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REVIEW

Strain improvement in actinomycetes in the postgenomic era

Richard H. Baltz

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Abstract With the recent advances in DNA sequencing technologies, it is now feasible to sequence multiple actinomycete genomes rapidly and inexpensively. An important observation that emerged from early *Streptomyces* genome sequencing projects was that each strain contains genes that encode 20 or more potential secondary metabolites, only a fraction of which are expressed during fermentation. More recently, this observation has been extended to many other actinomycetes with large genomes. The discovery of a wealth of orphan or cryptic secondary metabolite biosynthetic gene clusters has suggested that sequencing large numbers of actinomycete genomes may provide the starting materials for a productive new approach to discover novel secondary metabolites. The key issue for this approach to be successful is to find ways to turn on or turn up the expression of cryptic or poorly expressed pathways to provide material for structure elucidation and biological testing. In this review, I discuss several genetic approaches that are potentially applicable to many actinomycetes for this application.

Keywords Actinomycete · Cryptic genes · Genomics · Heterologous expression · Regulatory genes · Secondary metabolism · *Streptomyces*

Introduction

Methods for efficient chemical mutagenesis and recombination by protoplast fusion have been available for strain improvement in actinomycetes for over a quarter of a century [2, 45]. More recently, mutagenesis and protoplast fusion have been combined for whole genome shuffling to improve the production of antibiotics [22, 72, 75]. In addition, a number of molecular engineering strategies, often focused on optimizing precursor levels or expression of rate-limiting enzymes involved in secondary metabolite biosynthesis, have been summarized in other reviews [3–6, 51]. The mutagenesis, recombination, and molecular engineering approaches continue to be very useful tools for industrial-scale strain improvement.

As we have entered the postgenomic era, it has become apparent that actinomycetes with large genomes encode multiple secondary metabolite gene clusters, and that most of them are not expressed during standard fermentations [7, 14, 19, 23, 31, 36, 47, 50, 52, 68, 76]. The strategies for rapid strain improvement will likely be shifting from improved expression of well-known, highly productive, secondary metabolite fermentations to the expression of novel and often cryptic secondary metabolite pathways to solve the early-stage discovery problems of: (a) getting some level of expression of biosynthetic gene clusters identified by genome sequencing, and (b) rapidly increasing product yields to obtain enough material to characterize chemically and biologically. This review is not intended to be comprehensive, but is focused on recent examples of genetic approaches likely to be applicable to early-stage drug discovery, including expression of cryptic secondary metabolic genes identified in actinomycete genome sequencing projects.

Overexpression of positive regulatory genes

The overexpression of positive regulatory genes for yield enhancement is a concept that has been well established for

R. H. Baltz (✉)
CognoGen Biotechnology Consulting, 6438 North Olney Street, Indianapolis, IN 46220, USA
e-mail: rbaltz923@aol.com

some time [3, 4, 15, 16, 24, 51]. This approach continues to be productive, and new examples have provided further validation [9, 21]. A recent innovation is to decouple pathway regulation from complex or unknown regulatory cascades by the use of heterologous promoters to drive expression of pathway-specific positive regulatory genes [9]. The power of this approach was elegantly exemplified by the work of Cundliffe and colleagues on regulation of tylosin biosynthesis. Tylosin biosynthesis is regulated by a complicated interplay of at least three positive and two negative regulatory proteins [11, 12, 18, 24, 61–64]. At the top of the cascade, the TylP γ-butyrolactone receptor represses transcription of the *tylQ* repressor and the *tylS* activator genes, which in turn control the transcription of *tylU* and *tylR* activator genes. TylP repression is relieved by the production of a γ-butyrolactone. TylR expression is negatively regulated by TylQ and positively regulated by TylS and TylU. Disruption of *tylS* (*tylS*-KO) or *tylR* (*tylR*-KO) in wild-type *S. fradiae* C373.1 abolished tylosin production, whereas disruption of *tylU* (*tylU*-KO) greatly reduced tylosin production [12]. The regulation of *tylR* expression was bypassed by inserting an extra copy of *tylR* at the ϕC31 *attB* site under the transcriptional control of the *ermE** promoter. Insertion of *ermE**p-*tylR* into wild-type, *tylU*-KO, and *tylS*-KO strains resulted in 75–110% increases in tylosin production in a defined medium [12]. In another study using a complex medium, Stratigopoulos et al. [64] showed that addition of a second copy of *tylS* or *tylR* under transcriptional control of *ermE**p at the ϕC31 *attB* site of the wild-type strain caused increases in tylosin production from 40 mg/l to ~150 and ~200 mg/l, respectively. Similar duplications of *tylS* and *tylR* genes in the higher tylosin-producing strain *S. fradiae* C4 caused increases from 820 mg/l to 990 and 1,250 mg/l, respectively. These studies are important because they demonstrate that a complex regulatory cascade can be bypassed, and antibiotic yield can be enhanced, by simply expressing the main positive regulatory gene from the strong constitutive *ermE** promoter.

Some recent examples of decoupling pathway regulation from complex or unknown cascades by changing the promoter for positive regulators are: (a) the heterologous expression of the pladienolide biosynthetic gene cluster in a genome-minimized *S. avermitilis* strain by adding a second copy of the positive regulatory gene *pldR* expressed under transcriptional control of *ermE**p [38]; (b) the production of cephamycin C in *S. avermitilis* by adding a second copy of *ccaR* controlled by *ermE**p [38]; (c) the production of streptomycin in *S. avermitilis* by adding a second copy of the *strR* gene under transcriptional control of *aveR* or *rpsJ* promoters [38]; (d) the expression of the fredericamycin biosynthetic gene cluster in *S. lividans* under transcriptional control of the positive regulatory gene *fdmR1* expressed from *ermE**p [20]; and (e) the expression of the thiocoraline gene cluster from a marine *Micromonospora* species in *S. albus* J1074 by driving expression of the positive regulatory gene *tioA* with *ermE**p [43]. As demonstrated with tylosin [64], all of these examples required changing the promoter for a single pathway-specific positive regulatory gene to activate or improve expression. The example with thiocoraline expression is particularly important because it demonstrates that this approach can be used to express a secondary metabolite pathway from a nonstreptomycete actinomycete in a streptomycete host. This opens the possibility to explore and exploit the genomes of slow-growing, rare actinomycetes for novel secondary metabolite pathways.

Disruption of negative regulatory genes

The concept of improving productivity of a secondary metabolite by disrupting a negative regulatory gene was validated by the observation of Stratigopoulos and Cundliffe [61, 62] that the only mutation in the tylosin biosynthetic gene cluster in *Streptomyces fradiae* C4, a strain that contains several mutations associated with tylosin yield enhancement, was located in the *tylQ* gene. The mutation mapped to a helix–turn–helix region involved in DNA binding and repression. In a separate study, Zhang et al. [75] explored the same gene for mutation(s) after carrying out genome shuffling in *S. fradiae*, and found an independently derived *tylQ* mutation. The strain that was studied by Stratigopoulos and Cundliffe was used by Eli Lilly and Company for large-scale production of tylosin in the 1970s, and the original *tylQ* mutation was likely generated in the 1960s, decades before it was feasible to clone and sequence the tylosin biosynthetic gene cluster. So, empirical chemical mutagenesis revealed the vulnerable *tylQ* target in two separate instances over three decades after the original mutation was induced. With the technology now available, it is feasible to sequence the gene cluster of interest, search for genes that may encode positive or negative regulatory proteins, then carry out gene disruptions and duplications to confirm functions.

A recent example of improving product yield by disrupting a negative regulatory gene was described by Smanski et al. [59]. Platensimycin and platencin are recently discovered antibiotics produced by *Streptomyces platensis* with novel antibacterial targets (FabF and FabF plus FabH, respectively). The wild-type *Streptomyces platensis* produces these two compounds at ~1 mg/l. The negative regulatory gene *ptmR1* was disrupted, and individual mutants produced 323 mg/l and 255 mg/l of platensimycin and platencin, respectively.

Another example is the deletion of *scbR2* which encodes a γ-butyrolactone binding protein that represses the expression of the cryptic *cpk* gene cluster in *S. coelicolor* [29]. Deletion of *scbR2* resulted in the production of a new yellow pigment and antibiotic activities not previously observed in *S. coelicolor*, coupled with a substantial reduction in type II polyketide actinorhodin (Act) production. The latter observation may be due to competition for polyketide precursors, as the *cpk* pathway encodes a type I polyketide synthase [14]. The authors proposed that the yellow pigment (yCPK) may be derived from the antibiotic (abCPK), but the structures and yields of products were not determined. This may be the first example of activating the expression of an otherwise cryptic biosynthetic gene cluster in an actinomycete by deleting a negative regulatory gene located within the gene cluster [29]. Genes encoding γ-butyrolactone binding proteins can be identified bioinformatically, so this approach may be applicable to other orphan or cryptic secondary metabolite gene clusters identified in genome sequencing efforts.

Duplication of secondary metabolite biosynthetic pathway gene clusters

The duplication of genes encoding rate-limiting steps in antibiotic biosynthesis has been a successful method to improve antibiotic biosynthesis [3–6, 51]. The duplication or amplification of complete secondary metabolite biosynthetic gene clusters has dual advantages of improving product yield per se, and generating the biosynthetic capacity for further gains by improving production of key precursors, cofactors, etc., by mutagenesis or metabolic engineering. Whole pathway duplication or higher-order amplification has been validated to a limited extent by observations from industrial strain improvement programs; for instance, in *Streptomyces rimosus*, the oxytetracycline biosynthetic gene cluster is located about 600 kb from one end of the linear chromosome in a region prone to genetic instability [54]. Some spontaneous high-producing variants had three to four copies of a 200-kb tandem amplification containing the oxytetracycline gene cluster, and one variant had three to four copies of a 1-Mb linear plasmid containing one end of the *S. rimosus* chromosome including the oxytetracycline gene cluster recombined into an endogenous 387-kb linear plasmid. Although these variants produced elevated levels of oxytetracycline, they were too unstable for commercial production.

A lincomycin production strain of *Streptomyces lincolnensis* contains a duplication of a 450–500-kb DNA segment containing the lincomycin biosynthetic gene cluster [53]. A strain of *Streptomyces kanamyceticus* selected for increased resistance to kanamycin, which is a parent of current kanamycin production strains at Meiji Seika Kaisha, contains an average threefold tandem amplification of a 145-kb segment of DNA containing the kanamycin biosynthetic gene cluster [74]. The number of tandem repeats varied from cell to cell within the culture, thus reflecting the recombinational instability of tandem amplifications. The amplification was not maintained during sporulation, so strains were maintained as vegetative mycelia.

With the methods now available to clone large segments of actinomycete DNA in bacterial artificial chromosome (BAC) vectors [1, 8, 10, 25], it is now feasible to duplicate or triplicate large secondary metabolite gene clusters in a directed manner that avoids the instability associated with tandem amplifications. The genes can be inserted stably into one or more bacteriophage attachment sites (*attB*). There are several streptomycete bacteriophage attachment/integration (*att*/*int*) systems that can be used for stable integration, including φC31 [17, 39, 60], φBT1 [1, 30], TG1 [27, 46], and R4 [58]. The *attB* sites for these phages are unique; they are all located in genes widely conserved among *Streptomyces* species (R.H. Baltz, manuscript in preparation), and all reside within the stable core region of linear streptomycete chromosomes that contain primary metabolic functions. Many cryptic secondary metabolite gene clusters are located in the less stable chromosome ends, which are prone to deletion and amplification. *Streptomyces ghanaensis* produces the phosphoglycolipid moenomycin A. Makitrynskyy et al. [44] demonstrated that moenomycin production can be doubled by inserting a cosmid clone containing most of the moenomycin biosynthetic genes into the φC31 *attB* site of the *S. ghanaensis* chromosome, but production yields were not reported. Alexander et al. [1] deleted the complete lipopeptide A54145 biosynthetic gene cluster from the moderately productive *S. fradiae* XH125 that produced 472 mg/l of A54145 factors, and inserted the gene cluster present on a BAC vector into the φC31 or φBTI sites; the recombinants produced 513 and 583 mg/l of A54145 factors, respectively. In both cases, the A54145 gene cluster was transplanted from a potentially unstable region about 200 kb from one end of the linear chromosome to the stable core region. These data beg the question of what would happen if the A54145 biosynthetic gene cluster were left in the native site, then duplicated or triplicated by inserting additional copies into the φC31 and φBT1 sites. As a general concept, duplication or triplication of cryptic secondary metabolite gene clusters, particularly those mapping to potentially unstable regions of linear or circular actinomycete genomes, by inserting additional copies into the stable region of the chromosome, may improve the prospects of mining poorly expressed or cryptic pathways. This approach can also be coupled with the other

methods described in this review to further increase product yields.

Deletion or disruption of competing secondary metabolite pathways

In principle, actinomycetes that produce more than one secondary metabolite may encounter competition for some of the same precursors, cofactors, energy sources, reducing power, etc., thus limiting the potential yield of the most desired compound(s). A recent example is *S. avermitilis*, which produces two large polyketides, avermectin and oligomycin, and other secondary metabolites [36]. Komatsu et al. [38] generated a genome-minimized *S. avermitilis* strain deleted for many secondary metabolite gene clusters, and heterologous expression of the streptomycin biosynthetic gene cluster from *Streptomyces griseus* was improved from ~30 mg/l in a wild-type *S. avermitilis* strain to ~180 mg/l in the genome-minimized strain. Other heterologous expression hosts have been developed which have deleted or inactivated one or more key secondary metabolite pathways [9]. Some examples will be discussed in sections below.

Antibiotic selections for mutations that enhance transcription and translation of secondary metabolite genes in stationary phase

Selection for antibiotic resistance has several general advantages for rapid yield enhancement: (a) it is simple, rapid, inexpensive, and does not require automated high-throughput fermentation and analytical analysis; (b) all survivors of selection are mutants, so all possible antibiotic resistance mutations in a particular gene (e.g., *rpoB* or *rpsL*; see below) can be sampled by screening <100 individual mutants; (c) spontaneous mutations can be selected, so all possible base-pair substitutions (including transitions and transversions), deletions, and insertions can be surveyed; (d) chemically induced mutations can be avoided, thus eliminating the possibility for second-site negative mutations, while avoiding the limited spectrum of induced mutations [e.g., *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) induces GC to AT transitions almost exclusively].

Mutations in *rpoB*

Ochi and colleagues noted some time ago that certain rifamycin-resistant (RifR) mutations that mapped to the *rpoB* gene caused activation of production of Act, undecylprodigiosin (Red), and calcium-dependent antibiotic (CDA) in *S. lividans* [35, 40, 49]. They also showed that certain RifR mutations in *rpoB* reversed the deleterious effects of *relA* or *rplK* (*relC*) mutations on Act production [73]. They proposed that the mutant *rpoB* genes may encode RNA polymerase β-subunits that mimic the ppGpp-bound wild-type enzyme involved in activating the onset of secondary metabolism [45, 73].

Whereas *Streptomyces* species generally have a single copy of *rpoB*, some nonstreptomycete actinomycetes have two nonidentical *rpoB* genes [37, 65, 69]. The second *rpoB* gene, designated as $rpoB^R$ or *rpoB*(R) in *Nonomuraea* sp. strain ATCC 39727 (referred to as *Actinomadura* sp. strain ATCC 39727 before reclassification), has a mutation that confers a RifR phenotype [65, 69]. In the wild-type strain, the level of RifR was relatively low, but a derivative expressed high level RifR. The wild-type strain expressed *rpoB*(R) at low levels during early growth, but higher levels in transition and stationary phases, thus accounting for the relatively low level RifR phenotype. The more highly RifR derivative expressed *rpoB*(R) constitutively, initiated production of the glycopeptide antibiotic A40926 earlier than wild type, and achieved higher overall production of A40926 [69]. This suggests that constitutive expression of *rpoB*(R) facilitates early onset of antibiotic production as well as robust production in stationary phase.

Talà et al. [65] extended the latter observation by introducing the *rpoB*(R) gene from *Nonomuraea* sp. strain ATCC 39727 into the ϕC31 *attB* site of the *S. lividans* chromosome under transcriptional control of its native promoter. The recombinant produced elevated levels of Act, Red, and CDA relative to the wild-type parent. They demonstrated that a specific amino acid substitution N426 in RpoB(R), relative to the typical H426 found in RpoB(S) enzymes, was responsible for the elevated production of antibiotics. It will be interesting to see if expression of *rpoB*(R) activates cryptic secondary metabolite gene clusters in other actinomycetes.

Mutations in *rpsL*

Ochi and colleagues have been studying the effects of *rpsL*, *rsmG*, and other mutations that influence ribosome function on antibiotic production in *Streptomyces* species, and their early findings have been reviewed [49]. Several different *rpsL* mutations, primarily associated with amino acid substitutions at Lys residues at positions 43 and 88 of ribosomal protein S12, confer high level resistance to streptomycin (StrR) and are easily selected in a wide variety of actinomycetes. Mutations in *rsmG* express low level StrR, so the selection method may vary for different actinomycetes. For the purposes of this review, I focus on *rpsL* mutations.

In *S. coelicolor*, a mutation conferring a K88E substitution caused an increase in Act production from about 2 to 70 mg/l (Table 1). A mutation causing a K88E substitution in *S. antibioticus* resulted in an increase in actinomycin

production from 5.5 to 28 mg/l, and a K88R substitution in *S. parvulus* caused an increase in actinomycin production from about 6 to 33 mg/l. In *S. avermitilis*, a K88R substitution had little effect on avermectin production, whereas a K88E mutation reduced avermectin production from about 540 to about 40 mg/l, while increasing the production of oligomycin from about 10 to about 700 mg/l [67]. In the A54145-producing *Streptomyces fradiae*, a mutant with *rpsL* (K88E) produced 371 mg/l (88% of control), whereas one with *rpsL* (K88R) produced 472 mg/l (112% of control) (Table 1). Other *S. fradiae rpsL* mutants produced yields ranging from 27% of control (K43T) to 109% of control (K43N) [1].

A striking example of a productive *rpsL* mutation was the improvement of salinomycin production in an industrial strain of *Streptomyces albus* [66]. The wild-type *S. albus* JCM 4703 produces about 250 mg/l of salinomycin, whereas strain SAM-X selected after sequential ultraviolet (UV) and chemical mutagenesis of JCM 4703 produces about 10 g/l of salinomycin. An *rpsL* mutant KO-600 (K88R) produced 15 g/l (Table 1). *S. albus* KO-600 produced ribosomes that were much more active than those of the wild-type strain in stationary phase when salinomycin is produced.

The combined studies with different streptomycetes indicate that strains containing *rpsL* mutations causing K88E or K88R amino acid substitutions are the most likely ones to enhance secondary metabolite productivity. In some cases the enhancement was substantial, and in others modest. Productive mutations in *rpsL* can be combined with those in *rpoB* and others to further improve secondary metabolite production as discussed below.

Elevated production of ribosome recycling factor (RRF)

Hosaka et al. [32] demonstrated that enhanced protein synthesis in stationary phase and elevated Act production in a *S. coelicolor rpsL* mutant (K88E) are caused by increased expression of RRF encoded by the *frr* gene. This was confirmed by cloning the *frr* gene in a high-copy-number plasmid and introducing it into the wild-type strain. The recombinant produced 7–10 times as much Act as control.

Li et al. [42] showed that overexpression of *frr* in *S. avermitilis* causes increased avermectin biosynthesis in wild-type and industrial production strains. Elevated avermectin production was obtained by expressing *frr* from a single-copy insertion vector, pSET152, and from the multicopy replicating vector, pKC1139. Comparable yields were obtained using the native promoter or *ermE**p, and expression of *frr* from pKC1139 gave higher yields than expression from pSET152. In the wild-type strain, the avermectin yield was improved from about 190 to about 875 mg/l, whereas in the most productive strain the yield was increased from 6.2 to 7.8 g/l. They showed in the wild-type strain that overexpression of *frr* from pKC1139 caused more robust cell growth and enhanced transcriptional expression of the pathway-specific regulatory gene *aveR* and of four PKS genes. These results suggest that overexpression of *frr* may be a general method to boost the robustness of translation during stationary phase when secondary metabolites are being produced. The enhancement of production in the wild-type strain by nearly 700 mg/l and in the production strain by 1.6 g/l suggests that this approach may have applications to early- and later-stage yield enhancement projects with other actinomycetes. It is noteworthy that the yield enhancement in the *S. avermitilis* production strain by the addition of the *frr* gene expressed from its native promoter or from *ermE**p on pSET152 was increased from 6.2 to 7.5 g/l, suggesting that stable insertion of a second copy of the *frr* gene in the chromosome of high-producing strains may facilitate fermentation scale-up. It is not known if adding a heterologous second copy of the *frr* gene can activate cryptic secondary metabolite gene clusters in actinomycetes, or if

Table 1 Antibiotic production in *Streptomyces* species containing StrR (*rpsL*) mutations

Strain	Mutation	Antibiotic	Yield (mg/l)		Yield change (mg/l)	Reference
			control	mutant		
S. coelicolor	*rpsL* (K88E)	Actinorhodin	2	70	68	67
S. antibioticus	*rpsL* (K88R)	Actinomycin	5.5	28	22.5	67
S. avermitilis	*rpsL* (K88R)	Avermectin	540	500	−40	67
		Oligomycin	10	20	10	67
	rpsL (K88E)	Avermectin	540	40	−500	67
		Oligomycin	10	700	690	67
S. parvulus	*rpsL* (K88R)	Actinomycin	6	33	27	67
S. fradiae	*rpsL* (K88R)	A54145	423	472	49	1
	rpsL (K88E)	A54145	423	371	−52	1
S. albus	rpsL(K88R)	Salinomycin	10,000	15,000	5,000	66

constitutive expression of *frr* from *ermE**p or other promoters would be advantageous, so this is an area ripe for further investigation.

Combinations of antibiotic resistance mutations

Hu and Ochi [34] showed that, while certain StrR, RifR, and GenR mutants of *S. coelicolor* gave 1.6- to 3.0-fold increases in Act production, combinations of two resistance mutations gave additional 1.7- to 2.5-fold increases, and a combination of three gave an additional 3.5-fold increase. The final strain SGR-1 contained *rpsL* (K88E), *rpoB* (H347Y), and an unmapped GenR mutation. The authors noted that increased Act production was associated with increased expression of ActII-ORF4, a positive regulatory protein. They also noted that the triple mutant produced Act over a longer period of time, and that Act production was less sensitive to media differences than the wild-type strain.

Wang et al. [70] built on the StrR, GenR, RifR mutant SGR-1 by sequentially selecting for spontaneous mutations conferring resistance to other antibiotics that target the ribosome. A mutant strain C7 had four additional mutations conferring resistance to paromomycin, Geneticin, fusidic acid, and thiostrepton. C7 produced 1.63 g/l of Act compared with 9 mg/l produced by the wild-type *S. coelicolor* 1147 in GYM medium. High level Act production of C7 was associated with elevated ppGpp, elevated expression of ActII-ORF4, and robust protein synthesis late in the fermentation cycle.

Beltrametti et al. [13] showed that a combination of antibiotic resistance mutations causes elevated production of the thiopeptide GE2270 in the rare actinomycete *Planobispora rosea*. Individual StrR, RifR, and GenR mutations accounted for 10%, 10%, and 50% increases in GE2270 production, whereas sequential selection of StrR, GenR, and RifR mutations led to an 80% increase in titer, although the actual yields were not disclosed.

After showing that an *rpsL* mutation (K88R) caused an increase of 5 g/l (from 10 to 15 g/l) of salinomycin in *S. albus*, Tamehiro et al. [66] further selected for StrR-GenR, then StrR-GenR-RifR mutations. They showed that the double mutant produced 18 g/l and the triple mutant nearly 23 g/l of salinomycin. The GenR mutation was not mapped, but the RifR mutation mapped to *rpoB* (S442F). This remarkable yield enhancement by a combination of StrR-GenR-RifR mutations is the only published report using an industrial production strain, so it remains to be seen if the approach will be widely applicable for late-stage strain improvement.

Hosaka et al. [33] explored the use of RifR and StrR selections to activate silent antibiotic biosynthetic pathways in actinomycetes. In an agar plug assay, the majority of over 1,000 strains did not produce antibiotic activity. Among the antibiotic-resistant derivatives of apparent nonproducers, 43% of streptomycetes and 6% of non-streptomycetes produced antibacterial activities against *S. aureus*. They did further work on *Streptomyces mauvecolor*, a strain that produced no antibacterial activity in any medium tested. They isolated spontaneous StrR, RifR, and GenR mutants, and several RifR mutants, one RifR and StrR double mutant, and one GenR mutant produced antibiotic activity. The RifR and StrR mutations mapped to *rpoB* (H437L) and *rpsL* (K88R), respectively, and mutant strains produced a family of novel piperidamycin antibiotics.

Expression of secondary metabolite genes in heterologous hosts

For genomics applications, the cloning and heterologous expression of silent or poorly expressed secondary metabolite gene clusters provides another approach to produce sufficient yields for isolation and characterization of novel compounds. This approach may be particularly useful for the expression of cryptic pathways from rare actinomycetes that grow slowly or are not amenable to industrial-scale fermentation for other reasons. There are a number of laboratory strains that have been used to express heterologous secondary metabolite gene clusters [9, 28]. *S. lividans* and *S. coelicolor* have been used to express a number of secondary metabolite pathways, but the yields are not generally very high [9]. The yields in *S. lividans* ranged from 0 to 400 mg/l with a median of 20 mg/l, and yields in *S. coelicolor* ranged from 23 to 47 mg/l with a median of about 30 mg/l (Table 2).

Recently, Gomez-Escribano and Bibb [28] developed *S. coelicolor* M1154 that is deleted for the Act, Red, CPK, and CDA biosynthetic gene clusters. Strain M1154 also contains *rpsL* (K88E) and *rpoB* (S433L) mutations that enhance productivity of secondary metabolites, although they noted that the *rpoB* mutation accounted for most of the yield enhancement. *S. coelicolor* M1154 produced chloramphenicol and congocidine at 40- and 30-fold elevated levels relative to *S. coelicolor* M145 [28], and caprazamycin derivatives at an average of 152 mg/l [26]. *S. coelicolor* M1154 should be useful for expression of cryptic pathways because it has a simplified secondary metabolome as well as mutations to enhance secondary metabolite production in stationary phase. *S. albus* J1074 is another generally useful laboratory strain [9]. Heterologous production yields of iso-migrastatin and fredericamycin were 46 and 132 mg/l, respectively (Table 2).

Other expression hosts have been derived from industrial production strains or strains that have undergone some

Table 2 Heterologous expression of secondary metabolite gene clusters in *Streptomyces* hosts

Strain	Gene cluster	Product yield (mg/l)	Reference
S. lividans 66, 1326, K4-114, TK23, TK23 (*Δact*), TK24	Multiple	0, <1, 2.6, 10, 15, 20, 20, 25, 33, 50, 230, 400	9, 41
S. coelicolor M512, CH999	Multiple	23, 26, 31, 47	9
S. coelicolor M1154	Caprazamycins	152	26, 28
S. albus J1074	Iso-migrastatin	46	9
	Fredericamycin	132	9
S. avermitilis SUKA5, SUKA17	Multiple	80, 130, 180	9
S. roseosporus UA431	A54145	100	1
S. ambofaciens BES2074	A54145	385	1
S. fradiae K159-1	Midecamycin	1000	57
S. cinnamonensis C730.7	Tetracenomycin	4950	41

mutagenesis and selection for improved productivity [9]. For example, the complete A54145 lipopeptide antibiotic biosynthetic pathway was cloned in a BAC vector, conjugated from *E. coli* into *Streptomyces ambofaciens* BES2074 blocked in spiramycin biosynthesis, and inserted into the ϕC31 *attB* site; the transconjugant produced 385 mg/l of A54145 factors without fermentation optimization [1] (Table 2). The A54145 yield in *S. ambofaciens* was 4 times the yield of wild-type *S. fradiae* A54145 [1].

Strains derived from *Streptomyces fradiae* C373.1 by sequential mutagenesis and selection have been used for commercial production of the 16-membered macrolide antibiotic tylosin [24, 64]. The polyketide portion of tylosin is derived from malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA. Katz and colleagues developed a *S. fradiae* expression host derived from a moderately productive strain that is deleted for the *tylGI-V* polyketide synthase (PKS) genes, and have used it to produce hybrid polyketides in 1–2 g/l quantities [55, 56, 71]. They further modified the strain by adding five genes (*fkbG-K*) from the ascomycin producer *Streptomyces hygroscopicus*, under the transcriptional control of the *tylGI*p promoter to synthesize another polyketide precursor, methoxymalonyl-ACP [57]. They used this system to express the PKS genes from the midecamycin biosynthetic pathway from *Streptomyces mycarofaciens*, and the recombinant produced about 1 g/l of an analog of midecamycin, which required malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, and methoxymalonyl-ACP for polyketide assembly [57]. This strain should be generally useful for the heterologous expression of polyketide pathways requiring any of the four polyketide building blocks.

Another useful polyketide expression host is *Streptomyces cinnamonensis*, the commercial producer of nonglycosylated type I polyketide monensin. Reynolds and colleagues obtained two *S. cinnamonensis* strains from Eli Lilly and Company that produced moderate (strain C730.1) and elevated levels of monensin (strain C370.7). They cloned the gene cluster for the nonglycosylated type II polyketide tetracenomycin under the transcriptional control of the native *tcm* promoter into pSET152, and inserted the genes into the ϕC31 *attB* site of *S. cinnamonensis* of C730.1, C730.7, and two other strains. After fermentation in an oil-based medium in shake flasks, the strain derived from C730.7 produced the highest titer of tetracenomycins, 4.95 g/l [41], and normal levels of monensin. Another C730.7-derived strain deleted for monensin genes produced 4.18 g/l of tetracenomycins, so it appears that the oil-based medium provides ample precursors for the concurrent biosynthesis of tetracenomycin (requiring malonyl-CoA) and monensin (requiring malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA). As controls, the wild-type tetracenomycin producer and a recombinant *S. lividans* strain containing the cloned tetracenomycin gene cluster produced about 20-fold lower yields, 0.26 g/l and 0.23 g/l, respectively. It will be interesting to see if *S. cinnamonensis* C370.7 can produce other, more complex (e.g., glycosylated) polyketides, or non-PKS-derived secondary metabolites in high yields.

Other promising expression hosts (some included in Table 2) are *S. avermitilis* SUKA17 (deleted for major secondary metabolite pathways) [38]; *S. fradiae* DA1187 (*rpsL*[K88R], deleted for the A54145 gene cluster) [1], *S. roseosporus* UA431 (deleted for the daptomycin gene cluster) [48]; and *Saccharopolyspora erythraea* (erythromycin nonproducer derived from commercial production strain) [56]. These and other actinomycete expression hosts are discussed in more detail elsewhere [9].

Discussion

The approaches to strain improvement discussed in earlier reviews [2–6, 45, 51] remain valid for the generation of

strains for commercial production of antibiotics and other secondary metabolites. MNNG remains the mutagen of choice, followed by 4-nitroquinoline-1-oxide, methyl methanesulfonate, and ethyl methanesulfonate [2, 5]. Other approaches using transposon mutagenesis, targeted gene duplications, and linear replicon fusions were validated some time ago [5, 6]. In the present review, I have discussed several genetic approaches to product yield enhancement that should be generally applicable to early-stage projects, particularly those exploiting actinomycete genome sequencing. It is now feasible to sequence multiple actinomycete genomes inexpensively and to annotate potential novel secondary metabolite pathways, including pathway-specific positive and negative regulatory genes. Also, methods are available to genetically engineer many different strains of actinomycetes, particularly streptomycetes, using cosmid and BAC vectors with functions for conjugal transfer from *E. coli* to actinomycetes (RP4 *oriT*), λ-Red-mediated recombination for rapid engineering in *E. coli*, and bacteriophage integration systems (ϕC31, ϕBT1, and others) to generate stable recombinants [8, 10; Baltz, manuscript in preparation]. Furthermore, constitutive gene expression can be assured by using the *ermE** promoter, as exemplified in many studies described here and elsewhere [10]. These methods have provided the means to: (a) duplicate positive regulatory genes and express them constitutively, (b) delete or disrupt negative regulatory genes, (c) duplicate or triplicate other genes or complete secondary metabolite gene clusters, (d) delete or disrupt secondary metabolite pathways that compete for key precursors for secondary metabolite production, (e) overexpress the *frr* gene to improve translation during stationary phase, and (f) express silent or cryptic secondary metabolite gene clusters in heterologous hosts. In addition, product yields can often be enhanced by selecting for resistance to certain antibiotics that interfere with translation or transcription (e.g., StrR for *rpsL* mutations and RifR for *rpoB* mutations). These mutations can also be coupled with the molecular engineering approaches outlined above, and combinations of these approaches may facilitate the production of sufficient quantities of product for structure determination and biological profiling. Strains producing compounds that meet desired biological activity profiles can be further developed using more traditional chemical mutagenesis, recombination, and metabolic engineering approaches.

A number of questions remain regarding the application of the genetic approaches reviewed herein to the discovery and development of novel secondary metabolites. (1) Will the sequencing of multiple actinomycete genomes become a robust method for discovery of novel secondary metabolite pathways? (2) Can productive *rpsL* and *rpoB* alleles be cloned and transferred into multiple heterologous hosts for activation of cryptic pathways and yield enhancement as an alternative to selecting StrR and RifR mutations? The limited data on *Nonomuraea rpoB*(R) suggest that this might work. (3) Can a cloned and constitutively expressed *frr* gene activate cryptic secondary metabolite pathways in diverse actinomycete hosts? (4) Can the positive effects of overexpression of *frr* on translation be coupled additively or synergistically with the positive effects of certain *rpoB* mutations on transcription during stationary phase? If so, can they be transferred to multiple actinomycetes as a cassette delivered by conjugation from *E. coli* and insertion into phage *attB* sites? (5) Can a more robust set of expression hosts be assembled from the most productive but currently proprietary industrial strains? What will it take to convince industry to release the best strains to advance the discovery process? So far, only a small number of intermediate-level producers are in the public domain. (6) How robust is the approach to duplicate and triplicate cryptic or poorly expressed pathways in native or heterologous hosts? (7) Can the questions outlined above be addressed in a reasonable timeframe to reinvigorate the discovery and development of natural products from actinomycetes? Are the current mechanisms of funding natural products research adequate, or will it require a more coordinated, better funded multinational effort? The good news is that the genome sequencing and molecular genetic tools are available to answer many of these questions.

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