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The Impact of Genetic Engineering on the Commercial Production of Antibiotics by *Streptomyces* and Related Bacteria

C. RICHARD HUTCHINSON

School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, WI 53706

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ABSTRACT

Developments in *Streptomyces* genetics that have laid a foundation for this field over the past ten years are reviewed and discussed to suggest how this knowledge might useful for improving the commercial production of antibiotics. This brief analysis predicts a bright future for the application of *Streptomyces* genetics in antibiotic production.

Index Entries: Genetic engineering; antibiotics, commercial production of; streptomyces.

INTRODUCTION

Most of the commercially important antibiotics are fermentation products, but a wild-type microorganism rarely, if ever, makes enough antibiotic for the economic production of these valuable substances. Consequently, since about 45 years ago following the discovery of penicillin and streptomycin, the first antibiotics to be used widely in human medicine, much effort has been devoted to improving the antibiotic productivity of microorganisms. Until recently, this was possible only by using a combination of general mutagens (ultraviolet light, a chemical, or environmental stress) or selective agents (toxic amount of an antibiotic, a biochemical inhibitor, or a metal ion) and screening the resulting mutants for strains with improved productivity, and by optimization of the fermentation process to find the best combination of medium constituents and growth parameters (*1,2*). The advent of recombinant DNA technology in

the 1970s and its application to the study of antibiotic production by *Streptomyces* and related bacteria in the 1980s has changed the ways one can seek improved strains by sparking a hope that this goal can also be sought by genetic engineering. The new vista includes the discovery of antibiotics as well as the creation of higher producing strains and cheaper fermentation processes, but is clouded by lack of enough knowledge about the biology of antibiotic production to reach these goals nonempirically. Even though our understanding of the molecular biology of *Streptomyces* is growing at an increasing rate, it still is not possible to know exactly how to genetically engineer a strain to increase antibiotic production or to create a new antibiotic. In the present article, I review the developments in *Streptomyces* genetics that have laid a foundation for this field over the past ten years and discuss how this knowledge might be applied to the commerical production of antibiotics. This brief analysis predicts a bright future for the application of *Streptomyces* genetics in antibiotic production.

The Tools of *Streptomyces* Genetics

Recombinant *Streptomyces* were first constructed by conjugational mating, their natural process for gene exchange (*3,4*). In many cases plasmids act as fertility factors to promote intraspecific recombination at frequencies typically about 10^{-4}–10^{-5}, although values approaching 1 have been observed in the apparently unidirectional, "infectious transfer" of plasmid borne genetic markers in matings of some *Streptomyces coelicolor* strains or in certain crosses involving intergrated sex plasmids (*3*). Fusion of *Streptomyces* protoplasts also promotes genetic exchange often at more than an order of magnitude higher frequency (*5–7*). Neither technique is an expedient means for creating a desired combination of genes because of the current lack of tools for effecting efficient (particularly interspecific) DNA recombination in vivo. *Streptomyces* genes are therefore most often manipulated by in vitro recombinant DNA methods because these enable interspecies transfer in addition to gene analysis at the nucleotide level.

A large number of useful gene cloning vectors have been made from indigenous *Streptomyces* plasmids that occur widely in the genus (*8–10, 29*). Table 1 lists representative examples containing the thiostrepton resistance (*tsr*) gene, the marker most often used to select transformants since the growth of many *Streptomyces* is quite sensitive to this antibiotic. Many of these vectors are described in the indispensable laboratory manual prepared by the pioneering research group at the John Innes Institute in England (*10*). pIJ702 (*11*) has been used widely because of its broad host range and convenient visual indicator for the presence of cloned DNA (transformants carrying a recombinant plasmid usually do not produce melanin). pMT660 is a temperature-sensitive version of pIJ702 that does not replicate in *S. lividans* above 39°C (*12*). pIJ699, made from the same high copy number replicon as pIJ702 (*13*), permits positive selection for DNA inserts (*14*), and pIJ680 (*10*) contains the efficient promoter of the *S. fradiae* neomycin phosphotransferase gene, *aphI*, which should

Table 1
Typical *Streptomyces* Gene Cloning Vectors

Vector	Size, kbp	Genetic markers[a] *aph*	*apr*[b]	*tsr*	*other*	Main cloning site(s)
pIJ61	14.8	x		x		*Bam*HI, *Pst*I
pIJ486	6.2				*neo*[c]	7 in a polylinker
pIJ680	5.3	x		x		*Bam*HI
pIJ688	6.3				*neo*[c]	*Bam*HI
pIJ699	9.6			x		*Bgl*II, *Hind*III, *Xba*I
pIJ702	5.8			x	*mel*	*Bgl*III, *Sph*I, *Sst*I
pIJ941	25.0			x	*hyg*[d] Ltz$^+$	*Bam*HI, *Bgl*II, *Pst*I, *Xho*I
pHJL302	5.1			x		7 in a polylinker
pHJL400	5.8			x		7 in a polylinker
pKC505	18.7		x			*Bam*HI
pSK02	8.7			x	*galK*[e]	6 in a polylinker
pPS7	7.9			x	*β-gal*[e]	*Bam*HI, *Stu*I, *Bst*EII
pARC1	19.0	x		x	Brn[e,f]	*Bam*HI
pRS1105	9.4	x[e]		*x*	*luxAB*	*Bam*HI, *Bgl*II, *Xba*I
pJAS14	?			x	*ampC*[e,g]	*Bam*HI

[a] x = Selectable markers.
[b] Apramycin resistance.
[c] Neomycin resistance; *neo* gene lacks transcriptional and, in pIJ688, translational signals.
[d] Hygromycin resistance.
[e] Promoterless gene.
[f] Gene for formation of a brown pigment.
[g] Gene encoding a betalactamase.

enable the constitutive expression of DNA cloned into the unique *Bam*HI site (*15*). pIJ922, pIJ941, and several other vectors made from the low copy number SCP2* replicon also seem to have a broad host range (*16*), whereas vectors like pIJ61 (*10*) made from SLP1.2 (*see below*) work best in *S. lividans*, in which they have a copy number of 4–5 per cell. *E. coli-Streptomyces* shuttle vectors like pHJL400 have been made from SCP2* (*17,18*); one of these, pHJL302, was made by deleting part of the SCP2* replication origin (*18*) and is a runaway replicon in *S. lividans* TK23. Shuttle vectors may be less stable in *Streptomyces* than those constructed only from streptomycete plasmids, however (*8*). pKC505 is a shuttle cosmid containing the SCP2* replication origin (*19*); gene libraries in which each vector molecule will have an approx 35 kb pair (kbp) DNA insert can be made in pKC505 in *E. coli* and then transferred to a streptomycete for screening.

Among the special purpose *Streptomyces* vectors are ones useful for measuring promoter activity in cloned DNA fragments by assaying chloramphenicol (pSLP124 [*20*]) or kanamycin resistance (pIJ486 [*21*]), galactokinase (pSK02 [*22*]), betalactamase (pJAS14 [*23*]), or β-galactosidase (pPS7 [*24*]) activity, and brown pigment formation (pARC1 [*25*]). pRS1105 is a newly developed promoter-probe vector that permits the nondestructive, real-time assay of *Streptomyces* promoter activity by measuring the

light produced by the action of luciferase on *n*-decanal, a volatile aldehyde (*26*). Few *Streptomyces* expression vectors have been reported in the literature; transcriptional fusions in a derivative of pIJ32 (*15,27*) and translational fusions in pIJ688 (*28*) are two examples, but no vectors permitting the regulated expression of cloned genes are available yet.

A series of cloning vectors have been made from the broad host range *Streptomyces* phage, ϕC31 (*10,29*). Ones like KC515 (*30*), which lack the phage attachment site and can transduce a streptomycete to thiostrepton or viomycin resistance only if they carry a fragment of host DNA to provide the requisite homology for integration of the construct into the host genome, have found important uses in mutational cloning (*31*), homogentization (*32*), and the selective formation of deletion mutants (*33*).

With this versatile set of vectors, it is somewhat unfortunate that the polyethylene glycol-mediated transformation of protoplasts (*10*) still is the principal method for constructing recombinant *Streptomyces*. Since suitable conditions for each new host must be determined empirically (e.g., *34*), particularly for protoplast cell wall regeneration, it may not always be possible to transform or transfect (if phage DNA is used) a given strain. MacNeil has reported that *S. avermitilis* germlings (spores germinated and grown for 5 h) can be transformed with plasmid DNA (*35*), and that *S. lividans* protoplasts can be transformed with plasmid DNA by electroporation (*36*). The transformation frequency was $<10^4/\mu g$ of DNA in both cases, however, two orders of magnitude less than usually obtained by the standard methods (*10*). An efficient, generally useful way to make *Streptomyces* cells competent for direct transformation with DNA, or a versatile generalized transduction system would be valuable additions to the current repertoire of genetic tools in this field.

Fortunately, several good streptomycete strains are available as hosts for experiments with recombinant DNA. *S. lividans* 66 is the workhorse because of easily transformable strains with good growth characteristics, no detectable plasmids (*7*), and low to nonexistent restriction of unmodified foreign DNA (*10*). Its main drawback is the production of two diffusible antibiotic pigments, actinorhodin and undecylprodigiosin, that can complicate the analysis of the expression of certain foreign genes. Two other easily transformable hosts are *S. ambofaciens* (*34*) and *S. griseofuscus* (*17*), both of which also produce antibiotics. One can make highly transformable streptomycete strains by mutation (*37*), but the only *recA* streptomycete is a strain of *S. fradiae* (*38*). Although it is not known yet if the latter strain actually is recombination deficient, Tsai and Chen (*39*) have isolated an *S. lividans* mutant that lacks the ability to cause intramolecular recombination in a plasmid.

The Biology of *Streptomyces* Plasmids and Phages

Autonomously replicating DNA is frequently encountered in *Streptomyces* (*8*) and can be isolated by methods similar to those used for other bacteria (*10*). These plasmids usually are double stranded, circular DNA, but

at least one report has appeared about the occurrence of single stranded, circular DNA in *Streptomyces* (*40*), which is likely to be a replication intermediate. Linear *Streptomyces* plasmids are also known (*41,42*), and a method for isolating large, apparently linear plasmids from *Streptomyces*, including multimers of SCP1 and SCP2 cointegrants, has been described recently by Kinashi et al. (*43*). The plasmids may act as fertility factors (*9*), and SCP1 in *S. coelicolor* also carries the biosynthetic and resistance genes for the antibiotic methylenomycin (*44*). A diagnostic property of the self-transmissible plasmids is the transient inhibition of development of aerial mycelium when the plasmid enters a (plasmid-free) recipient, a phenomenon called "pock formation" and phenotypically designated Ltz^+ for lethal zygosis. Plasmid transfer results in the formation of a cleared zone surrounding the recipient strain on solid growth media superficially similar in appearance to phage plaques (*8,45*). Kendall and Cohen (*46*) have identified six plasmid-encoded loci involved in gene transfer and pock formation for pIJ101 (*13*): *kilA* and *tra* mutants produce no pocks, *kilB* and *spd* mutants are defective in pock formation, and the *korA* and *korB* genes seem to regulate the action of the *kil* genes. Integrated and autonomous forms of *Streptomyces* plasmids have been identified, of which the SLP1 series of plasmids are the best example (*47*). They appear in *S. lividans* after its mating with strains of *S. coelicolor* that contain an integrated copy of SLP1 (*47*) and are formed by reversible, site-specific recombinational events (*48*). Sequences homologous to SLP1 are common among streptomycetes (*48,48a*); Omer and Cohen suggested that they "may be involved in the acquisition and structural organization of genes encoding the diverse metabolic capabilities observed in different streptomycetes" (*48*). Yet, it is uncertain if the ubiquitous *Streptomyces* plasmids play any role other than sex factors in the bacterial life cycle.

Streptomyces phages resemble those of unicellular bacteria but have not been studied nearly as much (*49*). They can be found commonly in soil and may be functionally classified into two types, those that package their DNA into phage heads by site specific cleavage of concatemeric DNA or by the headful mechanism from a *pac* site in the concatemer. ϕC31 is the most thoroughly studied example of the first type (*45*) and SF1, discovered as its prophage in *S. fradiae*, is a good example of the second (*50*). The study of SF1 led to the discovery of the *Streptomyces* transposon Tn*4556* (*see following*). SF1 and SV1 are generalized transducing phages but only for *S. fradiae* (*51*) and *S. venezuelae* (*52*), respectively. SV1, in fact, was used for the transductional analysis of chloramphenicol biosynthetic genes in *S. venezuelae* (*53*).

Mobile Genetic Elements of the *Streptomyces*

Many of the phenotypes of *Streptomyces*, like sporulation, pigmentation, and antibiotic production or resistance, are quite variable and notoriously unstable in some strains, reflecting an underlying genetic instability. Thus, there is much interest in studying insertion sequences and transpo-

sons as possible mediators. IS*110* is a 1.6 kbp segment of *S. coelicolor* DNA that promotes integration of an *attP*-deleted derivative of ϕC31 into any of the several copies of IS*110* at several locations in the *S. coelicolor* genome (*54*). IS*110* has many of the characteristics of IS elements from enteric bacteria, although its transposition into ϕC31 DNA exhibits a strong site preference (*54*). Another segment of *S. coelicolor* DNA, the 2.6 kbp DNA minicircle, causes a predominantly site-specific integration of an *attP*-deleted ϕC31 derivative in *S. lividans* and several other streptomycetes (*55*). The only example of a *Streptomyces* transposon resembling those of other bacteria is Tn*4556*, a 6.8 kbp element found in *S. fradiae* that, as Tn*4560* (Tn*4556*::*vph*) [*vph* is the viomycin phosphotransferase gene, another selectable marker for *Streptomyces*], is able to transpose randomly between the genome of *S. lividans* and various plasmids (*56*). Tn*4560* deserves extensive study in different streptomycetes to see if it will be as useful a tool in *Streptomyces* genetics as other transposons have been in studies of enteric bacteria and bacilli. Perhaps further study of these and other mobile genetic elements of *Streptomyces* (*57*) will enable the development of a tool like the *E. coli* phage Mu derivatives, something greatly needed in this field.

DNA Amplification in the *Streptomyces*

Genetic instability in *Streptomyces* could have another basis: the frequent, spontaneous deletion or amplication of genomic DNA that seems to be widespread among the members of this genus (*57,58*). Detailed studies of this apparently unique phenomenon have been pursued with several streptomycetes. In *S. lividans* the high-frequency, spontaneous, and sequential formation of chloramphenicol-sensitive strains containing the *argG* mutation is associated with amplication of a tandemly duplicated 5.7 kbp amplifiable unit of DNA (AUD) in the wild-type strain (*59–61*). This unit can be amplified into tandem repeats of 200–500 copies per genome and its linkage to *argG* and the gene that determines chloramphenicol resistance is apparently responsible for their instability. The stability of the amplified DNA under nonselective conditions has been exploited in a new vector that appears promising for the amplification of commercially important genes (*62*). Transformation of an *E. coli* plasmid containing the *S. lividans* AUD joined to the *tsr* and tyrosinase (*mel*) genes into *S. lividans* resulted in integration of the construct into the host chromosome by homologous recombination; subsequent induction of DNA amplication caused co-amplification of the *tsr* and *mel* genes (*62*). Other streptomycetes contain different AUD segments that may mediate the loss of antibiotic (*63*) or melanin (*64*) production, and the loss of streptomycin resistance (*65*) by DNA amplification or deletion. In contrast, DNA amplification in *S. antibioticus* is associated with high oleandomycin-producing strains (*66*), in *S. tendae* with overproduction of an α-amylase inhibitor (*67*), in *S. achromogenes* subsp. *rubradiris* with the induction of high-level spectino-

mycin resistance (*68*), and in *S. griseus* NP1-1PR with the induction of kanamycin resistance (*69*).

Molecular Biology of *Streptomyces* Gene Expression

The notable features of *Streptomyces* metabolism like the formation of aerial mycelium, sporulation, and the production of excreted products (enzymes and antibiotics) that appear late in the bacterial growth cycle in laboratory cultures are governed by temporally regulated genes (*70*). *Streptomyces* thus are good laboratory models for the study of developmental biology that, together with the commercial importance of many of the associated products, has stimulated considerable interest in unraveling the mechanism of gene expression in these bacteria. *Streptomyces* promoters fall into two classes (Table 2): ones similar (A) and others dissimilar (B) to the consensus DNA sequence of typical *E. coli* promoters (*71*). These differences are part of the reason that very few *Streptomyces* genes can be expressed in *E. coli* (*71–75*), whereas several genes from *E. coli, Serratia marcescens,* or *Bacillus licheniformis* can be expressed in *S. lividans* (*72,73*). The RNA polymerase of *S. lividans* can recognize and use the transcriptional signal components of *E. coli* (*73*) and *Bacillus subtilis* (*76*), and the transcriptional organization and control of the *S. lividans* galactose utilization operon resembles its *E. coli* counterpart (*see following*). Transcription terminators of *Streptomyces* genes have been identified (*77,78*) and shown to function in *E. coli* (*77*). Selective expression of different gene sets in *Streptomyces* thus seems to depend on differences in promoter structure and is probably mediated in part by different RNA polymerase holoenzymes (*79*) as in *Bacillus* and other bacteria (*70*). Westpheling et al. have in fact identified two RNA polymerase holoenzymes in *S. coelicolor* that contain different sigma factors and recognize different promoter classes (*80*). Furthermore, several *Streptomyces* genes have multiple promoters (*82,83,88*), each of which could be recognized by different RNA polymerase holoenzymes (cf. *79*). The extent to which *Streptomyces* gene expression is controlled post-transcriptionally is unknown except for genes that confer resistance to the macrolide-lincosamide-streptogramin (MLS) antibiotics. Expression of the MLS resistance phenotype is thought to be governed by attentuation within a leader sequence upstream of the MLS structural genes (*81*); the same mechanism controls MLS resistance in several pathogenic bacteria. Thus the key features of *Streptomyces* gene expression uncovered so far appear to be special promoters and heterogeneous RNA polymerases.

Primary Metabolism Genes of *Streptomyces*

Since the production of secondary metabolites by *Streptomyces* is frequently repressed by glucose and other easily metabolizable carbon, nitrogen, and phosphate sources (*84*), studies of repressible and inducible

Table 2
DNA Sequences of Some *Streptomyces* Promoters

	Promoter	-25 to -45 region	Distance,[a] base pairs	-10 region	Distance,[b] base pairs	Ref.
	E. coli σ^{70}	TTGAC	16–18	TATAAT	6–8	*71*
	E. coli σ^{32}	T-tC-CCTTGAA	13–25	CCCCATtTa	5–8	*71*
A.	*glyP1*	CTCTTGACGCGCGCGT	10	GAGACT	8	*87*
	glyP2	TTGTCGAACACCTACC	12	TAGAGT	6	*87*
	gly"0.9kb"	CATTGGCGGGAGGTCG	11	TAGGTT	7	*87*
	pIJ101A	CCTACCCATTGCGCAACCTGA	11	CAGACT	7	*75*
	pIJ101B	AGTGTATGCCTTGACACAGCA	12	CAGTAT	7	*75*
	βgal	GACGGGGTAATTGATTCGGTT	12	TAGGGT	6	*24*
	ermEP1	ATGCTGTTGTGGGCTGGACAA	13	TAGGAT	6	*83*
	ermEP2	GCGGTCGATCTTGACGGCTGG	13	GAGGAT	6	*83*
	orfP1 (*ermE*)	GCGTGCACCTGCGATCGCCGA	11	TAGCAT	8	*83*
	sphP	TCCACGGACGGCGGGAGCCTG	13	TAGGTT	5	*130*
	orfP1 (*sph*)	TCCGTGGACGGTGGTGGGGTG	13	TACGGT	6	*130*
	orfP2 (*sph*)	GGGAGGGAGCGGGTGTTTCTC	12	GGAGAT	7	*130*
B.	*aphP1*	CGAAAGGCGCGCAACGGCGT	12	CATGAT	8	*83*
	aphP2	CGGTGGGGGATTCCGGCCGA	12	CCATGT	8	*83*
	endoH	ATTGACTGATTGACGC	12	CAGGGG	6	*80*
	hygP	CGTCCCCGACGTGGCCGACCA	12	CAACGC	6	*129*
	tsrP1	TTGCCGGTCAGGGCAGCCAT	14	TAGGGT	6	*71*
	tsrP2	GCTCGACGCAGCCCAGAAAT	14	AATACT	6	*71*
	vphP1	GCAGCGCCGTGTGCGGCCTG	7	CGGGAG	6	*71*

[a] Between the two promoter regions.
[b] From the transcription start site.

genes of primary metabolism may lead to ways to deregulate antibiotic production. Chater and coworkers, through their investigations of the molecular biology of glucose and glycerol catabolism in *S. coelicolor*, have identified two examples of such genes, the glycerol utilization operon (*gyl*) (*85*) and the glucose kinase (*glk*) gene (*86*). The *gyl* operon consists of at least two genes, *gylA* (probably encoding glycerol kinase) and *gylB* (encoding *sn*-glycerol-3-phosphate dehydrogenase), both of which may be regulated by the product of a 0.9 kbp region situated immediately upstream of *gylA* (*87,87a*). The *gyl* promoters resemble the typical prokaryotic consensus promoter sequence (Table 2) and both promoter regions contain related symmetrical operator-like sequences (*87*). About 30% of spontaneously generated 2-deoxyglucose resistant (Dog^r) *S. coelicolor glk* mutants contain deletions of the *glk* gene region (*33*). The Dog^r mutants have no detectable glucose kinase activity and, since phosphorylation of glucose by glucose kinase is the first step in glucose catabolism, Dog^r mutants of *S. coelicolor* either grow poorly (G$^\pm$) or not at all (G$^-$) on glucose (*88*). Glucose could no longer repress the utilization of various other carbon sources in the G$^-$ mutants. The cloned *glk* gene restored glucose kinase activity, Dog sensitivity, and glucose utilization and repression to the *glk* mutants (*86*), suggesting a possible role for glucose kinase in mediating glucose repression in streptomycetes.

Incidentially, the study of *S. coelicolor glk* mutants led to the development of a ϕC31::*glk* vector, KC573, that enabled selection of spontaneous deletions in regions of the *S. coelicolor* (*glk*) genome defined by the site of homologous recombination between the insert cloned in the *attP*-deleted vector and the chromosomal DNA (*33*). This method presumably could be used in other *Streptomyces glk* deletion mutants that can be lysogenized by ϕC31.

Galactose utilization by *S. lividans* is controlled by the products of the *gal* operon (*89,89a*), which consists of three genes, *galT*, *galE*, and *galK*, as in *E. coli*. The *galP1* promoter governs the galactose inducible expression of all three genes, whereas the *galP2* promoter, located downstream of *galP1* in front of *galE* and *galK*, is responsible for the constitutive expression of the latter two genes. Differences in gene order and promoter location noted for the *gal* operons from *E. coli* and *S. lividans* (*89,89a*) may reflect subtle differences in the physiology of galactose utilization by these two bacteria.

Streptomyces Extracellular Protein Genes

Streptomyces presumably evolved a diverse ability to produce extracellular enzymes in adapting to their niche as nonmotile soil bacteria whose primary carbon-source comes from the degradation of soil ingredients. Fungi are more prolific producers of extracellular enzymes, but this trait of *Streptomyces* has considerable commercial importance (*90*), which has resulted in numerous examples of the cloning of extracellular protein genes from the streptomycetes (Table 3). Valuable contributions to our

Table 3
Cloned Extracellular Protein Genes of *Streptomyces*

Protein	Source	Ref.
α-amylase	*S. hygroscopicus*	*131*
α-amylase	*S. limosus*	*132*
agarase	*S. coelicolor*	*133,134*
chitinase	*S. plicatus*	*135*
β-galactosidase	*S. lividans*	*24*
β-lactamase	*S. albus* G	*136*
cholesterol oxidase	*Streptomyces* sp.	*137*
endoglycosidase H	*S. plicatus*	*138*
serine *DD*-peptidase	*Streptomyces* R61	*139*
proteases A and B	*S. griseus*	*140*
tyrosinase	*S. lividans*	*11,141*
tyrosinase	*S. glaucescens*	*142*
xylanase	*Streptomyces* sp. 36a	*143*
xylanase	*S. lividans*	*144*

knowledge about *Streptomyces* gene expression and the mechanism of protein export by microorganisms will come from studies of these genes.

Streptomyces Genes Governing Morphological Development

The fungus-like, filamentous *Streptomyces* undergo a cycle of complex morphological changes on solid media following their vegetative growth (*70*). The availability of several types of morphological mutants of *S. coelicolor* has enabled investigations of the developmentally regulated genes that govern the formation of its morphological features. *BldA* mutants of this bacterium have malformed, prostrate hyphae and make none of the four antibiotics known to be made by the parental strain. Their bald phenotype is carbon source dependent; it appears in the presence of glucose, whereas several other carbon sources like maltose, galactose, or mannitol suppress it and permit sporulation. Piret and Chater (*32*) showed that the wild-type *bldA* gene, cloned in a phage vector, was dominant over six independently isolated *bldA* mutations through the use of double lysogens of the *bldA* hosts. They also found that presumably heterozygous *bldA*$^+$/*bldA* partial diploids could be converted to apparently homozygous *bldA/bldA* mutant diploids at a moderate frequency by some homogenetization process. In the latter case, the phages released from the bald lysogens carried *bldA* mutations, which led to the exploitation of homogenetization for the recovery of mutant alleles in another study by the same group (*91*). From the *bldA* DNA sequence Lawlor et al. (*92*) speculate that it encodes a

molecule like $tRNA^{leu}$ that can recognize the TTA anticodon, thus directing translation of genes with TTA codons only when *bldA* is expressed. *WhiG*⁻ mutants can form abundant aerial mycelia that show no signs of further development; the cloned *whiG* gene restored the wild-type phenotype to a collection of *S. coelicolor whiG* mutants and caused hypersporulation when present in extra copies (*91*).

The sequential formation of substrate mycelium, aerial mycelium, and spores during the *Streptomyces* life cycle must be governed by the expression of temporally regulated sets of genes. This intriguing example of developmental biology in a prokaryotic microorganism has been explored recently by the use of *Vibrio harveyi luxAB* operon fusions in RS1105 with the *bldA* and *whiG* genes and a third gene, *sapA*, whose product appears during the formation of aerial mycelium and is associated with purified spores (*26*). Evidence for both temporal and spatial expression of *S. coelicolor* genes was elegantly demonstrated by measuring light emission during the growth of colonies on solid medium. By this method it was possible to determine that transcription of *whiG* took place in the substrate mycelium at a time considerably before the WhiG⁻ phenotype was detectable in the aerial hyphae, suggesting that the *whiG* gene product (believed to be a sigma factor [*26*]) may be synthesized in a form that becomes active only later in the aerial hyphae.

Antibiotic Production Genes of the *Streptomyces*

Antibiotic production by *Streptomyces* has been the focal point of their commercial interest and of many of the studies of their genetics. Following the isolation of several antibiotic resistance genes from *Streptomyces*, which, as selectable markers aided the development of *Streptomyces* gene cloning vectors and were the object of the first detailed studies of *Streptomyces* gene expression, a large number of antibiotic production genes have been cloned (*see* Table 4, which also lists genes cloned from *Bacillus* and fungi). The following highlights what has been learned from this work.

The behavior of actinorhodin genetic markers in conjugational matings showed in 1979 that the *act* genes are clustered between the *guaA* and *hisD* genes on the *S. coelicolor* linkage map (*93*). This initial indication of the clustering of antibiotic production genes has been confirmed repeatedly by the results of cloning experiments. The first demonstration was the cloning of all of the *act* production and resistance genes in a 32 kbp segment of *S. coelicolor* DNA (*94*); equivalent findings have been made for tetracenomycin C (*95*) and possibly erythromycin A (*96*), which along with the results from studies of many other antibiotics (e.g., bialaphos [*97*], streptomycin [*98,100*], and tylosin [*101*]), show that antibiotic production genes are usually clustered in the genome of the producing strain. The known exceptions are oxytetracycline, whose genes reside in

Table 4
Cloned Antibiotic Production Genes

Antibiotic	Chemical class	Source	Ref.
actinomycin D	oligopeptide	*S. antibioticus*	*145*
gramicidin	oligopeptide	*B. brevis*	*146*
tyrocidin	oligopeptide	*B. brevis*	*147,148*
penicillin G	betalactam	*C. acremonium*	*125*
cephalosporin C	betalactam	*P. chrysogenum*	*126*
cephalosporin C	betalactam	*A. nidulans*	*149*
cephalosporin C	betalactam	*C. acremonium*	*150*
clavulanic acid	betalactam	*S. clavuligerus*	*151,151a*
actinorhodin	isochromane-quinone	*S. coelicolor*	*94*
bialaphos	peptide	*S. hygroscopicus*	*97*
candicidin	polyene	*S. griseus*	*152*
erythromycin	macrolide	*S. erythreus*	*96*
tylosin	macrolide	*S. fradiae*	*101*
methylenomycin	cyclopentane	*S. coelicolor*	*31,104*
oxytetracycline	tetracene	*S. rimosus*	*103*
puromycin	nucleotide	*S. alboniger*	*153*
streptomycin	aminoglycoside	*S. griseus*	*98–100*
tetracenomycin C	anthracycline	*S. glaucescens*	*95*
undecylprodigiosin	polypyrrole	*S. coelicolor*	*114,154*

two clusters in *S. rimosus* (*102,103*)*, and methylenomycin, whose genes are clustered (*104*) in the self-transmissible plasmid SCP1 (*44*).

Apart from the obvious need for self-resistance, the factors controlling expression of antibiotic production genes are largely unknown. *Streptomyces* antibiotic resistance genes certainly can be expressed in heterologous streptomycete hosts; this is also true for the biosynthetic genes in the case of actinorhodin (*94*), erythromycin (*96*), and tetracenomycin C (*95*). The finding of overlapping, divergently transcribed production and resistance genes for erythromycin (*83*), streptomycin (*105,106*), and possibly methylenomycin (*107*) argues for the coordinate expression of self-resistance and some production genes. This could ensure that a microorganism expresses antibiotic self-resistance and production simultaneously. Specific regions of the actinorhodin (*108*), bialaphos (*109*), methylenomycin (*104*), and streptomycin (*98,110*) gene clusters appear to control expression of the production genes, positively (*98,108*) or negatively (*104,110*), perhaps by encoding the formation of a sigma factor or another regulatory protein. Subclustering of genes governing sequential steps in antibiotic biosynthetic pathways (actinorhodin [*111*], tetracenomycin C [*112*], and tylosin [*99*]), and the presence of polycistronic tran-

*This has been invalidated recently: the oxytetracycline genes are a single cluster (c. Binnie et al., abstracts of the 7th International Symposium on Biology of Actinomycetes, May, 1988).

scripts (actinorhodin [*111*] and methylenomycin [*104*]) indicates that the expected tight coupling of transcription and translation in the prokaryotic bacteria ensures the correct sequence of production of pathway enzymes. The greatest current uncertainty concerns temporal regulation of antibiotic production genes: Is recognition of their promoters by unique RNA polymerase holoenzymes an important determinant governing the time of antibiotic production (which usually coincides with the period of stationary growth in laboratory cultures [*84*])? Are there instead (or in addition) other types of regulatory proteins? Does post-transcriptional regulation play a significant role in antibiotic production? Answers to these questions surely will be forthcoming now that several antibiotic production genes are available for study.

More specialized developments from studies of the genetics of antibiotic production during the past two years have simplified the cloning of polyketide antibiotic genes (*113*) and demonstrated the value of shuttle cosmid vectors in isolating the primary clones (*96*). From our experience in using the *actI, actIII,* and *tcmIa* "polyketide synthase" gene probes (*113*) and pKC505 (*19*) to clone the production genes for several polyketide antibiotics, it is clear that shuttle cosmids with large inserts ($\geq$ca 35 kbp) can be unstable in homologous or heterologous DNA backgrounds and difficult to isolate intact from a streptomycete host. Nonetheless, they still are useful for complementation of mutations and for expression tests in *S. lividans,* and offer an convenient way to obtain primary clones for subsequent evaluation by subcloning experiments.

The information reviewed up to this point shows that the forefront of *Streptomyces* genetics has been led by studies in one organism, *S. coelicolor.* This bacterium, like *E. coli* K12, certainly should be the testing ground for new research on the basic genetics of streptomycetes so that the field can advance rapidly. However, the commercially important products are produced by many other species; thus there probably will be an unfortunate but necessary duplication of work done first in *S. coelicolor* as each industrially important system is studied.

Impact of *Streptomyces* Genetics on the Production of Commercially Important Antibiotics

In discussions with industrial research scientists and managers, the most often heard query is "Can we by the genetic engineering of *Streptomyces* (or other microorganisms) make high producing strains, reduce the cost of fermentations, or discover new antibiotics at a fast enough pace to justify research on the genetics and biochemistry of antibiotic production in our company?". Knowing that it is impossible to predict or even guarentee research results, the following comments indicate that such queries should not be answered with promises due next year, yet with considerable enthusiasm for success in the foreseeable future if the work is supported at an adequate scale.

The simplest genetic approach to finding higher producing strains would be the random cloning of fragments of DNA from a producing organism into itself, or another strain that makes the same antibiotic, followed by screening for higher producing transformants (*114*). This method is entirely empirical, but practical if a convenient and semiquantitative screening procedure is available. Moreover, it has a finite chance of success through effects on gene dosage or the process that normally regulates antibiotic production.

Since expression of antibiotic production genes is apparently regulated by a gene(s) within the cluster of structural and resistance genes, one should see if mutations of this gene can enhance production in a given situation. Testing the effect of deletions (cf. *104*) or site-specific mutations of this region, or of the insertion of a strong promoter in the DNA controlling transcription of the regulatory gene would be worthwhile experiments. For this, a gene replacement method should be used to replace the wild-type sequence in the genomic DNA instead of plasmid or phage vectors that would necessitate continuous selection for their maintenance. Homogentization (*32*) by means of a ϕC31-derived vector carrying the mutated DNA or similar replacement by a double crossover recombinational event between the (mutant) DNA insert of a high copy number vector and its homologous segment in the chromosome (*115*) are demonstrated methods; and the use of integrating DNA species such as SLP1 (*115a*), ϕC31, Tn*4556*, or the *S. coelicolor* minicircle to insert the mutant DNA are alternatives worth testing.

A more straightforward approach to yield enhancement of course would be increasing the copy number of a positive regulatory gene or a structural gene encoding an enzyme that appears to catalyze the rate-limiting step in a biosynthetic pathway. For example, the presence on a plasmid vector of a few copies of the *afsB* gene, which somehow regulates the production of A-factor and pigmented metabolites in *S. coelicolor* and *S. lividans* (*116*), in an A-factor deficient *S. lividans* mutant caused a marked overproduction of its pigments (*117*). An increase in the gene dosage could also be accomplished by DNA amplification using the vector described by Altenbuchner and Cullum (*62*) or some modification of the spectinomycin-selectable system (*68*) if either method can be adapted to the desired strain. This approach could lead to deleterious effects from the high gene dosage and the recombinant strain might be unstable even under selective pressure. Two examples of decreased antibiotic production upon the introduction of antibiotic production genes on high copy vectors into homologous (*103*) or heterologous (*95*) backgrounds have already been reported. Furthermore, determining which step of a pathway is rate limiting is not a simple matter since in few cases do we understand what regulates enzyme titer or activity in the multistep pathways of antibiotic formation. (It may be invalid to assume that accumulation of a certain pathway intermediate in vivo indicates that its conversion to the next intermediate is the rate-limiting step, although this should be used

as a first approximation.) The outcome of increasing gene dosage therefore is unpredictable but the test is nonetheless justifiable.

The assumption that antibiotic production and self-resistance go hand-in-hand in determining the level of antibiotic produced by a microorganism underlies the success of selecting for higher antibiotic resistance in strain improvement programs (*1*). Crameri and Davies have tested the effect of increasing the copies of the gene encoding an aminoglycoside 6′-*N*-acetyltransferase, a modifying enzyme that provides aminoglycoside resistance, on aminoglycoside production (*118*). Strains of *S. kanamyceticus* and *S. fradiae* transformed with the gene cloned in pIJ702 exhibited increased antibiotic resistance and production. Thus, applying the approaches given above for a regulatory gene to a resistance gene could have beneficial results. In doing this one must be aware that a producing strain can have more than one antibiotic resistance gene, as demonstrated for the macrolide (*101,119,120*) and aminoglycoside (*121,122*) antibiotics. It will be especially interesting to see the effect on antibiotic production of the site-specific mutations of resistance genes where the promoters of resistance and biosynthetic genes overlap.

Another conceivable approach to yield enhancement is the introduction of whole antibiotic gene clusters into a heterologous host to see if this would result in deregulation of antibiotic production. If some component of primary (or secondary) metabolism plays a significant role in controlling antibiotic production by the normal producer, this control could be different, perhaps absent, in a completely different genetic and physiological background; enhanced antibiotic production could therefore result. Although this approach may seem to be a gamble and possibly impractical because the large amount of cloned DNA required (20–35 kbp) could be unstable in the recombinant strain, nevertheless the whole set of biosynthetic genes for actinorhodin (*94*), undecylprodigiosin (*114*), and methylenomycin (*114*) have been stably inserted into SCP2-derived vectors, and daunomycin (Engwall and Hutchinson, unpublished results) and tetracenomycin C (Motamedi and Hutchinson, unpublished results) into pKC505. In the case of methylenomycin, high production was observed when a small portion of one end of its gene cluster was deleted (*104,114*).

Besides purely genetic approaches, protein engineering offers a way to expedite antibiotic production for the simpler biosynthetic pathways. The impressive advances in our understanding of the relationship between protein structure and enzyme catalysis (*123*) suggest that it will eventually be possible to optimize the catalytic efficiency of a pathway enzyme by altering its primary amino acid sequence through genetic engineering. A promising case in point is the betalactam antibiotics where the enzyme isopenicillin *N* synthase (IPNS) catalyzes the formation of the key compound in a three- to five-step pathway and can convert a large number of analogs of its normal tripeptide substrate, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine, to unusual betalactams (*124*). The IPNS gene has been cloned

from *Penicillium, Cephalosporium,* and *Aspergillus* species (Table 4) and expressed in *E. coli* (*125,126*). Site-specific mutagenesis of the IPNS gene was used to probe the role of two cysteine residues in the catalytic activity of IPNS (*127*) and could be used to modify its activity positively once more knowledge about the mechanism of this biochemical reaction is gained. Reintroduction of the mutated IPNS gene, or the normal gene under the control of a more efficient promoter, into a fungal or bacterial host could lead to higher penicillin production. Alternatively, immobilization of the modified IPNS enzyme could be used for the large scale production of isopenicillin *N* analogs in vitro that could be converted chemically to penicillin or cephalosporin antibiotics. The protein engineering approach will certainly require more effort than the ones outlined above because it first requires the isolation and study of pathway enzymes, yet it should be given increasing credence.

There are other things that could be altered by genetic engineering of *Streptomyces* with the expectation that an improved antibiotic production process could result. These include the normally repressible genes encoding enzymes that metabolize carbon and nitrogen sources to avoid the inhibitory effects of easily utilizable nutrients on antibiotic production, genes that provide the precursors of the first intermediates in biosynthetic pathways to increase precursor availability through enhanced synthesis, genes encoding thermolabile regulatory proteins or pathway enzymes to permit fermentations at a higher temperature with a cheaper cost, and genes that control secretion of antibiotics into the fermentation medium or prevent their reuptake (prevention of reabsorption is a common resistance mechanism among antibiotic producers [*99,128*]), if the capacity of these processes is normally saturated. In any situation, however, including the potential of recombinant microorganisms for making new antibiotics (*124*), until we gain more knowledge about the biochemical, genetic, and physiological factors mediating antibiotic production, empirical methods will dominate all approaches to strain improvement that are based on the genetic engineering of *Streptomyces* and other antibiotic producing microorganisms.

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