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New genetic methods to improve secondary metabolite production in *Streptomyces*

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The most potent chemical mutagens commonly used for yield enhancement in streptomycetes induce a limited spectrum of base-pair substitutions, heavily dominated by GC to AT transitions. The AT to CG transversion pathway complements the GC to AT pathway, but no strong mutagens with this specificity are available. However, *mutT* mutations in *Escherichia coli* enhance spontaneous AT to CG transversions about 1000-fold, so such mutations in streptomycetes could be very beneficial for random mutagenesis. Rate-limiting steps in secondary metabolite biosynthesis, on the other hand, can be best addressed by cloning and insertion of extra copies of the appropriate gene(s) into a neutral genomic site. This approach has been used successfully to improve the production of tylosin and pristinomycin. Transposon mutagenesis can be used to identify and clone neutral genomic sites, as demonstrated in *Streptomyces fradiae* to improve tylosin yields. Transposon mutagenesis can also be used to activate or enhance the transcription of genes important for secondary metabolite production, and to identify and clone both positive and negative regulatory elements. In some cases it may be advantageous to amplify the complete biosynthetic pathway for secondary metabolite production. In *Streptomyces rimosus*, a highly productive oxytetracycline producer was shown to contain the complete biosynthetic pathway near the end of the linear chromosome, and on a hybrid linear plasmid that had undergone recombination with the chromosome. This concept of linear replicon fusion might be applicable to other secondary metabolite pathway genes that are located near one end of the linear chromosome.

Keywords: chemical mutagenesis; gene duplication; insertional cloning; transposon mutagenesis; linear replicon fusion

Introduction

Chemical mutagenesis continues to be a very successful method to improve secondary metabolite yields, but cannot be readily applied to solve specific rate limitations in biosynthesis. Gene cloning methods are best suited for this purpose, and the methods to construct stable recombinant strains containing extra copies of key genes inserted in the chromosome are now available. Transposable elements and linear replicon fusions may also be used to augment chemical mutagenesis and gene cloning. In this communication, recent examples are summarized of the application of several of these new methods to improve secondary metabolite yields, and a new approach suggested to broaden the specificity of base-pair substitution mutagenesis in streptomycetes.

Point mutations—can the distribution of base-pair substitutions be broadened?

The traditional method to improve secondary metabolite yields is by mutagenesis and fermentation analysis. Of the mutagens most widely used, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) is the most potent (Figure 1) and the most efficient at generating the optimal yield of desired mutants [1]. The major shortcoming of MNNG is its specificity; it produces GC to AT transition mutations almost exclusively [8,20]. Unfortunately, the other mutagens that are relatively potent (eg, EMS and NQO; see Figure 1) also show a strong bias toward this mutagenic pathway [8,20]. UV light, on the other hand, induces a broader spectrum of point mutations in *Escherichia coli* [8,20], but appears to be a rather weak mutagen in *Streptomyces fradiae* ([1]; Figure 1). I surveyed the distribution of potential amino

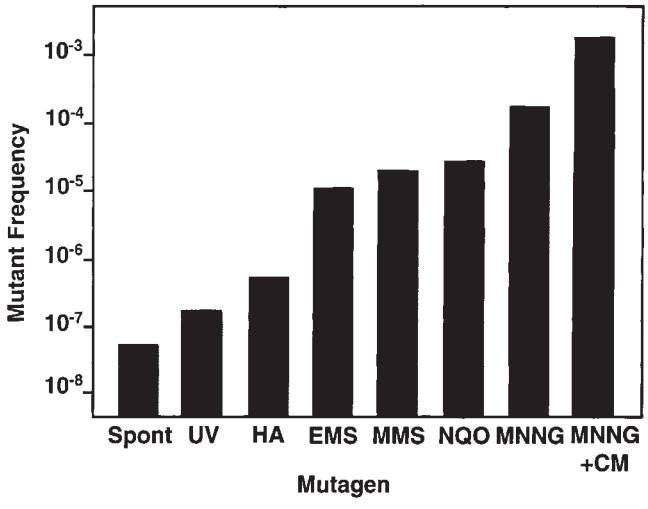

Figure 1 Frequency of spectinomycin-resistant mutants induced by different mutagens in *Streptomyces fradiae*. The mutant frequencies are those obtained at doses yielding approximately 10% survival of colony-forming units after treatment. Spont, spontaneous; UV, Ultraviolet light; HA, hydroxylamine; EMS, ethyl methanesulfonate; MMS, methyl methanesulfonate; NQO, 4-nitroquinoline-1-oxide; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; CM, chloramphenicol. Data calculated from Baltz [1].

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Table 1 Amino acid substitutions and chain terminations generated from streptomycete codons[a]

Starting codon	GC to AT	GC to TA	AT to CG
Phe	0	1	2
Leu	1	2	4
Ile	0	0	2
Met	1	1	2
Val	2	1	2
Ser	3	2(1)[b]	3
Pro	4	2	0
Thr	2	2	2
Ala	4	4	0
Tyr	0	(1)	2
His	1	2	1
Gln	(1)	2	1
Asn	0	1	2
Lys	0	1	2
Asp	1	2	2
Glu	1	1(1)	1
Cys	1	1(1)	1
Trp	(2)	1(1)	1
Arg	5	5	0
Gly	4	4	0
end	0	2	4

[a]Codons ending in G or C.
[b](), mutation leads to chain termination.

acid substitutions and chain termination mutations in the G+C rich streptomycetes that can be generated by different mutational pathways from the most prevalent codons (those containing G or C in the third position; [30]). Table 1 shows the number of potential mutations from streptomycete codons by three different mutational pathways. The GC to AT transition pathway cannot generate mutations at Phe, Ile, Tyr, Asn or Lys prevalent codons, whereas the AT to CG transversion pathway can generate all five of these. Table 2 shows the potential mutations that can generate new codons. The GC to AT pathway cannot generate codons for Pro, Ala or Gly. Similarly, the GC to TA transversion pathway cannot generate these amino acid substitutions, but the AT to CG transversion pathway can. A more thorough comparison of the three pathways generally indicates that of the two transversion pathways, the AT to CG pathway is strikingly more complementary to the GC to AT transition pathway. Analysis of potential mutations at the 31 dominant codons for streptomycetes indicates that 30 amino acid substitutions can be induced by the AT to CG pathway resulting in 25 different amino acid substitutions. Similarly, the GC to AT pathway gives 30 potential amino acid substitution mutations, 26 of which represent different amino acid substitutions. None of the amino acid substitutions induced by either of these mutational pathways can be induced by the other pathway. Therefore, one way to broaden the spectrum of point mutations in streptomycetes would be to develop a method to generate AT to CG transversions. Since this pathway is not induced effectively by any of the mutagens that work well in streptomycetes [1,8], a different approach will be required.

Table 2 Amino acid substitutions that generate new codons in streptomycetes[a]

New codon	GC to AT	GC to TA	AT to CG
Phe	2	2	0
Leu	3	5	2
Ile	3	4	0
Met	2	1	0
Val	2	2	2
Ser	3	4	2
Pro	0	0	4
Thr	2	2	2
Ala	0	0	5
Tyr	2	3	0
His	1	1	1
Gln	1	2	1
Asn	2	3	0
Lys	2	3	0
Asp	1	3	1
Glu	1	2	0
Cys	1	1	1
Trp	1	1	1
Arg	1	0	4
Gly	0	0	4

[a]Calculations made from codons ending in G or C.

In *E. coli*, a number of strong mutator mutations have been identified, and of these the *mutT* mutation causes a specific strong enhancement of spontaneous mutations by the AT to CG transversion pathway [21]. Approximately 1000-fold increases in mutation rates have been observed [25]. To implement this methodology in streptomycetes, the *mutT* gene needs to be identified, cloned and disrupted. With the *rpsL* system for selection and counter selection [12], a system could be set up to allow selection of a mutant containing a disrupted *mutT* gene, and spontaneous mutants containing AT to GC transversions could be generated and analysed in this strain. The wild type *mutT* gene could be regenerated by recombinational excision, selecting for streptomycin resistance encoded by a chromosomal allele of the *rpsL* gene [12]. The identification of the *mutT* gene should be facilitated by the *Streptomyces coelicolor* genome sequencing effort now underway.

Cloning and neutral insertion of genes involved in rate-limiting steps in secondary metabolite production

It is well documented that the introduction of self-replicating plasmids can cause the inhibition of product formation in streptomycetes [2,3,5]. Thus, to address a rate-limiting step in secondary metabolite biosynthesis, it is necessary to clone, express and insert the appropriate gene(s) into a neutral site in the genome [3–5]. There are several ways to insert DNA into the chromosome, including the use of plasmid or bacteriophage site-specific insertion elements, the use of transposable elements to generate regions for homologous transposon exchange, and the use of cloned neutral genomic sites for homologous insertion of plasmid DNA or for insertion of individual genes [3–5]. In *S. fradiae*, the producer of the macrolide antibiotic tylosin, Tn*5099* transposition mutagenesis has been used to locate neutral genomic sites. The transposon in a neutral site and the neutral site *per se* have been used to introduce a second copy of the macrocin *O*-methyltransferase gene (*tylF*) by homologous recombination [6,29]. In both cases,

recombinants produced substantially more product by converting the precursor macrocin to tylosin more efficiently. In *Streptomyces pristinaespirallis*, the *snaA* and *snaB* genes that encode a heterodimeric protein that oxidizes pristinamycin IIB to pristinamycin IIA, have been fused with the strong constitutive promoter *ermEp** and inserted into the plasmid pSAM2 *attB* site in the chromosome [26]. The recombinant produced a higher yield of the desired pristinamycin IIA. These two examples illustrate the value of neutral chromosomal insertion systems to construct stable production strains.

Other transposon applications

Tn*5099* and related transposons have outward reading promoter activity [7]. Therefore it is possible that insertions upstream of certain key genes might change the level or timing of transcription to enhance the production of a desired secondary metabolite. Also, since Tn*5099* and related transposons insert relatively randomly in streptomycetes [6,18,27,29], insertion into negative regulatory genes might enhance productivity. Insertion into positive regulatory genes for secondary metabolite biosynthesis would cause a reduction in yield. In either case, the regulatory gene could be cloned, selecting for the hygromycin resistance gene present on the transposon that expresses in streptomycetes and in *E. coli* [11]. McHenney and Baltz [18] surveyed a number of Tn*5099* transposition mutants of *Streptomyces roseosporus* that expressed different levels of red pigment for their levels of production of the cyclic lipopeptide antibiotic daptomycin. Figure 2 summarizes some of the key findings. A number of the mutants that produced normal levels of red pigment also produced control levels of daptomycin. These are neutral insertions, and therefore define potential sites to insert genes. Normal levels of red pigment were produced by strain MM93, which contains an insertion in a daptomycin peptide synthetase subunit, blocking daptomycin production [19]. This indicated that red pigment and daptomycin production can be decoupled.

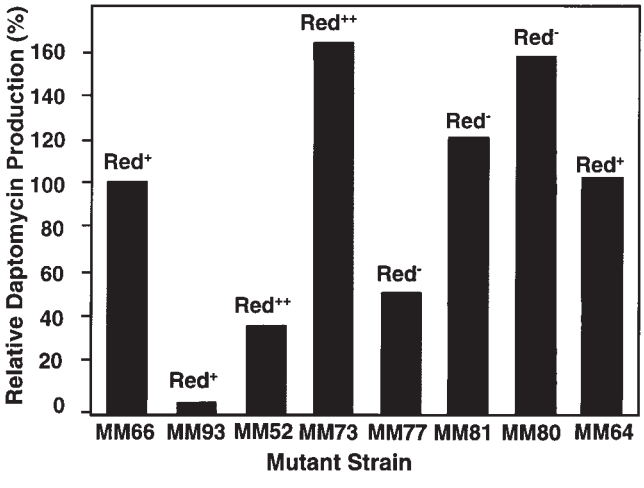

Figure 2 Levels of daptomycin produced in Tn*5099* transposition mutants of *Streptomyces roseosporus* that produce different amounts of red pigment. Red$^+$, wild-type level of red pigment; Red^{++}, elevated level of red pigment produced; Red$^-$, no red pigment produced. Data from McHenney and Baltz [18].

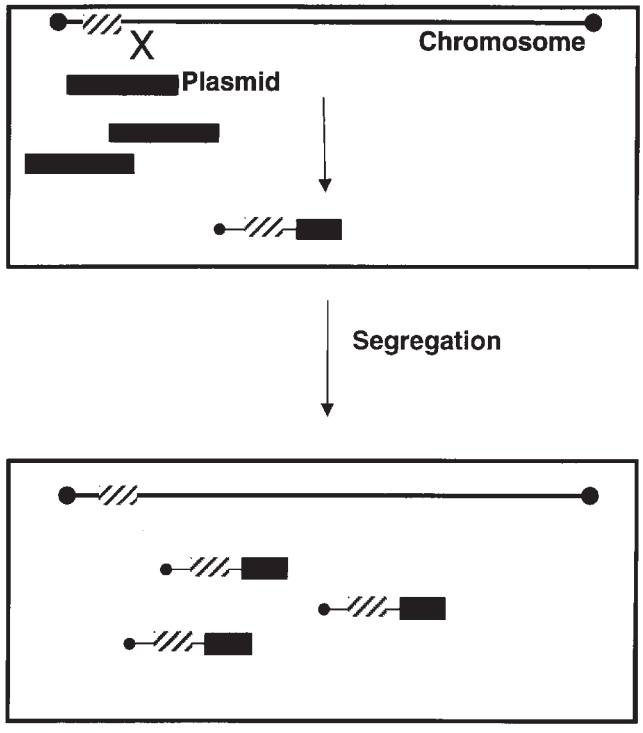

Figure 3 Linear replicon fusion or streptomycete artificial chromosome (SAC) concept. Crosshatched region indicates secondary metabolite genes.

Among the red pigment overproducers, they observed daptomycin overproduction or reduced production. The same was true with the red pigment nonproducers (Figure 2). Some of these effects might be explained by insertions into regulatory genes or to changes in expression due to transcriptional fusions. Cloning and sequencing the sites of insertion should help define the nature of the genetic changes, and define the steps needed to further exploit the observations (eg, delete a negative regulatory gene or add a second copy of a positive regulatory gene). This example demonstrates that transposition mutagenesis can be used to induce mutations that enhance product yield, and might aid in determining which genes are critical in controlling the levels of secondary metabolite production in streptomycetes.

Linear replicon fusions or streptomycete artificial chromosomes (SACs)

The chromosomes of streptomycetes are generally linear [13–17,19,22–24]. In addition, many streptomycetes harbor linear plasmids [2]. There is a growing body of evidence indicating that many antibiotic biosynthetic pathway genes are clustered near one end of the linear chromosome [13,19,22], a region generally prone to deletion and amplification [14,22,23], and often devoid of genes required for normal growth and primary metabolism. In *Streptomyces rimosus*, the 30-kb oxytetracycline biosynthetic gene cluster is located in a region 550–670 kb from one end of the linear chromosome and close to the 550-kb terminal inverted repeat, a region prone to deletion and amplification [9,10,22]. *S. rimosus* also harbors a 387-kb linear plasmid

[10]. Cullum and co-workers have analysed a number of spontaneous variants of *S. rimosus* that differ in pigmentation, sporulation, tetracycline resistance or tetracycline biosynthesis [9,10]. One high-producing variant of particular interest contained a 1-Mb linear plasmid composed of part of the 387-kb plasmid and one end of the linear chromosome, including the complete oxytetracycline biosynthetic gene cluster [10]. Since the plasmid was present in three or four copies per linear chromosome, the antibiotic yield enhancement was likely due to the increased expression of the complete oxytetracycline biosynthetic pathway. Since the hybrid plasmid appears to have been generated by a single crossover between the 387-kb plasmid and the chromosome, this suggests that dissimilar plasmid and chromosomal ends (teleomers) are compatible with normal replication of linear elements. Similar recombinational events might be feasible in other streptomycetes, particularly those containing secondary metabolic genes near one end of the chromosome (Figure 3). The recombinational events could be enhanced by providing regions of homology to direct the precise location of the crossover. Alternatively, a more random set of hybrid plasmid primes might be generated by crossing plasmid and chromosomal elements each containing random transposon insertions to provide the homology.

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