry.

6. Conclusions

Although *Streptomyces* species have long been recognized and employed as a source of medically useful compounds, they still have many biochemical mysteries to be elucidated. As such, the search for novel secondary metabolites and their biosynthetic gene clusters is the primary objective of currently ongoing studies. Systems biological approaches with high-throughput techniques have also been useful in the discovery and engineering studies of *Streptomyces* species as they allowed inspection of correlations among components of primary and secondary metabolisms at the genome scale. Moreover, because *Streptomyces* species have very conserved reactions in biosynthetic pathways of secondary metabolites, it is possible to make predictions about final chemical structures of polyketides or nonribosomal peptides, or the set of genes involved in their biosynthesis. This unique feature progresses relevant genomic databases on *Streptomyces* species towards knowledgebases, enabling one to explore experimentally

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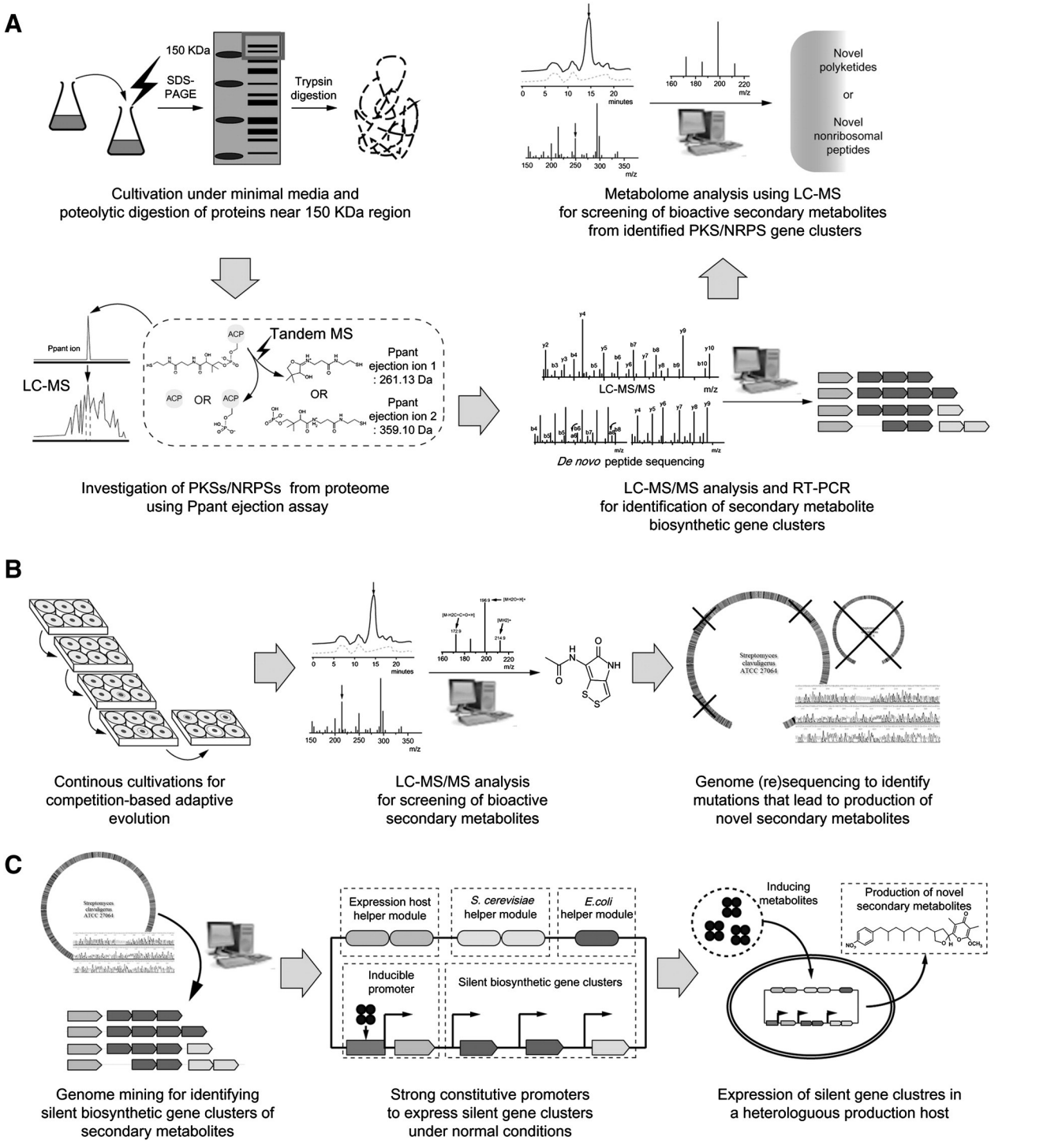

Fig. 3. Use of mass spectrometry-based proteomic approach, PrISM, (A), competition-based adaptive evolution platform (B), and synthetic biology-based plug-and-play scaffold (C) for the identification of novel secondary metabolites. (A) Phosphopantetheinyl (Ppant) ejection assay is critical in PrISM, which detects ACPs bound to the PKSs and NRPSs, thereby allowing them to separate from the rest of the proteins. (C) Genome mining to identify silent gene clusters for novel secondary metabolites may be employed first. Also, in the scaffold, helper modules contain genes necessary to maintain and replicate plasmids, mainly origins of replication and selection markers. Promoters without the sign of inducing metabolites indicate strong constitutive ones.

unrecognized biological spaces of secondary metabolism. With these tools and insights, it is anticipated that systems metabolic engineering of *Streptomyces* species will more precisely be performed to overproduce secondary metabolites or identify novel ones, as revealed in the form of mass spectrometry combined with genome mining, competition-based adaptive laboratory evolution platform and synthetic biological approach.

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