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REVIEW ARTICLE

Towards a new science of secondary metabolism

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Secondary metabolites are a reliable and very important source of medicinal compounds. While these molecules have been mined extensively, genome sequencing has suggested that there is a great deal of chemical diversity and bioactivity that remains to be discovered and characterized. A central challenge to the field is that many of the novel or poorly understood molecules are expressed at low levels in the laboratory—such molecules are often described as the 'cryptic' secondary metabolites. In this review, we will discuss evidence that research in this field has provided us with sufficient knowledge and tools to express and purify any secondary metabolite of interest. We will describe 'unselective' strategies that bring about global changes in secondary metabolite output as well as 'selective' strategies where a specific biosynthetic gene cluster of interest is manipulated to enhance the yield of a single product.
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INTRODUCTION

Secondary metabolites are biologically active small molecules that are not required for viability but which provide a competitive advantage to the producing organism. The bacterial secondary metabolites are a source of many of the antibiotics, chemotherapeutic drugs, immune suppressants and other medicines.[1–3] Of bacteria, the actinomycetes and, in particular, the streptomycetes produce the greatest number of chemically diverse secondary metabolites.[4–7] Other major sources include soil Bacilli,[8] Myxococci[9,10] and Pseudomonads.[11] Two major structural classes of secondary metabolites are the polyketides[3] and the nonribosomal peptides,[12] both of which are produced by multienzyme biochemical pathways encoded in discrete genomic clusters.

A classical approach to the discovery of secondary metabolites having medical utility has involved screening culture supernatants for the modulation of growth of a target organism, extracting and fractionating the supernatants with organic solvents and then characterizing purified molecules using NMR, X-ray crystallography and MS. Antimicrobial activity against *Staphylococcus aureus* has been a commonly sought-after biological activity. Using this approach, those strains that produced antimicrobial compounds were typically found to generate, at most, one or two molecules of interest. However, the sequencing of streptomycete genomes suggests a much greater secondary metabolic potential than had been expected; it turns out that streptomycete genomes generally have the genetic capacity to produce as many as 30 distinct secondary metabolites per strain, including polyketides, nonribosomal peptides and other classes of compounds.[13] It is not known how many of these pathways generate novel compounds or compounds of medicinal utility; however, these 'cryptic' pathways have generated considerable interest as they represent an enormous reservoir of new chemical matter and may include important drug leads.

Many secondary metabolites are expressed at low levels during laboratory growth. The factors that limit production are unknown; however, they are likely to include low expression of the biosynthetic genes or limited precursor availability during standard laboratory culture. The biological signals and regulatory networks that control the secondary metabolic genes are slowly coming into focus as a result of targeted research in this area.[14–19] So too are the metabolic networks that provide the precursors necessary for the biosynthesis of individual molecules. This knowledge has provided new strategies for tapping into this metabolite reservoir. Vital to elucidating these strategies have been the workhouse actinomycetes (Table 1). Investigations into these organisms has led to an understanding of the biochemical processes of secondary metabolite biosynthesis, as well as a growing appreciation for the extensive regulatory network that controls the expression of the metabolic genes. Information gained from studying these model systems is potentially transferable to many bacterial secondary metabolite producers.

Streptomyces coelicolor is a powerful model for secondary metabolism as it produces two pigmented secondary metabolites: actinorhodin (blue)[20] and the prodiginines (red) (Figure 1).[21] These compounds have facilitated genetic analysis of biosynthetic mutants, leading to the discovery of important regulators as well as many biosynthetic genes.[18] The *S. coelicolor* genome sequence has been available for more than a decade and is well-annotated.[22] There are

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excellent tools for chromosomal manipulation,[23] reporter systems[24–27] as well as a growing understanding of the bacterium's stress response mechanisms[28–30] and sporulation pathway.[18,31–35] At present *S. coelicolor* has the most well-understood secondary metabolome of any streptomycete (Figure 1 and Table 2).[13,18,22,36] Of the 29 predicted secondary metabolites, the structures of 17 are known and there is a growing understanding of their biochemical and biological roles. We will focus on how this organism has served the field. There have been several excellent reviews of this topic generally that deal with related aspects of secondary metabolism. Given the availability of this information, we have not sought to be comprehensive in this review but have instead summarized general concepts arising from work in *S. coelicolor* with a particular emphasis on applying this knowledge to the discovery and characterization of cryptic secondary metabolites in other streptomycetes. We describe 'unselective' strategies that bring about global changes in secondary metabolite output and 'selective' strategies where a specific biosynthetic gene cluster of interest is manipulated to enhance the yield of a compound and illustrate how these differing approaches can be integrated into an overall strategy.

Table 1 Model actinomycete secondary metabolite producers

Species	*Secondary metabolite studied*	*Reference*
Amycolatopsis orientalis (ATCC 19795)	Vancomycin	111
Streptomyces avermitilis (ATCC 31267)	Avermectins	112
Streptomyces clavuligerus (ATCC 27064)	Clavulanic acid	113
Streptomyces coelicolor (A3(2))	Actinorhodin	114
	Prodiginines	21
Streptomyces griseus (IFO 13350)	Streptomycin	115
	A-factor	51
Streptomyces venezuelae (ISP5230)	Chloramphenicol	116
	Jadomycin B	50
Saccharopolyspora erythraea (NRRL23338)	Erythromycin A	117
Salinispora tropica (CNB-440)	Salinosporamides	118

BIOSYNTHESIS OF PIGMENTED ANTIBIOTICS IN *S. COELICOLOR*

Actinorhodin is an aromatic polyketide synthesized by enzymes encoded in a 22-kb gene cluster (Figure 2a).[37] Aromatic polyketides are an important class of medically relevant secondary metabolites: the anticancer agent daunorubicin and the tetracycline antibiotics belong to this class.[38] Their production occurs using a type II, or iterative, polyketide synthase. The hallmark of iterative polyketide synthesis is the initial synthesis of the carbon backbone by the minimal polyketide synthase (*actI-orf1/2/3* in the case of actinorhodin), followed by tailoring to create the final product (see Figure 2b for details). Actinorhodin production draws heavily on primary metabolism as the carbon backbone is produced entirely from fatty acid precursors, acetyl-CoA and malonyl-CoA (Figure 2b). The actinorhodin biosynthetic cluster also encodes a pathway-specific activator (*actII-orf4*) that activates the biosynthetic genes. This activator gene is in turn subject to the action of global regulators that can either activate or repress its expression and which presumably serve to integrate environmental and metabolic cues.

The red, cell wall-associated, pigment produced by *S. coelicolor* is a mixture of prodiginines—undecylprodiginine and the cyclized derivative streptorubin B being the major products.[39] Prodiginines are a widespread and structurally related group of tripyrrole antibiotics

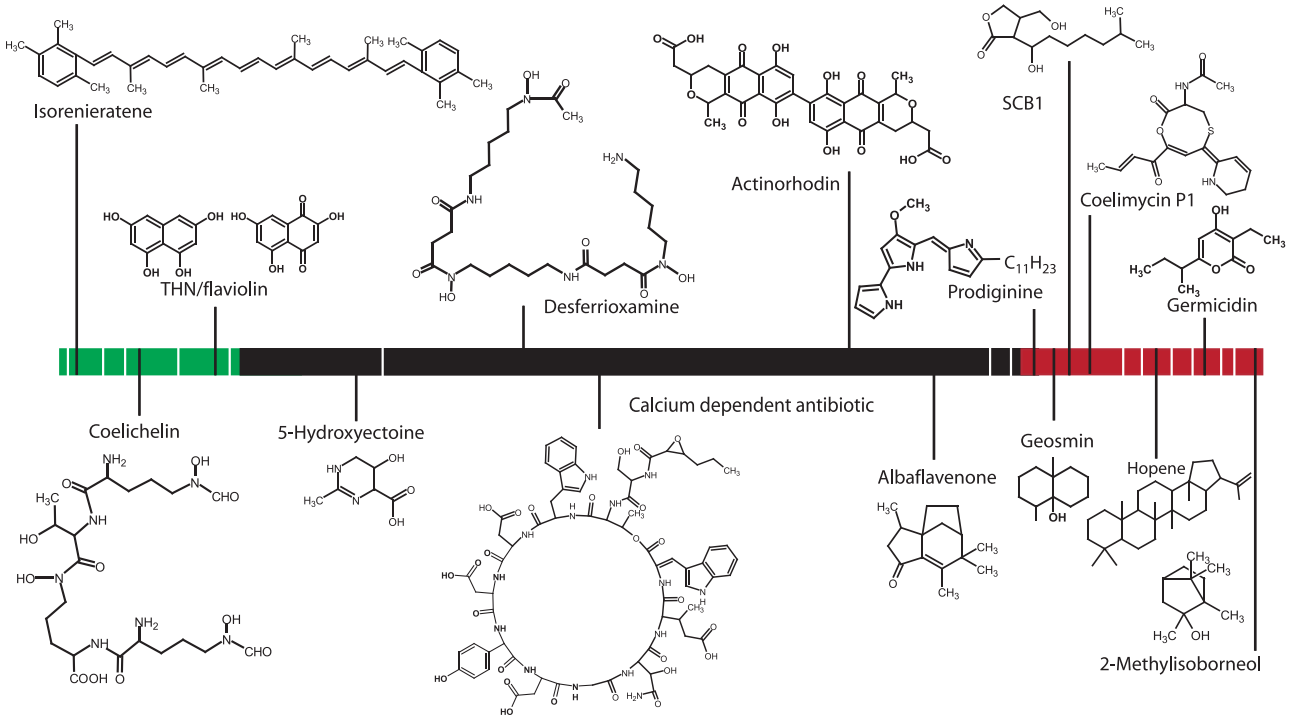

Figure 1 *Streptomyces coelicolor* secondary metabolites. Secondary metabolites are depicted in relation to their chromosomal location, emphasizing the fact that the majority of biosynthetic genes are located outside the highly conserved core region. The left arm (corresponding to the first 1.3 Mb of the chromosome) is highlighted in green and the right arm (last 2.3 Mb of the chromosome) is highlighted in red. The core chromosome region (4.9 Mb) is in black. Secondary metabolites without structures are shown in white (see Table 1 for details), while those with structures are in black.

Table 2 ***Streptomyces coelicolor*** **secondary metabolites**

Secondary metabolite	*Location*	*Type*	*Identification method*	*Reference*
Identified structures				
Isorenieratene	SCO0185-0191	Terpenoid	Blue light induction	63
Coelichelin	SCO0489-0499	NRP	Genome mining	62
THN/flaviolin	SCO1206-1208	PK—type III	Genome mining	119
5-Hydroxyectoine	SCO1864-1867	Cyclic amino acid	Salt or high temp	64
Desferrioxamine	SCO2782-2785	Tris-hydroxymate	Genome mining	61
CDA	SCO3210-3249	NRP	Antimicrobial activity	120
Actinorhodin	SCO5071-5092	PK—type II	Blue pigment	20
Albaflavenone	SCO5222-5223	Terpenoid	Odor/genome mining	121
Prodiginine	SCO5877-5898	Tripyrrole	Red pigment	21
Geosmin	SCO6073	Terpenoid	Odor/genome mining	122
SCB1	SCO6266	γ-Butyrolactone	Genome mining	123
Coelimycin P1	SCO6273-6288	PK—type I	Yellow pigment/genome mining	36,124,125
Hopene	SCO6759-6771	PK—type III	Genome mining	126
Germicidin	SCO7221	PK—type III	Genome mining	127
2-Methylisoborneol	SCO7700-7701	Terpenoid	Odor/genome mining	128
Methylenomycin	SCP1.228c-246	Cyclopentanoid	Antimicrobial activity	129
Methylfurans	SCP1.228c-246	Methylfurans	Genome mining	130
Developmental secondary metabolites				
SapB	SCO6681-6685	Lantibiotic	MS/phenotype	131
Predicted structures (untested)				
Eicosapentaenoic acid	SCO0124-0129	Fatty acid	Genome prediction	13
Melanin	SCO2700-2701	Melanin	Genome prediction	13
Bacteriocin	SCO0753-0756	Bacteriocin	Genome prediction	13
Coelibactin	SCO7681-7691	NRP	Genome prediction	22
Unable to predict structures				
Lantibiotic	SCO0267-0270	Lantibiotic	Genome prediction	13
Lantibiotic	SCO6927-6932	Lantibiotic	Genome prediction	13
PKS	SCO1265-1273	PK—type II	Genome prediction	13
PKS	SCO6826-6827	PK—type II	Genome prediction	13
PKS	SCO7669-7671	PK—type III	Genome prediction	13
Siderophore	SCO5799-5801	—	Genome prediction	13
Dipeptide	SCO6429-6438	—	Genome prediction	13
Gray spore pigment	SCO5314-5320	PK—type II	Gray pigment	132

Abbreviations: NRP, nonribosomal peptide; PK, polyketide.

currently being explored for use as chemotherapeutics. Their biosynthesis in *S. coelicolor* is directed by a 30-kb gene cluster (Figure 3a). Two pathway-specific transcriptional activators RedZ and RedD are required for the activation of prodiginine gene expression: RedZ is a direct activator of RedD, which then acts on the biosynthetic genes. The biosynthetic pathway itself is complicated—a bifurcated process requiring the production of two specialized precursors, 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC) and 2-undecylpyrrole (see Figure 3b for details). The enzymes required for prodiginine synthesis are encoded within the biosynthetic cluster; however, proline, serine, glycine, acetyl-CoA and malonyl-CoA must be drawn from primary metabolism and the creation of the lipid moiety requires enzymes from fatty acid biosynthesis as well (Figure 3b).[40]

A REGULATORY NETWORK GOVERNING SECONDARY METABOLISM

Secondary metabolism is subject to diverse regulatory inputs. Most of these pathways have been discovered through the analysis of mutations that alter yields of actinorhodin, and/or prodiginines. There does not appear to be a universal regulatory network for secondary metabolism.[18,19,41] There are, however, many shared regulatory mechanisms, some of which are widely conserved and the general principles are similar in all streptomycetes. For example, it is very common for the expression of secondary metabolite pathway genes to be controlled by a pathway-specific regulator (Table 3), typically encoded in the cognate biosynthetic gene clusters, and these regulators are in turn under the control of the more globally acting pleiotropic regulators (Table 4).

Pathway-specific regulators

Many biosynthetic clusters encode one or more pathway-specific activators (Table 3). The *Streptomyces a*ntibiotic *r*egulatory *p*roteins or SARPs, characterized by a winged helix–turn–helix motif at their N terminus,[42,43] are a common type of pathway-specific regulator. For example, ActII-4, the pathway-specific activator of actinorhodin biosynthesis, binds two of the three intergenic regions (*actVI-orfA*/*actVI-orf1* and *actIII*/*actI-orf1*) found within the biosynthetic cluster (Figure 4). These ActII-4 binding sites overlap the −35 regions of the promoters facilitating recognition by RNA polymerase.[44]

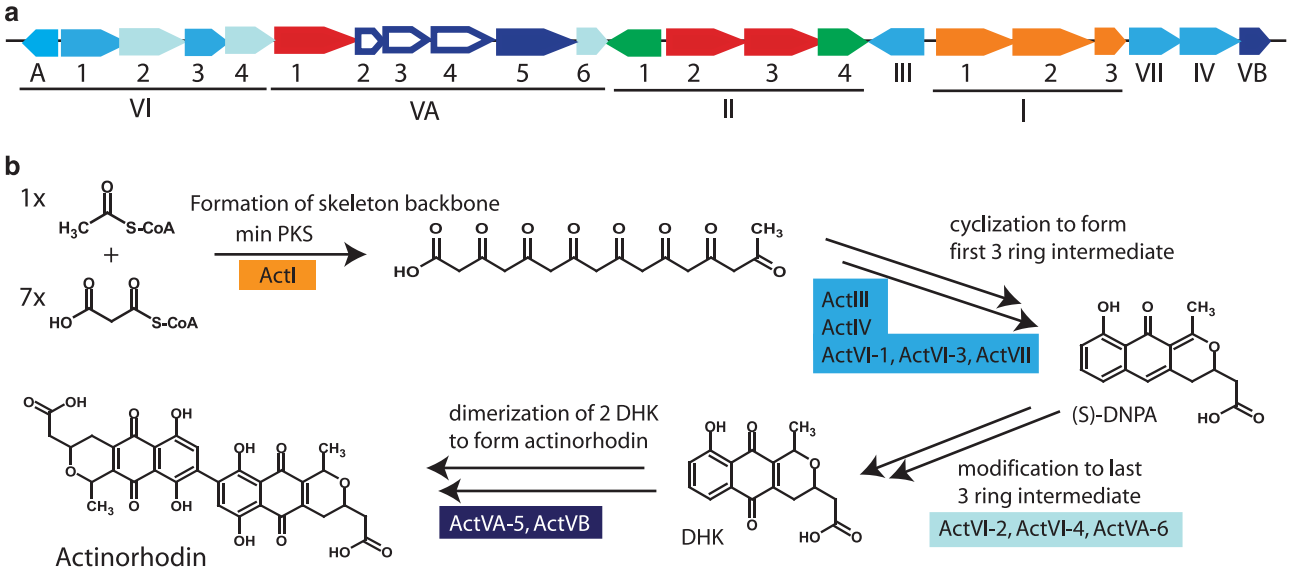

Figure 2 Actinorhodin biosynthesis. (**a**) Organization of the actinorhodin biosynthetic cluster. Regulatory genes are highlighted in green and putative resistance genes in red. The minimal PKS (ActI) is orange. Tailoring genes are colored depending on their role in forming actinorhodin. Genes that have not been characterized are filled with white. (**b**) 1x Acetyl-CoA and 7x malonyl-CoA are condensed to form the carbon skeleton by ActI. This carbon backbone is cyclized to form a three ring intermediate (*s*)-DNPA (by ActIII, ActVII, ActIV, ActVI-1 and ActVI-3) followed by modification to DHK (ActVI-2, ActVI-4 and ActVA-6). Dimerization of 2 DHK molecules results in the formation of actinorhodin (by ActVA-5 and ActVB). The involvement of ActVA2-4 has yet to be characterized.

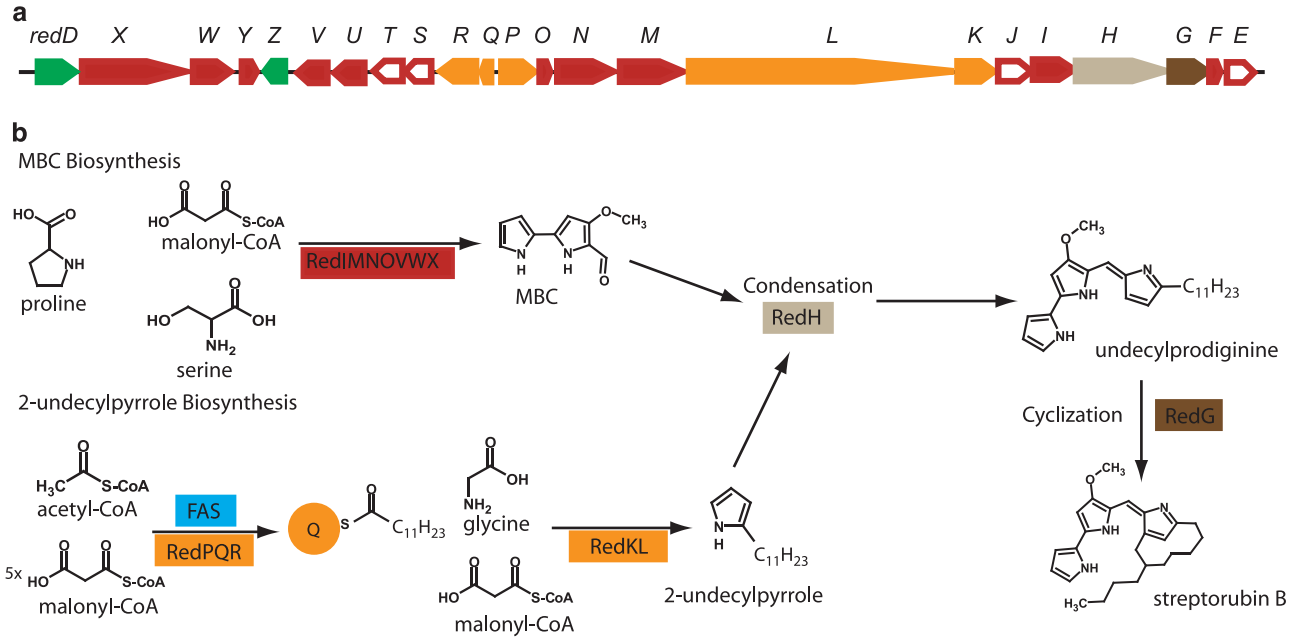

Figure 3 Prodiginine biosynthesis. (**a**) Organization of the prodiginine biosynthetic cluster. Regulatory genes are in green, genes for MBC synthesis are in red and genes for 2-undecylpyrrole are in orange. Genes for condensation of MBC and 2-undecylpyrrole and subsequent cyclization are in gray and brown, respectively. Genes with unknown function have white centers. (**b**) Prodiginine synthesis requires the production of a dipyrrole, MBC, and a monopyrrole, 2 undecylpyrrole, from separate enzymatic reactions, which are subsequently condensed together to form the final tripyrrole. MBC synthesis requires proline, malonyl-CoA and serine as substrates and is catalyzed by RedMNOVWX. 2-Undecylpyrrole begins with the formation of a 12 carbon lipid, which is synthesized by RedPQR with the aid of the enzymes from fatty acid biosynthesis (FAS). This lipid is transferred to RedL, where glycine and another malonyl-CoA are added to the chain. Once released from RedL, RedK performs the final modifications to form 2-undecylpyrrole. MBC and 2-undecylpyrrole are condensed by RedH to form undecylprodiginine. Further cyclization by RedG occurs to ~1/3 to form streptorubin B.

Table 3 Pathway-specific regulators in *Streptomyces coelicolor*

Regulator	*Type*	*Metabolite*	*Reference*
ActII-4	SARP	Actinorhodin	133
CdaR	SARP	CDA	134
CpkO/KasO	RR	Coelimycin P1	54
ScbA	GBL synthase	Coelimycin P1	54
ScbR	TFR	Coelimycin P1	54
EcrA1/A2	SK(his)/RR	Prodiginine	135
RedD	SARP	Prodiginine	136
RedZ	RR	Prodiginine	137
MmyR	TFR	Methylenomycin	45
MmfR	TFR	Methylenomycin	45
MmfB	Xre-like	Methylenomycin	45

Abbreviations: GBL, γ-butyrolactone; RR, response regulator; SARP, *Streptomyces* antibiotic *r*egulatory *p*roteins; SK(his), histidine kinase; TFR, tetR-like protein.

Some biosynthetic clusters encode pathway-specific repressors. For example, production of the *S. coelicolor* metabolite methylenomycin is regulated by an activator (MmyB) and two repressors (MmyR and MmfR) that repress the *mmyB* promoter. Repression by MmyR and MmfR is relieved by autoregulatory methylfuran signaling molecules, leading to methylenomycin production.[45]

Global regulators

The pleiotropic regulators influence more than one secondary metabolite. *S. coelicolor*'s pigments have been used as indicators for the identification and characterization of over 55 pleiotropically acting loci, most of which encode regulatory proteins (Figure 4 and Table 4). These regulators include many signal-transduction systems, suggesting that they sense and respond to the cellular environment (Table 4 and Figure 4).

One signal-transduction pathway that illustrates the complexity of sensory inputs to secondary metabolism has, at its core, the serine/threonine kinase AfsK. AfsK phosphorylates the DNA binding protein AfsR,[46] an activity that is modulated by binding of the protein KbpA,[47] although little is known about how this interaction is itself controlled or what purpose it serves. Phosphorylation of AfsR enhances its interaction with the promoter of the *afsS* gene activating its expression. AfsS, which exhibits sequence similarity to domain 3, the RNA polymerase binding moiety of the σ-factor proteins, then serves to enhance the expression of the actinorhodin and prodiginine biosynthetic genes,[48,49] although again the mechanistic details of its action are obscure.

A metabolic input that controls *afsS* expression is phosphate limitation,[17] which is sensed by the sensor kinase PhoR leading it to phosphorylate the response regulator PhoP. PhoP∼P has a large number of targets, most of which are concerned with phosphate uptake and management; however, the *afsS* gene is a member of the Pho regulon, and the expression of AfsS is increased in phosphate-limiting conditions leading to increased production of actinorhodin and the prodiginines.[17] The biological advantage of linking secondary metabolism to phosphate availability is unknown.

Another activating signal for the AfsK pathway is *S*-adenosyl-L-methionine (SAM),[14,15] an important metabolite and the methyl group donor in all organisms. Among many other roles, SAM provides a methyl group to the MBC biosynthetic pathway, a critical component in prodiginine synthesis.[40] This therefore links the activation of the AfsK kinase activity to the availability of this primary metabolite.

In addition, recent work has revealed a role for AfsK in responding to cell wall stress: in response to bacitracin-induced cell wall damage, AfsK phosphorylates a cytoskeletal protein to modulate cell wall biosynthesis. A link between cell wall damage and AfsK-mediated activation of secondary metabolism has yet to be demonstrated, but may represent another avenue to improve production of secondary metabolites. It is known that yields of some metabolites, for example, jadomycin B production by *S. venezulae*,[50] can be enhanced by heat shock—perhaps this is influenced by damage to the cell wall via this arm of the AfsK pathway?

γ-Butyrolactones: Pathway-specific and global regulators

The γ-butyrolactone (GBL) signaling molecules are produced by many streptomycetes and usually impinge directly on secondary metabolism. Their effects can be pathway specific or global. For example, in *S. griseus*, sporulation and secondary metabolism is controlled by the production of a single GBL, A-factor, making it a global regulator.[51] In *S. avermitilis*, its GBL, avenolide, elicits production of the avermectins but is not believed to influence other metabolites: it therefore serves as a pathway-specific regulator.[52] The GBL of *S. lavendulae* IM-2 is also a global regulator but has more complex effects on secondary metabolism, in that it reduces D-cycloserine production and increases nucleoside antibiotics and a blue pigment.[53]

In some cases, the mode of regulation by GBLs is less clear. For example, the molecule SCB1 of *S. coelicolor*, synthesized by ScbA, is a pathway-specific regulator of coelimycin P1, the polyketide product of the *cpk* gene cluster.[54] However, deletion of ScbA causes a strong stimulation of both prodiginine and actinorhodin through an unknown mechanism. It is unclear whether this makes ScbA a global regulator[55] or whether the loss of activation of coelimycin P1 simply favors yields from a competing pathway (ScbA is listed in both Table 3 and Table 4).

STRATEGIES TO IMPROVE SECONDARY METABOLITE PRODUCTION AND DETECTION

Classical screening of cell culture supernatants containing many metabolites for activities of interest is appealing because it is simple and inexpensive. The problem with the established methods is that they result in the frequent rediscovery of common metabolites; such as, streptomycin, streptothricin, tetracycline and actinomycin.[56] However, growing evidence suggests that we can take advantage of regulatory mechanisms to alter the spectrum of secondary metabolites produced by a strain and thereby rejuvenate this straightforward approach.

The strategies that have been taken to tap into the cryptic secondary metabolites can be described as 'selective', in that a single metabolite is targeted, or 'unselective', in that secondary metabolism is generally perturbed to enhance yields of multiple metabolites (Figure 5).

UNSELECTIVE STRATEGIES

Manipulation of media and stress responses

The classical method for activating secondary metabolites involves the manipulation of culture conditions or biological stress responses. The outcomes of this approach are unpredictable in that different streptomycetes respond in different ways to each manipulation. Production of pure daptomycin, the clinically relevant form of the antimicrobial 'calcium-dependent' lipopeptide antibiotics produced by *S. roseosporus*, requires feeding with decanoic acid.[57] Efficient production of jadomycin B by *S. venezulae* requires induction by

Table 4 Regulators involved in *Streptomyces coelicolor* secondary metabolism

Regulator	*Type*	*Notes*	*Reference*
Multicomponent systems			
abeR regulon			
AbeABCD (α-abeA)		↑ *abeABCD* (α-*abeA*)↑ACT	138
		Four gene operon with antisense RNA (α-*abeA*)	
AbeR	SARP	↑*abeR* ↑ACT	
		Δ*abeR* ↓ACT	
absA regulon			
AbsA1	SK(his)	Phosphorylates AbsA2	139
		Δ*absA1* ↑ACT/RED	140
		↑*absA1*↓ACT/RED	141
AbsA2	RR	AbsA2∼P global repressor	93
		Directly binds to pathway-specific promoter	
		Δ*absA2* ↑ACT/RED	141
afsK regulon			
AsfK	SK(ser)	Phosphorylation stimulated by SAM	14,15
		Phosphorylates AfsR	46
		Δ*asfK* ↓ACT	46
AfsL	SK(ser)	Phosphorylates AfsR	142
PkaG	SK(ser)	Phosphorylates AfsR	142
AfsR	RR(SARP-like)	Integrates signals from AsfK/AfsL/PkaG	142
		Δ*asfR* ↓ACT	143
KpbA		Represses AfsK phosphorylation	47
		Δ*kpbA* ↑ACT	
AfsS/AfsR2	σ-Like	Δ*asfS* ↓ACT	48
		Integrates signals from AfsR∼P and PhoR∼P	
Pho regulon			
PhoR	SK(his)	Phosphorylated in low phosphate	17
		Phosphorylates PhoP	
PhoP	RR	PhoP∼R binds promoters of *pho* regulon	17
		afsS is part of the *pho* regulon	
		↑ACT/RED in ↓P_i	
afsQ regulon			
AfsQ2	SK(his)	↑*afsQ1/Q2* ↑ACT/RED/CDA in *S. lividans*	144
AfsQ1	RR	Glutamate as carbon source required to see mutant phenotypes	
		Δ*afsQ1/Q2* ↓ACT/RED/CDA	
σ^Q	σ-Factor	Antagonizes AfsQ1–Q2	
AbaR1/R2	SK(his)/RR	Δ*abaR1R2* ↑ACT	145
AbaC1/C2/C3	SK(his)/SK(his)/RR	Δ*abaC1C2C3* ↓ACT	
DmdR1/Adm	DmdR	Antiparallel overlapping genes	146
		Senses Fe^{2+}	
		Δ*dmdR1/adm* ↓ACT/RED	
		Δ*adm* ↑ACT/RED	
DraK/R	SK(his)/RR	Δ*draK/R* ↓ACT/↑RED/CPK	147
		Directly with pathway-specific promoter ACT/CPK	
		Indirect effect on RED	
RapA1/A2	RR/SK(his)	Δ*rapA1*/A2 ↓ACT/CPK = RED	148
One-component systems			
AtrA	TFR	Δ*atrA* ↓ACT	149
AbsC	MarR-like	Δ*absC*+ low Zn no ACT/RED	150
CprA	TFR	Δ*cprA* ↓ACT/RED	151
		↑*cprA* ↑ACT/RED	
CprB	TFR	Δ*cprB* ↑ACT = RED	151
DasR	GntR	DasR represses pathway-specific promoters	152
		GlcNac relieves DasR repression	
NdgR	IclR-like	Δ*ndgR* ↑ACT	153
		Binds intergenic region of ScbA/R	
RrdA	TFR	Δ*rrdA* ↑RED ↓ACT	154
		↑*rrdA* ↓RED ↑ACT	
SigT	ECF σ-factor	Δ*sigT*+ low N ↓ACT	155

Table 4 (Continued)

Regulator	*Type*	*Notes*	*Reference*
BldD regulated			
BldD	DNA binding	Δ*bldD*↓ACT	156,157
CdgA	c-di-GMP	↑*cdgA* ↓ACT	156
CdgB	c-di-GMP	↑*cdgB* ↓ACT	158
NsdA	TPR-like	Δ*nsdA* ↑ACT/CDA/MMY	159
NsdB	TPR-like	Δ*nsdB* ↑ACT/CDA	160
SsgR/SsgA	SALP	Δ*ssgA*↓ACT	161
		ssgR positively regulated *ssgA*	162
Translation			
AdpA/bldA	tRNA–leu	Developmentally regulated	163
		Prevents translation of genes with TTA codons until expressed	
		redD, *actII*-4 and *cdaR* contain TTA	
AbsB	RNAseIII	Δ*absB* ↓ACT/RED	164–166
		Degrades double-stranded mRNA	
Stringent response			
ppGpp synthesis ↑ACT/RED			
AfsB/HrdB	σ-Factor	Mutations interfere with ppGpp synthesis resulting in lowering pigment production	167
EshA	ppGpp	Δ*eshA* reduce ppGpp production	168
RelA	ppGpp	Nitrogen limited ppGpp production	67,73
RelC/RplK	ppGpp	Mutations reduce ppGpp production	169
RshA	ppGpp	Phosphate limited ppGpp production	170
Miscellaneous			
CmdABCDEF		Δ*cmdABCDEF* ↑ACT	171
HmgA	Homogentisate 1,2-dioxygenase	Δ*hgmA* ↑ACT	172
SarA	Unknown	Δ*sarA*↓ACT	173
ScbA	GBL	Δ*scbA* ↑ACT/RED	55,174

Abbreviations: ACT, actinorhodin; GBL, γ-butyrolactone; RED, prodiginine; SALP, ssgA-like protein; SAM, *S*-adenosyl-L-methionine; SARP, *Streptomyces a*ntibiotic *r*egulatory *p*roteins; SK(ser), serine/threonine sensor kinase.

ethanol shock or growth at high temperature (42 °C) and can be improved further by combining these two growth conditions.[50,58] This is exemplified by the OSMAC (one strain, many compounds) approach, which involves selectively changing easily accessible growth parameters to probe the secondary metabolic potential of a strain. Initial proof-of-principle work identified 20 metabolites from a single strain by changing growth conditions.[59]

Many secondary metabolites offer selective advantages to the producers and are only produced during these specific conditions. Siderophores are secondary metabolites that sequester iron and are expressed in low iron conditions.[60–62] The carotenoids of *S. coelicolor* are expressed in the presence of blue light consistent with their protective role against photodamage.[63] Production of ectoine and 5-hydroxyectoine protect against dehydration and thus are expressed under high salt or high temperature conditions in *S. coelicolor*.[64] Thus, starvation for certain elements and stresses can therefore be expected to elicit some of these compounds.

Random mutagenesis using chemical mutagens or UV light has been employed to generate strains optimized for industrial production. The producer is subject to rounds of mutagenesis involving either UV or chemical mutagens, with surviving clones screened for improved activity. For example, yields of clavulanic acid from *S. clavuligerus*[65] and rapamycin from *S. hygroscopicus*[66] were both improved through random mutagenesis. Again, the effects of mutagenesis and multiple rounds of screening are unpredictable. Although this approach can be applied to enhancing yields of compounds produced at low levels, it is not a suitable screening platform as it cannot be easily adapted to high throughput.

Ribosomal engineering

One strikingly successful perturbation of secondary metabolite production has been developed through the observation that resistance to antibiotics enhances yields of some cryptic secondary metabolites. In particular, resistance to antibiotics that target the ribosome (for example, streptomycin, paromycin and gentamicin) frequently involving ribosomal protein S12[67] or rifampicin via RNA polymerase β-subunit mutations prove effective.[68] The effects of these mutations can be combined for increased effects on secondary metabolism and have been demonstrated by developing stepwise resistance for up to eight ribosomal antibiotics in *S. coelicolor*, with a concomitant increase in the production of actinorhodin.[69]

The mechanism of this fascinating effect is not entirely clear[70] but involves the upregulation of pathway-specific regulators; such as, *actII-orf4*.[71,72] One possible explanation that has been advanced is that the alteration of ribosome function mimics the stringent response, upregulating the production of ppGpp, which is known to increase the production of some secondary metabolites.[73] Regardless of how ribosome engineering actually works at the molecular level,

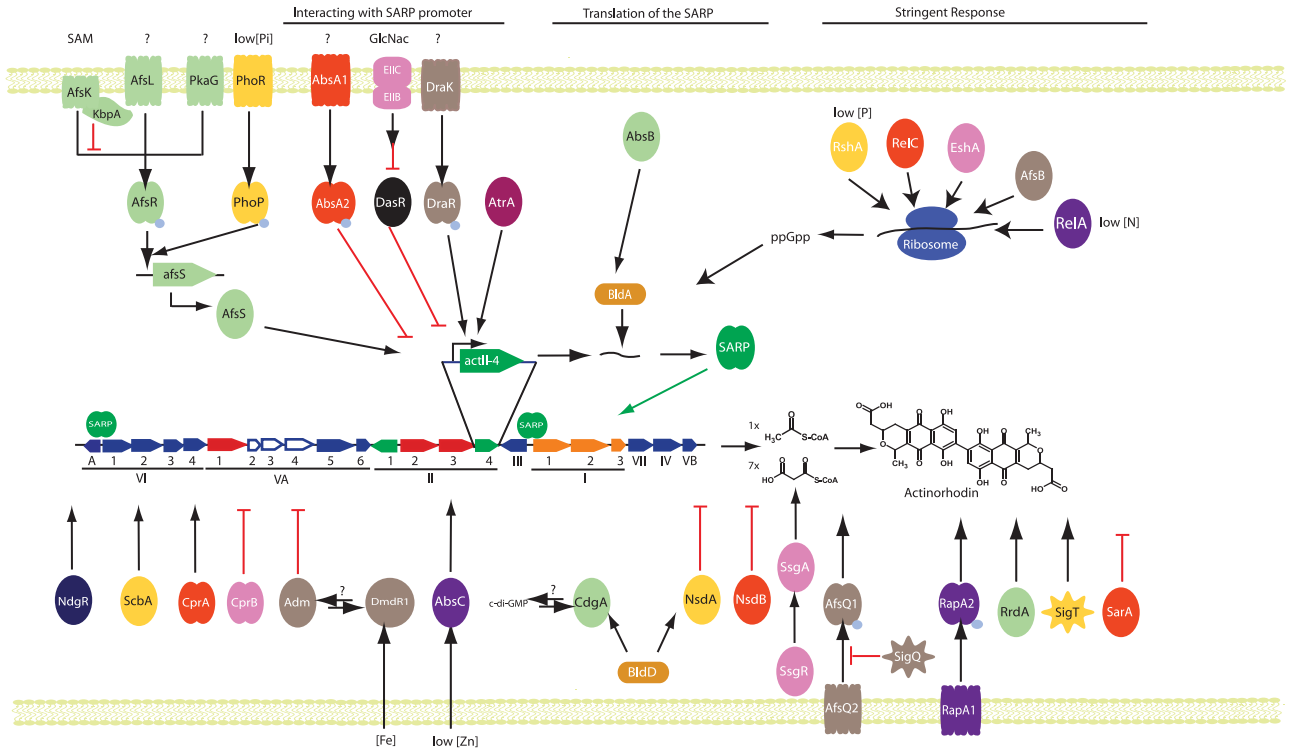

Figure 4 Complex regulation of actinorhodin production in *Streptomyces coelicolor*. The pathway-specific activator ActII-4 integrates many of the global regulators. Repression in the network is illustrated in red and activation in black. Regulators are grouped as follows: those with experimental evidence of binding to the promoter region of *actII*-4 are under 'interacting with *Streptomyces* antibiotic regulatory proteins (SARP) promoter', those regulators affecting translation are under 'translation of the SARP' and those that influence the stringent response are under 'stringent response'. All the remaining regulators influence actinorhodin production but their mechanism has yet to be elucidated. A complete list of regulators can be seen in Table 3. AbeABCD(α-abeA), AbeR and CmdABCDEF are not depicted because of their complexity.

this approach to strain improvement is advantageous in that there is no requirement for genetic engineering. Libraries of resistant mutants have been successfully screened, resulting in the discovery of novel piperidamycins.[74]

Small-molecule probes

The first synthetic molecule reported to influence secondary metabolism was an inhibitor of phosphopantetheinyl transferase.[75] Phosphopantetheinyl transferases activate the acyl carrier protein of fatty acid biosynthesis and secondary metabolism by 'priming' the carrier protein. Priming occurs by the addition of a phosphopantetheinyl group and provides the reactive sulfhydryl group that tethers the new metabolites and is essential for these processes to occur. Specifically, it was found that a phosphopantetheinyl transferase inhibitor developed against *Bacillus subtilis* enhanced actinorhodin production when added to *S. coelicolor*. The mechanism for this increase is unclear as *S. coelicolor* encodes many phosphopantetheinyl transferases;[75] however, it was proposed that the inhibition of fatty acid biosynthesis could improve precursor availability or that it could activate a stress response and that one or both of these pathways enhance actinorhodin production.[75] There is at present no experimental evidence of either mechanism.

More recently, a collection of synthetic small molecules that alter secondary metabolism in *S. coelicolor* was identified by specifically looking for enhanced blue pigmentation.[76] Of 19 compounds identified in this screen, four molecules referred to as the ARC2 series were found to be related to the antibiotic triclosan. Similarly, they appear to act by inhibiting the enoyl reductase FabI, which plays a critical role in fatty acid metabolism. It was proposed that the mechanism of action of the ARC2 series of molecules involves a buildup of fatty acid precursors that are shunted from fatty acid biosynthesis to secondary metabolism as a result of the inhibition of FabI. Consistent with the overall conservation of the fatty acid biosynthetic machinery in prokaryotes, the ARC2 series also alters the secondary metabolic profiles of many actinomycetes. Initial work demonstrated increased production of desferrioxamine B/E in *S. pristinaespiralis*, doxorubicin and baumycin in *S. peucetius* and an unknown metabolite (252.175 $[M+H]^+$) in *Kutzneria* sp. 744. Other molecules from this screen had strikingly different properties. For example, one of these compounds, ARC6, elicited enhanced yields of a differing though overlapping set of secondary metabolites as ARC2. The effect of ARC6, however, appears to be restricted to *S. coelicolor*, indicating that it acts more as a species-specific synthetic signaling molecule.[77] While this molecule is less likely to serve as a valuable screening tool, it could be used to probe the genetic and/or metabolic pathways that control and limit secondary metabolism in *S. coelicolor*.

Chemical manipulation of secondary metabolism is an advantageous strategy as it negates the need for genetic manipulation, which can limit the application of genetic strategies as many streptomycetes possess systems that restrict introduction of foreign DNA.[78–84] It is hoped that this will serve as a valuable screening tool by activating the

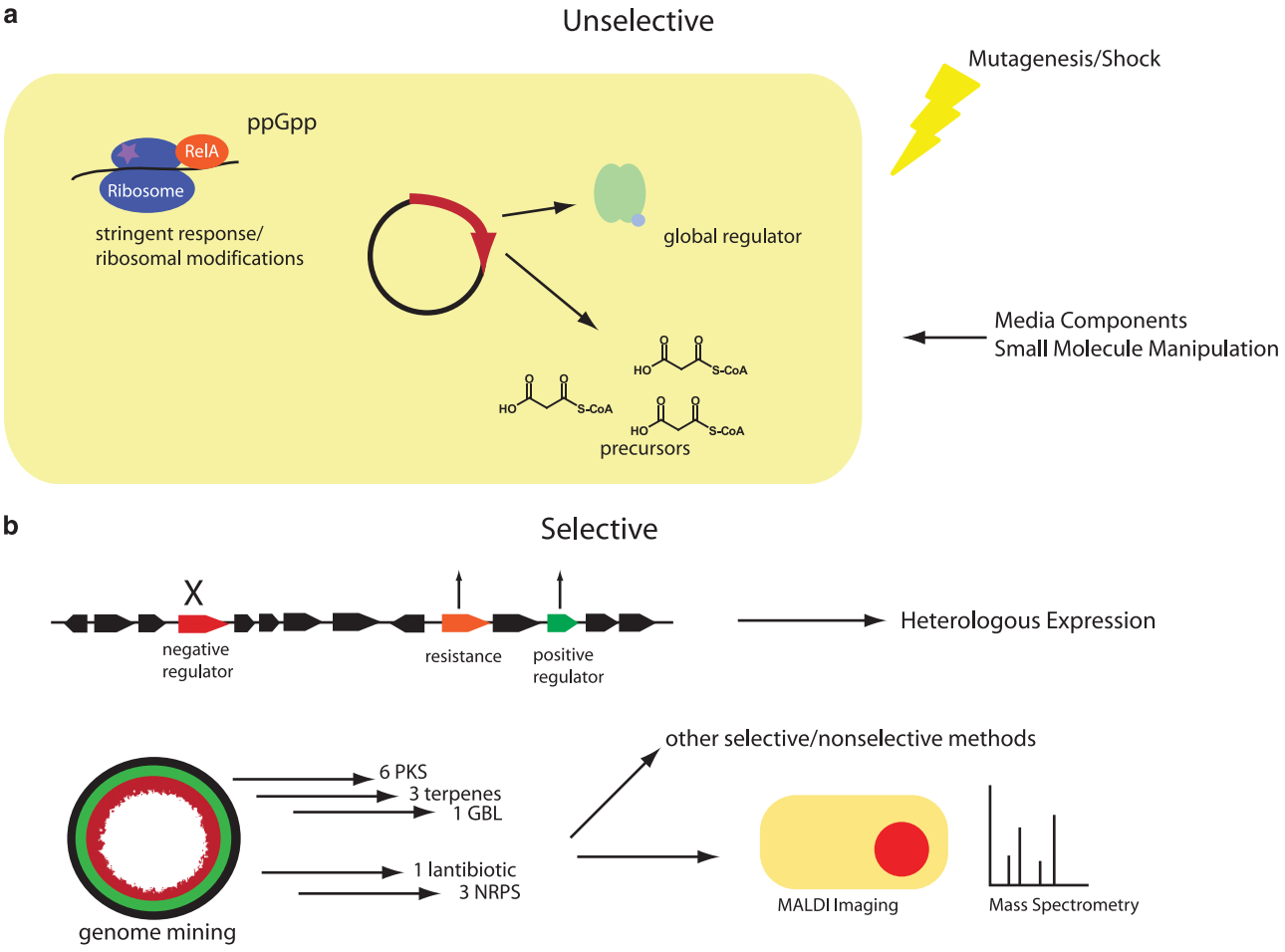

Figure 5 Strategies to improve secondary metabolism. (**a**) Unselective methods include eliciting the stringent response (through ribosomal modifications), overexpressing global regulators or precursor metabolites, changing media composition or by stressing the cell through mutagenesis or shock. (**b**) Selective methods involve manipulating an individual biosynthetic cluster by either overexpressing a pathway-specific activator or resistance determinant, deleting a pathway-specific repressor, or by heterologous expression. Genome sequences can be analyzed for secondary metabolites based on homology to genes known for the production of different classes of metabolites. Following genomic identification, the other selective/unselective methods can be used for detection and structural elucidation. Matrix-assisted laser desorption/ionization (MALDI) imaging may also be used to aid in identifying the secondary metabolite.

yields of cryptic molecules that can be purified and characterized structurally and biochemically.

Metabolic engineering

Metabolic engineering involves genetically modifying the producer organism to elevate the available levels of certain metabolic precursors. Acyl-CoA precursors are important for the production of a number of secondary metabolites; acetyl-CoA, malonyl-CoA and methylmalonyl-CoA are common building blocks of polyketide synthesis and must be available for efficient yields of the secondary metabolic products. This precursor pool can be improved by manipulating the biochemical pathways that produce or consume them; fatty acid biosynthesis, fatty acid degradation, branched chain amino-acid degradation and glucose metabolism are prevalent examples of these pathways.[85] For example, overexpression of the methylmalonyl-CoA mutase pathway (*mutAB*) elevates the availability of methylmalonyl-CoA (by isomerization of succinyl-CoA from the tricarboxylic acid cycle) and thereby enhances the production of FK606 in *S. clavuligerus*.[86] Disruption of *zwf1* or *zwf2* from the pentose phosphate pathway improves the production of acetyl-CoA and malonyl-CoA, resulting in increased production of actinorhodin in *S. coelicolor*[87] and oxytetracycline in *S. ambofaciens*.[88]

Engineering global regulators

Strains can be genetically engineered to overexpress global regulators to elicit overall changes in secondary metabolites within the host organism or when heterologously expressed in other streptomycetes.[42] For example, overexpression of various alleles of AbsA2 results in the overproduction of actinorhodin, prodiginines and CDA in *S. coelicolor*.[89] This same allele has the capacity to enhance secondary metabolites in other streptomycetes, demonstrated by increased production of streptomycin in *S. griseus* and blasticidin S in *S. griseochromogenes*. In addition, introduction into *S. flavopersicus* resulted in the production of pulvomycin, previously unreported in this strain and undetectable in the absence of the AbsA2 mutant allele.[89]

SELECTIVE METHODS

The biosynthetic gene clusters can themselves be targeted to enhance yields of their products. Genes that enhance production can be

Table 5 Heterologous hosts

Strain	*Relevant modifications*	*Reference*
Streptomyces coelicolor		
M1154	Δ*act*/*red*/*cda*/*cpk* *rpoB[C1298T]*/*rpsL[A262G]*	100
M512	Δ*redD*/*act*II-4	175
CH999	Δ*act*	176
S. avermitilis		
SUKA17	Δ*sav71–1286(ave/pte)/olm* Δterpenes (sav2161–2168, *sav2990–3002*, *sav7456–7491)*	100
S. venezuelae		
DHS2001	Δ*pikAI–IV*	177
YJ309	DHS2001 + *matBC*	178
S. albus		
J1074	Δ*sal*GI (restriction system)	179
S. lividans		
66/1326	Wild type	180
TK24	1326 derivative *str*^{R}	180

overexpressed (resistance genes and pathway-specific activators) and those genes that repress production can be deleted. Secondary metabolite gene clusters of interest can be moved to alternate hosts for improved expression (heterologous expression). As the cost of high-throughput DNA sequencing drops, the strategy of simply activating novel gene clusters in newly isolated streptomycetes becomes increasingly appealing and feasible (Figure 5b).

Engineering self-resistance

A growing body of evidence suggests that self-resistance mechanisms, which are often encoded in secondary metabolic gene clusters, can influence yields of some secondary metabolites. While the mechanisms by which this occurs are not well understood, they may include limiting toxicity or preventing product inhibition of biosynthesis. It is also possible that some export proteins or other resistance determinants participate directly in biosynthesis. For example, upregulation of the resistance genes *drrABC*, *avtAB* and *actAB* has been applied, respectively, to improve yields of doxorubicin and daunorubicin in *S. peucetius*,[90] avermectin production in *S. avermitilis*[91] and actinorhodin yields in *S. coelicolor*.[92]

Regulatory engineering

As many biosynthetic clusters encode pathway-specific activators, overexpression of these activators can elevate yields of a desired metabolite and induce expression of cryptic clusters. Overexpression of the pathway-specific activators of the actinorhodin and prodiginine biosynthetic gene cluster in *S. coelicolor* enhances yields of the cognate metabolites.[93] Similarly, overexpression of AveR and StrR enhances yields of avermectin in *S. avermitilis*[94] and streptomycin in *S. griseus*,[95] respectively. More importantly, overexpression of the predicted pathway-specific activator, SamR0484, was recently used to activate the previously cryptic biosynthetic gene cluster for stambomycin A–D, a family of 51-membered glycosylated macrolides, in *S. ambofaciens*.[96]

Conversely, some biosynthetic clusters encode pathway-specific repressors, which when deleted improve production. Thus, deletion of the pathway-specific repressor *cmmRII* in *S. griseus* resulted in the overproduction of chromomycin[97] and similarly the deletion of AlpW in *S. ambofaciens* results in constitutive expression of alpomycin.[98]

Heterologous expression of biosynthetic gene clusters

The availability of cloning methods for managing large DNA fragments has made it possible to clone entire biosynthetic clusters, some of which are very large.[99] By modifying these clones to include site-specific integration sites, it is possible to then move them into heterologous expression strains (Table 5). *Streptomyces lividans* and *Streptomyces albus* J0174 were originally used to this end because of their low secondary metabolite output and limited restriction barriers. *S. venezulae* has also been adapted for heterologous flavonoids biosynthesis (Table 5).

More recently, chassis strains of *S. coelicolor* and *S. avermitilis* have been developed for the expression of heterologous metabolites with very exciting results.[100,101] These chassis strains lack their own biosynthetic clusters and this reduces the metabolic competition for precursors by heterologous metabolites. These strains also greatly simplify the detection of heterologous metabolites as the LC/MS spectra of their culture supernatants are otherwise devoid of secondary metabolites. The *S. avermitilis* chassis, SUKA17, was created by removing ~1.4 Mb of DNA, including biosynthetic gene clusters for the avermectins, filipin, oligomycin and terpenes. Heterologous expression of streptomycin in the resulting strain was enhanced fourfold relative to the expression in wild-type *S. avermitilis* (Table 5).[101]

The *S. coelicolor* chassis lacks 1.73 Mb of DNA, including the biosynthetic gene clusters for actinorhodin, the prodiginines and two other prominent metabolites. In addition, secondary metabolite-stimulating mutations in *rpoB* and *rpsL* (see 'Ribosomal engineering' above) were introduced to further improve yields. The resulting strain, M1154, was used for heterologous expression of the biosynthetic genes for chloramphenicol (*S. venezuelae*) and congocidine (*S. ambofaciens*), with yields enhanced by 20- to 40-fold relative to the parent *S. coelicolor* strain M145.[100]

These chassis strains may offer a general solution to the production of compounds of interest at high levels.

Genome mining

Increasingly, new secondary metabolites are being identified through mining genomes for novel biosynthetic cluster. In spite of the extraordinary structural diversity of the secondary metabolites, the enzymes that produce them are highly conserved, making it possible to identify and explore novel clusters.[62,102,103] Non-iterative assembly as occurs in nonribosomal peptide synthesis and type I polyketide biosynthesis facilitates the prediction of pathway product structures with considerable precision.[104–106] This is more difficult with the iterative processes; such as, type II and type III polyketides; however, it may still be possible to use cluster features to predict products that are likely to be distinct from known compounds.[107] The genomes of many streptomycetes are now available and have been mined for their secondary metabolites; *S. coelicolor* is predicted to encode 29, *S. avermitilis* 37 and *S. griseus* 36 potential secondary metabolites.[13]

The first metabolite identified through genome mining was the nonribosomal peptide siderophore, coelichelin in *S. coelicolor*.[62] Genome prediction aided greatly in the structural elucidation of

coelichelin, as it suggested culture conditions and detection methods. While a complete structural prediction could not be made from the genomic information, accurate prediction of substrate specificity was achieved, and paved the way for genome mining for nonribosomal peptide clusters.[62] The rapidly decreasing cost associated with genome sequencing suggests that this approach will gain momentum in the coming years.

Matrix-assisted laser desorption/ionization imaging

Another recent advancement involves the use of scanning MS[108] to visualize directly the metabolite output of growing colonies: this approach is particularly well suited for peptides (including both nonribosomal and ribosomally produced molecules). Detection of metabolites can occur by either analysis of extracted metabolites by MS or by direct detection of the growing strain using matrix-assisted laser desorption/ionization imaging.[108,109] Metabolites of interest are fragmented and these fragments, or sequence tags, are used to deduce the identity of the amino-acid sequence, and ultimately, the identity of the peptide natural product.[108] Although this technique shows considerable promise in the identification of peptide natural products, to date it has proven less effective for identifying other classes of molecules. Furthermore, metabolites produced at low levels may be less amenable to this approach. Indeed, one of the biggest successes of matrix-assisted laser desorption/ionization imaging has involved perturbation of producer organisms through, for example, co-culture with other organisms.[110] Initial proof of principle demonstrated correct accurate of the previously identified ribosomal peptide AmfS from *S. griseus*, the nonribosomal peptide stendomycin from *S. hygroscopicus* and nine new ribosomal proteins including their biosynthetic clusters.[108] In addition, the production of the nonribosomal peptide arylomycin was detected in the daptomycin producer *S. roseosporus* using imaging MS.[109]

Conclusion: New momentum in secondary metabolite discovery

These developments, in our view, make it possible to purify and characterize virtually any secondary metabolite encoded in any microorganism. Unselective approaches including the application of synthetic small molecules[76] (SM Pimentel-Elardo and JR Nodwell, unpublished), the introduction of antibiotic-resistant mutations[74] or the introduction of regulatory genes[89] (Hameed, Socko and Nodwell, unpublished) can be used to enhance and alter the spectrum of secondary metabolite output in new streptomycetes: these can then be used to screen for novel activities of interest. Alternatively, selective methods including regulatory engineering or the introduction of gene clusters of interest into chassis strains can be used to investigate single molecules of interest.

Indeed, we imagine that these approaches go hand in hand. For example, if, as a result of an unselective screening procedure, a new compound is discovered, a logical next step would be the sequencing of the cognate genome and identification of the corresponding genes. Movement of these genes into a chassis strain could then be used to scale up yields of the molecule so that its mechanistic and, perhaps, clinical utility can be assessed.

We suggest that there has therefore never been a better time for a concerted effort to identify and understand the structural and functional diversity of microbial secondary metabolites, and to seek applications for these newly identified molecules.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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