Streptomyces Cloning: Possible Construction of Novel Compounds and Regulation of Antibiotic Biosynthetic Genes

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INTRODUCTION

Another article (56) provided an overview of the systems available for performing gene-cloning experiments. It also delineated those genes and pathways that people have cloned. The potential application of these systems and the knowledge derived from them to modify therapeutically useful organic compounds compose the content of this article. The limited but rapidly expanding information about regulation in streptomycetes is also covered.

CLONING AND MODIFICATION OF BIOSYNTHETIC GENES AND OPERONS

Several approaches have been taken in cloning genes of antibiotic biosynthetic pathways: by complementation of blocked mutants (36), by cloning antibiotic resistance genes (40), or by detection of enzymatic activity (30). In the last case, DNA fragments from *Streptomyces antibioticus* which do not encode the structural gene for phenoxazinone synthase (30) for some as yet unknown reason can elicit *S. lividans* to produce this enzyme (31). The second approach, cloning of the antibiotic resistance gene(s), has great appeal since data have accumulated to validate the hypothesis that this gene(s) is embedded or contiguous with the antibiotic biosynthetic operon(s) (e.g., see references 17 and 59). This review omits discussion of primary metabolic operons, such as glycerol (7) and galactose (1, 18).

Most of the streptomycete biosynthetic genes are reported in reference 56. These include the entire biosynthetic pathways for bialaphos and for actinorhodin. Those biosynthetic genes which were not listed are involved in the biosynthesis of streptomycin (16, 22), erythromycin (51), tylosin (13), and tetracenomycin (39). Of these, the entire pathways for erythromycin and tetracenomycin have apparently been cloned.

For streptomycin, three genes were shown to flank the resistance gene: one codes for an aminocyclitol amidinotransferase (*strB*); another possibly encodes a hexose-nucleotidylating enzyme (*strD*); and the last, *strR*, appears to be a regulatory gene. In this region, three promoters have been identified, one of which lies within the regulatory gene and is constitutive; another appears to function later in cell growth (17). Biosynthesis of the *strB* gene product appears to absolutely require the regulatory *strR* gene product. Interestingly, no biosynthesis occurs (17) in an A-factor (23) mutant. Also, the resistance *aphD* gene (i.e., *strA*) which encodes streptomycin-6-phosphotransferase is flanked by different G+C-rich inverted repeat sequences (16).

For tylosin biosynthesis, a variety of genes have been cloned by complementation of blocked mutants. These genes reside in a 42-kilobase DNA fragment obtained from *S. fradiae*. Their gene products participate in the modification of the tylosin aglycone (tylactone), involving various

oxidation and glycosylation steps (13). No complementation of mutants in tylactone formation (*tylG*) have been reported yet.

The cloning of the erythromycin pathway utilized a cosmid shuttle vector that can exist in both *Escherichia coli* and *Streptomyces* spp. The *E. coli* cosmid library was probed for clones containing the erythromycin resistance gene plus contiguous DNA; after transformation into *S. lividans*, isolates were identified that produced erythromycin A (51). To verify that most, if not all, biosynthetic genes were present on the 35-kilobase insert, the cosmid was transformed into several *S. erythraeus* mutants and clones that complemented and produced the antibiotic were obtained.

As for erythromycin and tylosin, complementation of *S. glaucescens* mutants that do not make tetracenomycin, in combination with selection for tetracenomycin resistance, led to the entire cloned biosynthetic pathway for this compound in *S. lividans*. The pathway was cloned as two overlapping subsets on separate, identical replicons, neither of which individually produces the antibiotic (39). However, one of the constructs prevented formation of the normal pigments produced by *S. lividans*. Unlike actinorhodin biosynthetic genes (see below), this pathway was cloned on a high-copy-number plasmid. The instability seen on this plasmid was localized to an approximately 1-kilobase region of the clone.

For the tripeptide antibiotic bialaphos, whose entire pathway has been cloned (Table 2 in reference 56), the antibiotic resistance gene (52) is also part of the biosynthetic pathway (55), acetylating both phosphinothricin and demethylphosphinothricin. The resistance gene, as well as five other biosynthetic genes, is under the transcriptional control of the *brpA* gene product that maps to the immediate right of the gene biosynthesis cluster (3), similar to regulatory genes for other biosynthetic pathways (see reference 3 for references).

By introducing the entire cloned actinorhodin biosynthetic pathway into organisms which produce structurally similar compounds, Hopwood and his colleagues generated strains that coproduce both antibiotics (36). When clones without the entire actinorhodin pathway were used, the recombinants produced novel antibiotics (21, 41). Preliminary reports by others indicate that they, too, have successfully made novel compounds by this technique (J. Tuan, J. Majer, and L. Katz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, H22, p. 131). Even though these compounds were less efficacious than the parent compound, they prove that by manipulating DNA from one species into another species one can make a new compound.

Recombinant DNA techniques obviously allow the increased yield of compounds from a given source by cloning the rate-limiting step (if known) or entire pathway, or a regulatory gene, followed by mutation if it regulates negatively. However, this method also provides a means to

create novel compounds from natural sources. Apparently, the entire pathway is not required for this approach to work and may even be disadvantageous (see above). However, this may apply only for antibiotics that are synthesized as part of an enzyme complex with no freely diffusible substrates, such as the polyketides (e.g., actinorhodin, the aglycone of macrolides) or the cyclic peptides (e.g., gramicidin S). In addition to the biosynthetic genes, the adjacent (e.g., bialaphos and streptomycin) and distant (e.g., A-factor) regulatory genes also need to be present, either in the recipient host or also cloned.

Even if the strategy succeeds (i.e., obtaining a clone of all necessary structural and possibly regulatory genes), limitations do exist with this approach. The first limitation is that the novel compound might be so radically different that neither resistance determinant of either parent compound would provide protection; i.e., the novel compound would destroy its producing host. Another limitation may be the chemical reactions which the cellular enzymes perform, even though a vast array of modifications is possible (38).

A third consideration concerns "silent" genes (as mentioned earlier for phenoxazinone synthase). In this case, an endogenous silent gene becomes activated in *S. lividans* by a cloned fragment to synthesize this enzyme in which little or no activity is present normally. In regeneration of *S. griseus* protoplasts, a kanamycin resistance gene became spontaneously expressed in a clone whereas the parent strain was susceptible to this antibiotic (24). This gene was cloned, characterized, and shown to exist on the chromosome of the parent even though it was not expressed (25). Thus, obtaining clones that become resistant or synthesize a compound of interest after gene-cloning experiments does not guarantee that the genes of interest have been cloned (discussed further in reference 29). Lastly, all the genes needed to make an antibiotic may not cluster contiguously in an operon but may be dispersed in two or more clusters, as for oxytetracycline (45).

Even with these limitations, this approach may be the most logical route for novel antibiotic discovery since the number of useful compounds, although still present from soil microorganisms, are discovered at an ever diminishing rate. The limitations delineated above could in fact be advantageous, similar to the requirement of needing only a partial biosynthetic pathway. For other therapeutic agents these procedures can be utilized *ab initio*.

Other applications of cloned genes could be screening for organisms that make related activities. The probe would be of a gene essential and unique to the biosynthesis of the compound of interest which then would be used in colony hybridization experiments. For example, actinorhodin is a polyketide antibiotic (48), as are macrolides and tetracycline (48). By probing with the gene encoding the actinorhodin polyketide synthetase, it is possible to identify regions in different genomes where various antibiotic biosynthetic genes reside (35). In this example, a probe for one pathway can obviously detect a structurally related compound (i.e., another actinorhodin) or a totally different class of compounds (e.g., macrolides).

Recombinant DNA techniques also allow modifications to a compound to be performed *in vivo* (e.g., the conversion of kanamycin to amikacin, as suggested in reference 53). Besides these applications of recombinant DNA techniques for detecting, making, or modifying known or novel compounds, other approaches are possible and will undoubtedly be used.

REGULATORY REGIONS OF STREPTOMYCETES

Preliminary results on transcriptional regulatory sites have been reported. Promoters were identified and characterized either from genes which had been sequenced (4, 54) or by using promoter probe plasmids (5, 8, 19, 22) to select directly for promoters (9, 22, 37, 57). A recent promoter probe utilizes bacterial luciferase genes and has been used to detect not only temporal expression of luciferase with various streptomycete promoters but also their spatial location in this differentiating species (50).

Streptomycete promoters (reviewed in reference 26) are diverse: some appear similar to the *E. coli* -10 and -35 consensus sequence regions (47), while others are completely different (26). Many of the regions upstream of the translational start region have two (or more) putative promoters; it is unclear whether both promoters function *in vivo*. Except that the distance between the two promoters is greater, the arrangement appears similar to that of promoters found in *E. coli* stable RNA operons (e.g., see reference 49) which are subject to several types of regulation. In *Bacillus subtilis*, genes exist under contiguous promoters that are recognized by different RNA polymerases (11, 28) and may be expressed at different times in the cell cycle. Different forms of RNA polymerase have also been demonstrated for *Streptomyces* spp. (60). Four promoters have been identified for the agarose (*dagA*) gene of *S. coelicolor* A3(2), and the gene responds to at least three different RNA polymerase holoenzymes, i.e., sigma factors (10). This paper also reports that the galactose P1 and P2 promoters respond to different RNA sigma factors; the latter promoter causes constitutive expression of two downstream genes (18).

Several nonstreptomycete genes, e.g., pBR322 *tet* (12) and *E. coli* *lacZ* (32) and *ampC* (27), appear to be expressed in streptomycetes from their own promoters, albeit at low levels. In addition, a *Staphylococcus aureus* gene encoding an APH(3')-III phosphotransferase (14) and a *Salmonella typhimurium* gene encoding a β -lactamase (2) have been expressed in *S. lividans*. In both cases, the genes appear to be expressed from their own promoters although an undefined upstream promoter on the vector cannot be ruled out. Interestingly, the *S. typhimurium* gene product appears to be excreted almost completely in *S. lividans*.

The promoter probe plasmids can order promoters with respect to their transcriptional strengths. One probe which utilizes the *E. coli* galactokinase (*galK*) gene (8) has been shown to complement the equivalent streptomycete function and thus can be used to select genetically for both up and down promoters. These mutations would be useful to define which regions are essential for expression in streptomycetes under a variety of physiological growth conditions. Also, mutations can help to determine which sequences are important for optimal expression in this host.

The *E. coli* *ampC* promoter which expresses in streptomycetes (see above) was cloned, as were several of its up promoter derivatives (27). This study showed that mutations which made the promoter closer in sequence to the *E. coli* consensus promoter increased the promoter strength in streptomycetes as well as in *E. coli*, although the increased transcriptional activity was not as pronounced as for *E. coli*. However, the insertion of an extra GC base pair between the -10 and -35 region had a more pronounced effect on increased promoter strength in streptomycetes than in *E. coli*.

In addition to the plasmid promoter probes, Φ C31 promoter probes have also been constructed (32, 46). Some of

these actinophage promoter probes can be used for transcriptional fusion experiments, such as those performed with the Mu *d lac* transpositional operon fusions. Translational fusion phage vectors have also been constructed (32). The reporter gene used for the fusion vectors is the *E. coli lacZ* (β -galactosidase) gene. Unfortunately, streptomycetes have an enzymatic activity(ies) which is capable of hydrolyzing the substrates for the *E. coli* enzyme. These vectors are essentially restricted to use in streptomycete mutants deficient in this activity. Fortunately, the Φ C31 cloning vectors with the *vph* gene lack the viomycin promoter (46), and this viomycin resistance must be determined by an upstream promoter, probably from the *tsr* gene. This fact was utilized to make transcriptional fusions with the *vph* gene (46). Sequencing of several antibiotic resistance genes showed structures (4) located 3' to the translation termination site which were similar in secondary structure to the *rho*-independent transcriptional termination sites found in *E. coli* (47). However, these structures lack the run of thymidine residues seen in *E. coli*.

Since *E. coli* genes can be expressed in *Streptomyces* spp., this genus must have a full complement of all tRNA molecules. However, the relative abundances of these molecules remain to be determined. One tRNA molecule (34), a product of the *bldA* gene of *S. coelicolor*, is involved in the differentiation of this organism and has been cloned (42). This tRNA recognizes the codon UUA, which occurs at a very low frequency because streptomycetes have a high G+C content. The possibility of translational control has been raised for this molecule (34).

Of the genes sequenced to date, translation initiation began with ATG or GTG, and translation termination occurred at the normal nonsense codon TAA or TAG. Other translational information, specifically the 3' end of the 16S rRNA corresponding to the Shine-Delgarno sequence, was determined for *S. lividans* and found to be identical to that found for *B. subtilis* (5). The formylmethionine initiation codon tRNA molecule obtained from *S. griseus* appears to be more eucaryotic in nature than procaryotic (33). For *S. rimosus*, two initiator genes were detected, one of which may be a pseudogene (20). As for the streptomycin gene (17) and the galactose P2 promoter (18), a potential stem-loop structure occurs in this tRNA promoter region (20). The significance of this putative structure remains to be determined. Such structures downstream of genes have been shown to have transcription termination capabilities, e.g., from the 3' end of the *aph* gene (43) and from plasmid pIJ101 (15), with the latter functional in both *E. coli* and *S. lividans*. Transcription termination sequences from *B. subtilis* (44) and from an *E. coli* bacteriophage (58) have also been shown to function in streptomycetes.

Since streptomycete DNA contains a very high G+C content (6), codon usage might be important in gene expression for this genus, especially for heterologous DNA. Analysis of DNA sequences demonstrates that either a G or C nucleotide is found at a high frequency in the third codon nucleotide (>90%) and in the first codon nucleotide (ca. 70%). This high G+C content permits analysis of nucleic acid sequences for potential open reading frames (6).

CONCLUDING REMARKS

Recombinant DNA techniques have played a significant role in understanding the primary and secondary metabolic genetic organization in *Streptomyces* spp. These approaches also facilitated the discovery of transposable elements. The

information gleaned from this work will ultimately prove beneficial to both the aesthetic researcher (morphogenesis and differentiation) and the practical one (yield and modification of therapeutic compounds). This area complements nicely the approach taken for proteinaceous therapeutic agents. *Streptomyces* spp. may well be used for the expression and production of these protein products as well.

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