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Perspective

Drug Discovery and Development through the Genetic Engineering of Antibiotic-Producing Microorganisms

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Most scientists recognize that molecular biology now pervades the study of all biological systems. This is due largely to the explosion of knowledge in molecular biology during the last 15 years, which has been fueled by developments in recombinant DNA technology. The influence of molecular biology on medicinal chemistry is increasing dramatically, in spite of the fact that medicinal chemists still receive little exposure to this field during their years of formal training. This perspective examines one area—antibiotic discovery and production—where molecular biologists and medicinal chemists are collaborating to drive the quest for new drugs and to improve production of old ones. Other areas, such as the use of genetic engineering to develop small proteins into drugs[1] or to produce new and more specific vacccines,[2] are not covered here. Further possibilities where interaction between medicinal chemists and molecular biologists should lead to valuable new drugs include the search for small molecules with the ability to modulate gene expression or genetic regulatory circuits in cell biology. Some of these possibilities are mentioned later. Hopefuly, such opportunities will attract researchers with chemical expertise to make the leap into genetically based endeavors, or at least will help awaken educators and research managers to the need for more exposure of medicinal chemists to the concepts and tools of molecular biology.

Antibiotic Discovery: Old and New Ways

Since the discovery of penicillin and streptomycin about 50 years ago, antibiotics have been one of the most avidly sought-after products of microbial metabolism. Initially, the simple screening of culture broths for antibiotic activity against common pathogens or related Gram-negative or Gram-positive bacteria sufficed to uncover a large number of compounds, many of which became widely used antiinfectives (e.g., the erythromycins, cephalosporins, and tetracyclines). As the clinical use of antibiotics became widespread, however, resistant forms of the targeted pathogens became an increasing problem. This necessitated the search for new antibiotics with a broader spectrum of activity or effectiveness against the resistant microbes (e.g., the 7α-methoxycephalosporins and carbapenems) by using more sophisticated screens with greater sensitivity or focused only on the problematic pathogens.[3,4] The chemical and biological modification of existing antibiotics also resulted in more effective analogues of naturally occurring antibiotics, with the β-lactams representing the zenith of such activity since hundreds of their analogues have been studied and a large number have been developed into important antiinfective drugs.[5] All of these developments have offset the diminishing rate of discovery of new antibiotics since the early 1970s, yet it is common today to screen 10 000 isolates before a truly promising new antibiotic is discovered.[6] While pharmaceutical companies and a few academic laboratories are vigorously pursuing the search for new microbial products by the use of more directed antibiotic screening methods coupled with ones

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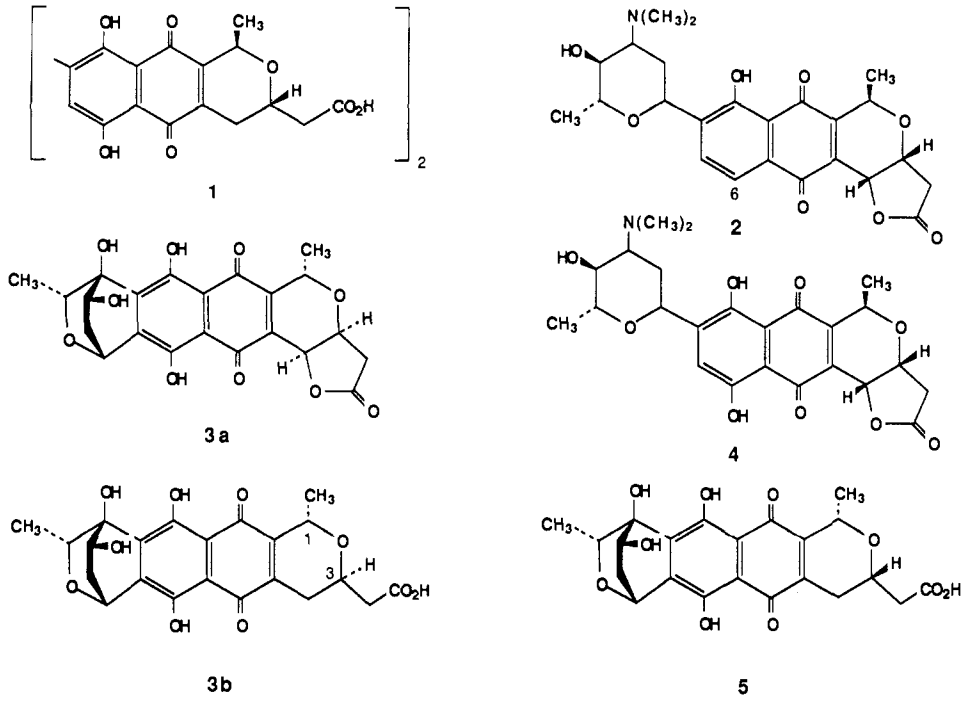

Figure 1. The natural (1–3) and hybrid (4 and 5) isochromane quinone antibiotics produced by *S. coelicolor* A3(2), *S. violaceoruber*, and *Streptomyces* AM7161.

designed to look for other biological activities (e.g., inhibitors of HMGCoA reductase or proteins that regulate cellular growth, or immunosuppressants),[3,4,6] a different and versatile approach to drug discovery and development would be enthusiastically welcomed.

A clue to a new approach comes from the realization that the spectrum of isolable microbial metabolites is predicated by the existing genetic diversity of microorganisms, but that this can be altered almost without limitation by recombinant DNA methods. Thus, if it is possible to genetically engineer a microorganism to produce new antibiotics (or just new metabolites with their categorization to follow), then it should be feasible to design a new type of drug screening or development strategy tailored toward a particular therapeutic goal that has a high potential of success, or that is at least highly versatile. Three approaches are discussed below: each appears to meet this need but none have yet been tested thoroughly.

Hybrid Antibiotic Production

The utility of modifying antibiotics by fermentation methods has been known since 1948 when penicillin V, an acid-stable analogue of benzylpenicillin, was produced by adding sodium phenoxyacetate to the *Penicillium chrysogenum* fermentation.[7] Since a wild-type microorganism often produces both the normal metabolite and the desired analogue, this so-called "directed fermentation" method was later modified by the use of mutants blocked in some step of antibiotic biosynthesis and supplementation of the fermentation with an analogue of a normal intermediate of the pathway. Since the mutation prevents the biosynthesis of the normal intermediate, this latter method results in the production of only the desired modified antibiotic. "Mutasynthesis" and a variant called "hybrid biosynthesis", where the requisite metabolic blockade is achieved by biochemical rather than genetic inhibition, represent improved ways of making analogues of known antibiotics.[8,9] Both methods rely on a satisfactory precursor uptake and plasticity of the enzymes of microbial secondary metabolism, i.e., metabolism that is dispensible with respect to cellular growth. Because these demands are seldom well-met in practice, both methods suffer from inefficient conversion of an unnatural precursor to the desired metabolite. Formation of the modified precursor in vivo would circumvent one of these problems and perhaps others. This has now been achieved by using genetically engineered *Streptomyces*, the ubiquitous soil bacteria that produce about three-fourths of the commercially important antibiotics. [The principles of genetic engineering and the techniques of recombinant DNA research are thoroughly explained in ref 10–12.] The recombinant bacteria were able to produce "hybrid antibiotics", a name coined to reflect the production of new, antibiotically active compounds by genetic hybrids rather than the fact that these metabolites may contain structural elements of different types of known antibiotics.

The pioneering discovery was made in 1985 by David Hopwood and his collaborators in Japan and the USA who examined the effect of introducing some or all of the genes for the production of actinorhodin (Figure 1: 1), which had been cloned from *Streptomyces coelicolor* a year earlier,[13] into other streptomycetes that make the closely related isochromane quinone metabolites medermycin (2) and granaticin/dihydrogranaticin (3).[14] The production of the

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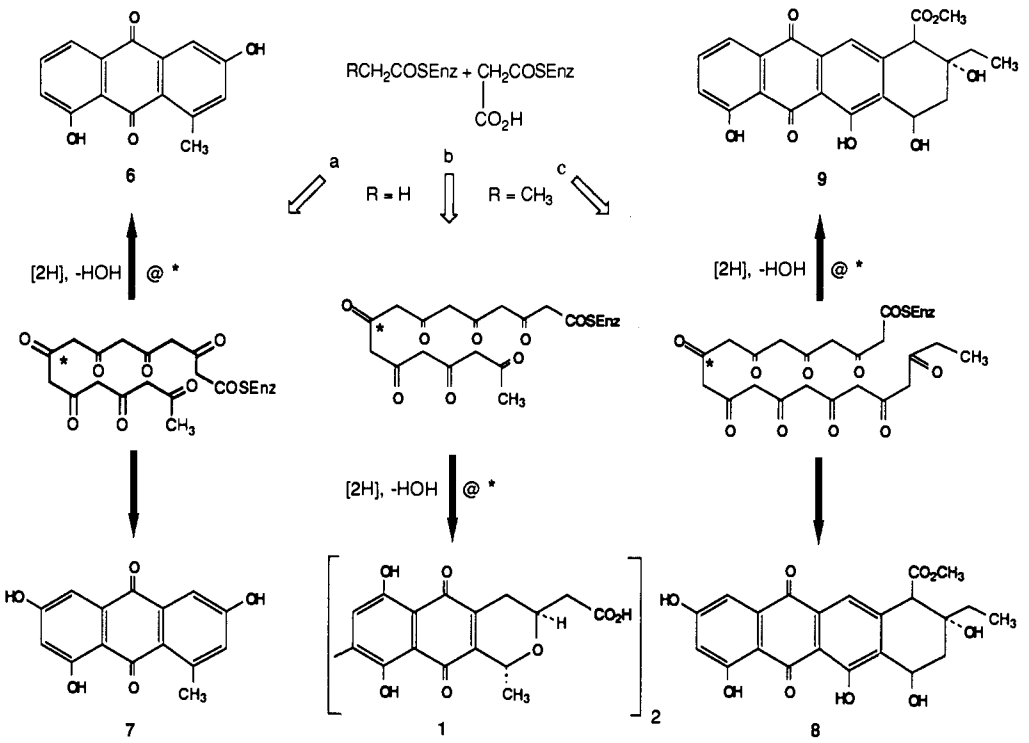

Figure 2. The polyketide metabolites produced by expression of the *S. coelicolor actI, III, IV,* and *VII* genes in other *Streptomyces*. Acetate and malonate through their enzyme-bound forms are used to make **1** in *S. coelicolor* by path b that involves the folding of the octaketide intermediate shown. In several other streptomycetes, the same precursors make **6** through the same polyketide that is folded in a different manner (path a) if all four *act* genes are present, or **7** if only the *actI* and *VII* genes are present. (The COOH group present in the cyclized polyketide is lost by decarboxylation in forming **6** and **7**.) *S. galilaeus* ATCC 31671, which normally uses propionate and malonate to make **8** by way of a decapolyketide intermediate (path c), makes **9** in the presence of the *actIII* gene. The carbonyl in each polyketide intermediate that undergoes reduction followed by dehydration when the *actIII* gene is present is indicated by an asterisk. Not all of the steps required for the conversion of the fatty acid precursors to the final metabolites are shown.

new metabolites, mederrhodin A (**4**) and B (dihydromederrhodin **A**)[15] and dihydrogranatirhodin (**5**) by the respective interspecific transformants[14] reflects the ability of enzymes normally involved in actinorhodin formation to act on similar intermediates in the medermycin or granaticin pathways, or vice versa. Medermycin was hydroxylated at position 6,[15] which is believed to have been catalyzed by the *actV* gene product (a putative hydroxylase[14,16]). [Genes are designated by three lower-case letters followed by capitalized Arabic letters or Roman numerals, all of which are italicized.] The stereochemistry of **3b** was inverted at position 1 or 3,[17] which could have been due to the utilization of an actinorhodin precursor[16,18] in the granaticin pathway since the wild-type strain produced **5** only in the presence of all of the *act* genes.[14] Both of the new metabolites were produced in large amounts compared with the yields of **2** or **3**, but it is interesting that the *act* genes behaved differently in the two host strains. In the medermycin producer, they acted independently and caused the production of **4** only when one class of *act* genes was introduced; in the granaticin/dihydrogranaticin producer, the gene products "cooperated"[14] with the granaticin pathway enzymes to produce **5** almost exclusively. The reason for this outcome is not yet clear; it may reflect several factors, including such things as enzyme specificity, metabolite channeling, and regulation of gene expression, all of which will have to be investigated individually to understand the complex interactions possible.

Subsequent investigations of the interspecific behavior of the *act* genes by the Strohl and Floss groups have shown that the *actI, III, IV,* and *VII* genes cause the production of aloesaponarin (Figure 2: **6**) when they are introduced by transformation into several other *Streptomyces* species.[19a] These results are consistent with the finding that **6** is a shunt product of the actinorhodin pathway in *S. coelicolor actVI* mutants.[19a] In *S. galilaeus* 31133, only the *actI* and *VII* genes are required for the production of **6** since it contains a functional equivalent of the *actIII* gene[19a] that is believed to encode the reductase that reduces the β-keto group at position 9 in the nascent octa-(poly)ketide chain.[20] In fact, when *S. galilaeus* ATCC 31671 is transformed with the *actI* and *VII* genes, it produces 2-hydroxyaloesaponarin (desoxyerythrolaccin, **7**) because this strain lacks such a β-keto reductase, consistent with the fact that it normally produces 2-hydroxyaklavinone (**8**). Introduction of the *actIII* gene into *S. galilaeus* 31671 by transformation therefore shifted its primary metabolite from **8** to aklavinone (**9**), the product of reduction of C-9 in the decaketide precursor.[19a]

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Why the *act* genes specify one folding pattern of the octaketide intermediate that leads to 1 in *S. coelicolor* normally, but a slightly different one leading to **6** or **7** in *S. galilaeus* and other *Streptomyces* (Figure 2, path a vs b), is a curious outcome of these experiments. Since **6** is a shunt product of the actinorhodin pathway in *S. coelicolor*, it may be that the putative "octaketide cyclase" of this pathway is not absolutely specific. This could be a useful attribute in attempts to create new polyketide metabolites by genetic engineering. Furthermore, the recent discovery that the polyketide metabolites of *Streptomyces* are made by a multienzyme complex whose constituents are encoded by separate genes or cistrons in genes with multicistronic transcripts[21] makes it possible to explore systematically the possibility of forming new polyketide-derived metabolites by constructing recombinant bacteria with different combinations of "polyketide synthase" genes.

The successful generation of hybrid macrolide antibiotics by mutasynthesis and hybrid biosynthesis[9] implies that new macrolides should be even more accessible with cloned genes. Macrolide production genes have been cloned recently from the erythromycin[22,23] and tylosin[24] pathways. An erythromycin-like compound was produced in very low yield by *S. lividans* upon its transformation with a clone containing about 35 kilobase pairs of *ery* DNA including the gene conferring erythromycin resistance in this host.[22] It is unfortunate that the exact nature of this antibiotically active compound was not determined because more recent evidence shows that this clone does not carry all of the *ery* production genes;[23] therefore, the substance produced by *S. lividans* could actually have been a hybrid macrolide antibiotic.

An antibiotic nonproducing mutant of *S. erythreus* (now called *Saccharopolyspora erythraea*) that could not make 6-deoxyerythronolide B, the earliest known intermediate of the erythromycin pathway, produced an antibiotic after transformation with a gene library from *S. antibioticus*, the bacterium that produces oleandomycin, a 14-membered macrolide closely related to erythromycin. The material isolated from an antibiotic-producing recombinant strain was identified as a mixture of the 2-norerythromycins A, B, C, and D which lack the methyl group normally at position 2 of the lactone ring in the erythromycins.[25] Because of insufficient knowledge about the enzymology of 6-deoxyerythronolide B biosynthesis and no information about the function of the transforming DNA [or even its identity for technical reasons (L. Katz, personal communication)], this result cannot be clearly interpreted but, nonetheless, demonstrates the validity of the approach.

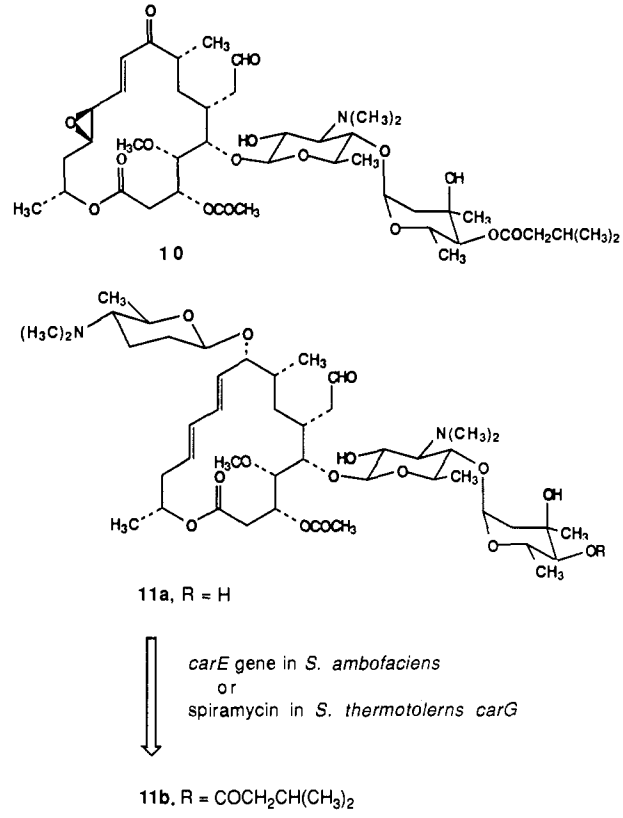

Figure 3. The 16-membered macrolide antibiotics produced by *S. thermotolerans* (**10**), *S. ambofaciens* (**11a**), and *S. ambofaciens* transformed with the *carE* gene from *S. thermotolerans* (**11b**). The *carE* gene presumably directs formation of a 4″-mycarosyl isovalerylCoA transferase in the recombinant strain.

In a subsequent and better understood case, Epp et al.[26] cloned some of the genes for the production of carbomycin (Figure 3: **10**), a 16-membered macrolide, from *S. thermotolerans* and moved the gene (*carE*) believed to encode an isovalerylCoA transferase into *S. ambofaciens*, which produces the related 16-membered macrolide spiramycin (**11a**). The interspecific transformant produced 4″-isovalerylspiramycin (**11b**) due to the ability of the carbomycin isovalerylCoA transferase to recognize the equivalent position on mycarose in spiramycin. The same compound was produced by adding spiramycin to the fermentation of an *S. thermotolerans carG* mutant that is blocked early in the carbomycin biosynthetic pathway. Other types of hybrid antibiotics could be sought in the same way with genes from aminoglycoside,[27] tetracycline,[28] anthracycline,[19,29] polyether,[30] and several other pathways since cloned genes governing the production of many types

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of antibiotics are now available.[31]

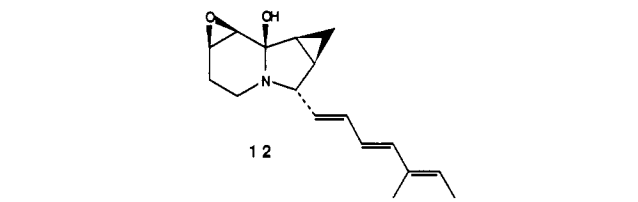
Yet the quest for new (hybrid) antibiotics in genetically engineered microorganisms can encounter technical problems or limitations as highlighted by the following four considerations. It may not be possible to transform certain desirable hosts with heterologous DNA due to restriction barriers (although these can be bypassed by mutation[32] or transduction[33]) or to the lack of a suitable vector. The introduction of genes on high copy number vectors may repress antibiotic production in homologous[19,28] and heterologous (E. Wendt-Pienkowski and C. R. H., unpublished results) genetic backgrounds. Providing a host microorganism with the gene(s) needed for self-resistance to a new antibiotic may be difficult. Finally, the expression of the temporally regulated antibiotic production genes may require host-specific factors (regulatory proteins: see below and ref 34) that are not provided in heterologous situations. In spite of these and other problems yet to be revealed, the genetic engineering of antibiotic-producing bacteria is a powerful method for the discovery of new drugs.

New Antibiotics by Expression of Latent Genes

There are many instances where a microorganism produces more than one type of antibiotic or secondary metabolite, and the formation of families of closely related compounds by the same or different species is a hallmark of secondary metabolism. This and the well-known variability in antibiotic production by a given strain suggests that a microorganism can have more capacity for the production of biologically active substances than is immediately apparent. A way to trigger the expression of such hidden or latent potential would be quite useful for drug discovery.

One approach is to bring two antibiotic-producing microorganisms together under conditions that promote random genetic recombination between their genomes, hoping that this will cause the expression of new genes and thereby the formation of new antibiotics. Studies of representative species within the *Actinomycetales*,[35] the order in which most antibiotic-producing bacteria are classified, using Southern hybridization to probe the structural relatedness of the genomic DNA, indicate that such interspecific recombination may occur at a detectable frequency in vivo. (The DNA of taxonomically divergent organisms would normally be expected to undergo recombination very infrequently, if at all, due to lack of sequence identity and barriers to genetic exchange like restriction.) For example, *S. bikiniensis* (zorbamycin-producer) and *S. fradiae* (tylosin-producer) yielded interspecific recombinants at frequencies of 7.8×10^{-8} (mating) to 1.7×10^{-5} (protoplast fusion).[36] Protoplast fusion has given interspecific recombinants at frequencies as high as 8%,[37] but it would be incorrect to claim that this approach has led to the discovery of new (hybrid) antibiotics. The closest someone has come to this is the finding that a stable fusant of *S. fradiae* TBM (blocked in the glycosylation of protylonolide, the macrolactone precursor of tylosin) and *S. narbonensis* NA12US3 (blocked in the formation of narbonolide, the aglycon of the 14-membered macrolide antibiotic narbomycin) produced narbomycin.[38] This is believed to have resulted from interspecific recombination but there was no direct proof that recombination actually occurred between the antibiotic-production genes in the two strains.

Since antibiotic-producing microorganisms have evolved specific resistance mechanisms to the antibiotics they produce,[39] it is not likely that a different antibiotic will be produced by the expression of latent genes or new combinations of genes without the host also having or acquiring self-resistance to that compound. This requirement, which will obviously limit what can be achieved by the genetic engineering of antibiotic-producing microorganisms, was investigated by Umezawa and his co-workers who fused antibiotic nonproducing mutants of *S. griseus* (streptomycin producer resistant to this aminoglycoside) and *S. tenjimariensis* (istamycin producer resistant to kanamycin). Apparent recombinants exhibiting resistance to streptomycin and kanamycin were isolated at a frequency of 10^{-6},[40] and one of them produced indolizomycin (**12**), a new antibiotic unrelated to the aminoglycosides.[41,42] It is likely that protoplast fusion caused expression of normally silent indolizomycin production genes and acquisition of sufficient resistance to **12** in one the of parental strains rather than the production of a hybrid antibiotic in the sense previously illustrated. A similar explanation can be invoked for the production of curromycin (**13**) by *S. hygroscopicus*, which normally produces the polyether antibiotic carriomycin, upon its treatment with ethidium bromide.[43,44] The production of new antibiotics as a result of random genetic recombination or mutagenesis has been seen in several other cases.[9,45]



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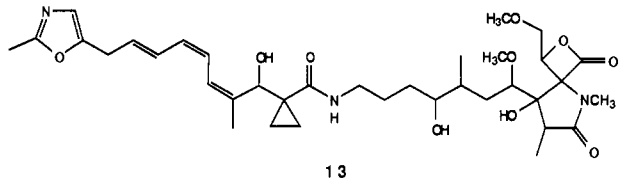

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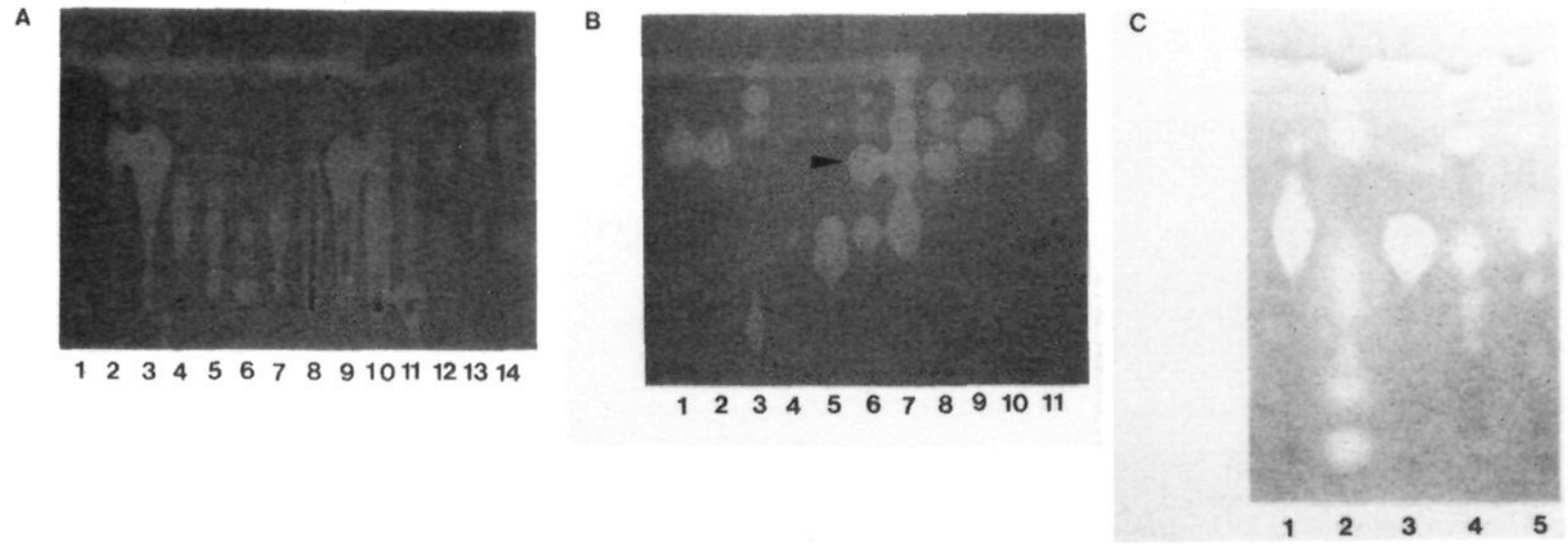

Figure 4. Bioautograms of extracts of the culture broths from recombinant *Streptomyces* strains containing clones that hybridize to the *actI*, *actIII*, or *tcmIa* genes.[48] (A) Lane 1, daunorubicin†; 2,ϵ-rhodomycinone†; 3, maggiemycin (7-keto-ϵ-rhodomycinone); 4, *S. lividans* TK24; 5, *S. lividans* TK24(pKC505); 6, *S. lividans* TK24(pNJ1); 7, *S. lividans* TK24(WHM301); 8, *S. lividans* TK24(pWHM317); 9, *Streptomyces* strain A21; 10, *Streptomyces* strain A21(pWHM309); 11, *S. lividans* TK24(pWHM308); 12, *S. peucetius* ATCC 29050; 13, *S. peucetius* H6125; 14, *S. peucetius* H6125(pWHM317) († = compounds produced by *S. peucetius*). The *E. coli–Streptomyces* shuttle cosmid pKC505[47] was used for clone pWHM301 and pNJ1[23] for clones pWHM309 (this clone contains the fragment in pWHM308), pWHM308, and pWHM317. The DNA was cloned from *S. peucetius* ATCC 29050. Strains were grown as 25-mL cultures in the medium described by Dekleva et al.[49] in 250-mL baffled flasks at 30 °C and 300 rpm for 7 days. The cultures were acid hydrolyzed by the addition of oxalic acid (30 mg/mL) and heating at 55 °C for 45 min, then the pH of the cultures was adjusted to 8.5 by adding 10 N NaOH, and each was extracted with an equal volume of chloroform. After centrifugation at 3000 rpm for 10 min, the chloroform layer was removed, concentrated 10-fold, and spotted on TLC plates that were developed in chloroform–methanol (95:5) and bioautographed with *Bacillus subtilis*. (B) Lanes 1 and 11, salinomycin†; 2, *S. albus* ATCC 21838; 3, azalomycin†; 4, *S. lividans* TK24; 5, *S. lividans* TK24(pKC505); 6, *S. lividans* TK24(pWHM210); 7, *S. lividans* TK24(pWHM204); 8, 18,19-dihydrosalinomycin†; 9, 20-ketosalinomycin†; 10, 20-deoxysalinomycin† († = compounds produced by *S. albus*). pKC505 was used for clones pWHM204 and pWHM210; the DNA was cloned from *S. albus* ATCC 21838. Strains were grown as 50-mL cultures in SCVM medium (glucose, 5 g; soy flour, 15 g; dextrin, 20 g; yeast extract, 2.5 g; and $CaCO_3$ 1 g per liter) in 250-mL baffled flasks at 30 °C and 300 rpm for 7 days. The cultures were each shaken for 2 min with 50 mL of ethyl acetate, and the solvent layer was removed and concentrated 50-fold. A 20-μL aliquot of the concentrate was spotted on TLC plates that were developed in chloroform–methanol (93:7) and bioautographed with *B. subtilis*. The arrow indicates that this substance had a weak ionophoric property. (C) Lane 1, *S. mycarofaciens* 1748; 2, *S. lividans* TK24(pWHM405); 3, midecamycin A1†; 4, *S. lividans* TK24(pWHM400); 5, *S. lividans* TK24(pWHM401) († = compound produced by *S. mycarofaciens*). pNJ1 was used for clones pWHM400, pWHM401, and pWHM405 (this clone contains the fragment in pWHM400); the DNA was cloned from *S. mycarofaciens* 1748. Strains were grown as 50-mL cultures (glucose, 35 g; soybean flour, 30 g; KH_2PO_4, 1.4 g; $MgSO_4$, 1 g; $CaCO_3$, 3 g per liter) in 250-mL baffled flasks at 30 °C and 300 rpm for 2–3 days. The pH of the cultures was adjusted to 8 by adding 1 N NaOH and each was extracted with an equal volume of ethyl acetate. The solvent layer was removed, concentrated 50-fold, and spotted on TLC plates that were developed in chloroform–methanol (97:3) and bioautographed with a thiostrepton-resistant strain of *S. aureus*.

All of these findings could have resulted from induced expression of silent genes rather than creation of new combinations of antibiotic production genes; therefore, a more direct way to test the hypothesis that expression of latent genes can lead to the production of new antibiotics would be to clone large segments of DNA into different genetic backgrounds where the controls on the expression of the cloned genes might be different, and where there is an opportunity for interaction of the gene products with those of the host organism. The discovery that normally silent *Streptomyces* genes encoding phenoxazinone synthase, an enzyme of actinomycin D biosynthesis, are expressed in heterologous hosts when DNA from an actinomycin producer is cloned into them[46] supports the validity of this approach to drug discovery. Antibiotic biosynthetic and self-resistance genes are clustered in all of the cases studied, frequently within a region of approximately 35 kilobases of DNA;[31a] in some cases, however, the gene clusters may span up to 100 kilobases.[26,31b] This suggests that a good method would be to clone this amount of DNA with cosmid vectors[11] or other vectors like plJ922[13] that can carry large DNA inserts, transform the DNA into other microorganisms that permit expression of the cloned genes, and look for the production of new biologically active substances by a suitable screening method. This approach is limited by the things already mentioned for the production of hybrid antibiotics as well as by another factor, the uncertain stability of large plasmids in a recombinant microorganism, but is potentially more versatile since the type of antibiotic formed is not limited to the class normally produced by the cloned genes as in the hybrid antibiotic production method.

Instead of testing this idea by shotgun cloning, which certainly is possible albeit laborious, we decided to screen for new antibiotics by using clones made in *E. coli–Streptomyces* shuttle cosmids[47] and that hybridized to the *actI* and *actIII* genes. The knowledge that the occurrence of the latter genes is frequently correlated with the production of polyketides,[48] the largest class of *Streptomyces* secondary metabolites and the biochemical origin of many known antibiotics, pointed to the possibilty that such clones might make new antibiotics in a different genetic background. This belief has been validated by finding that clones from three different streptomycetes which produce anthracycline (*S. peucetius*), macrolide (*S. mycarofaciens*),

(45) Okami, Y.; Hotta, K. In *Actinomycetes in Biotechnology*; Goodfellow, M., Williams, S. T., Mordarski, M., Eds.; Academic Press: New York, 1988; pp 33–68.
(46) Jones, G. H.; Hopwood, D. A. *J. Biol. Chem.* **1984**, *259*, 14158.
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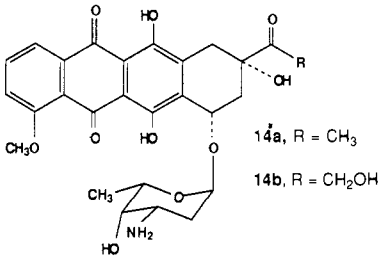
(48) Malpartida, F.; Hallam, S. E.; Kieser, H. M.; Motamedi, H.; Hutchinson, C. R.; Butler, M. J.; Sugden, D. A.; Warren, M.; McKillop, C.; Bailey, C. R.; Humphreys, G. O.; Hopwood, D. A. *Nature (London)* **1987**, *325*, 6107.

or polyether (*S. albus*) antibiotics, caused *S. lividans* to produce substances active against *Bacillus subtilis* that were not produced by the *Streptomyces* species from which the DNA had been cloned under their normal fermentation conditions (Figure 4: The production of new antibacterial substances is most evident in A, lanes 8–10; B, lanes 6 and 7; C, lanes 2, 4 and 5). Whether or not these new compounds, having resulted from the expression of silent genes or the production genes for the known antibiotics from these strains, remains to be determined by chemical characterization of the unknown substances.

We have uncovered further evidence for the existence of possibly latent secondary metabolism genes in *S. peuectius* strains that produce the antitumor drugs, daunorubicin (14a) and doxorubicin (**14b**).[19] Cosmid clones



from the ATCC 29050 strain that hybridized to the *actI*, *tcmIa*, and *actIII* polyketide synthase gene probes were classified on the basis of restriction mapping and DNA hybridization into four different groups, representing nonoverlapping regions of the bacterial genome. Three of these regions were also present in the ATCC 27952 strain, the doxorubicin producer derived from the 29050 strain by mutagenesis. One of the regions that is present in both strains contains most if not all of the *dnr* production genes on the basis of the results of complementation experiments with *S. peucetius* blocked mutants and the production of intermediates of daunorubicin biosynthesis by *S. lividans* transformants. Clones from the three other regions, however, modulate expression of the genes in the fourth region or cause the production of new secondary metabolites in *S. lividans* transformants. Such transformants also exhibit daunorubicin resistance and the abilty to convert a shunt metabolite of daunorubicin biosynthesis into one of the key pathway intermediates. Thus, it seems that *S. peucetius* contains at least four multigene families that are somehow involved in the production of anthracycline and other (polyketide?) metabolites, but we do not know if all of these genes are normally expressed.

Increasing Antibiotic Production

A vital part of the commercial production of microbial drug products is the economics. An exciting new antibiotic may never reach the clinic if it cannot be produced in an amount sufficient for its complete biological evaluation. As a wild-type microorganism seldom, if ever, makes enough antibiotic for such needs, considerable work goes into the development of high-producing strains. This has traditionally been done by mutagenesis and random screening for higher producing mutants, plus the artful variation of growth medium constituents and fermentation conditions.[31,45,50] Recent information about the genetics of antibiotic production indicates that overproducing strains could now be constructed by genetic engineering.

Table I. Genes That Regulate Secondary Metabolism in the *Streptomyces*

gene	functions
actII	increases actinorhodin production 30–40-fold when introduced into *S. coelicolor*;[51] positively regulates expression of the *S. coelicolor actII* gene that encodes the polyketide reductase of actinorhodin biosynthesis and maybe other *act* genes[20]
mmy	inactivation of a region at one end of the cluster of methylenomycin biosynthetic and resistance genes causes overproduction of methylenomycin in *S. coelicolor*[52]
afsB	greatly increases actinorhodin and undecylprodigiosin production by some type of indirect mechanism when introduced into *S. lividans*[53,54]
redD	increases undecylprodigiosin production ~30-fold when introduced into *S. coelicolor* (F. Malpartida, personal communication)
strR	increases streptomycin production 5–7-fold when introduced into *S. griseus*; enhances 3–6-fold the amidinotransferase activity required for streptomycin production when introduced into *S. griseus* or two of its Str$^-$ mutants[55]
brpA	controls expression of the bialaphos resistance and at least six biosynthetic genes of bialaphos production in *S. hygroscopicus*[56]

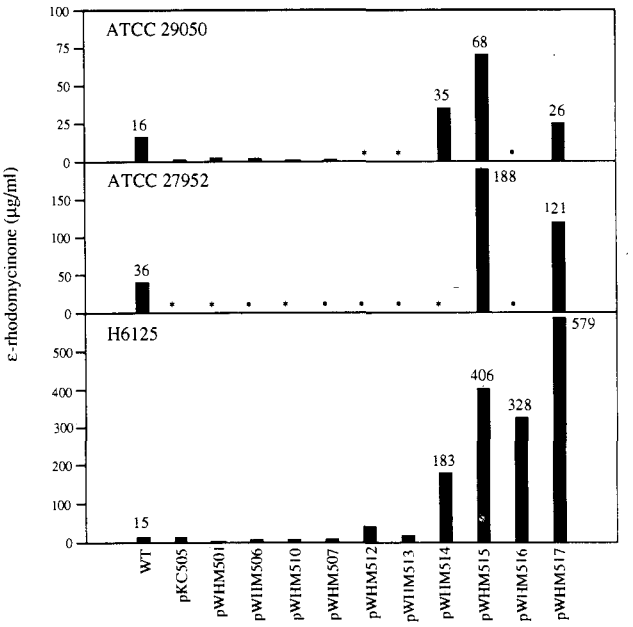


Figure 5. Production of ε-rhodomycinone by wild-type and recombinant *S. peucetius* strains. DNA cloned from *S. peucetius* 27952 in pKC505[47] that hybridized to the *actI* or *tcmIa* genes[48] was used to transform *S. peucetius* wild-type (ATCC 29050) and mutant (ATCC 27952 or its H6125 mutant that accumulates ε-rhodomycinone) strains. The strains were grown as described in Figure 4A, and ε-rhodomycinone production was quantitated by high-performance liquid chromatographic analysis. The data are the average values from at least four replicate cultures, and the numbers atop some bars are the amount produced: WT = wild-type or parental strain; * = not determined.

Antibiotic production, in fact secondary microbial metabolism in general, is controlled at several levels, few of which are well understood. It is clear, however, that key regulatory genes are embedded in the gene clusters governing antibiotic formation by many *Streptomyces*. Table I lists the cases known at the time of writing where a specific gene or DNA segment can influence the amount of metabolite produced. Also, some of the cosmid clones from *S. peucetius* can stimulate ε-rhodomycinone (Figure 5) as well as daunorubicin production. In most of these

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(50) Normansell, I. D. In Antibiotic-Producing Streptomyces. *The Bacteria: A Treatise of Structure and Function*; Queener, S. W., Day, L. E., Eds.; Academic Press: New York, 1986; Vol. 9, pp 95–118.

cases, the regulatory gene presumably affects metabolite production by some type of positive control mechanism since introduction of the gene back into the wild-type strain from which it was cloned results in metabolite overproduction. These genes could encode a DNA binding protein that stimulates transcription of one or more genes directing the formation of biosynthetic enzymes, which is a well-established mechanism for controlling bacterial gene expression.[57] For example, the nucleotide sequence of the *afsB* gene (which indirectly controls secondary metabolism and possibly other temporarily regulated genes in *S. coelicolor*) suggests that it encodes this type of protein,[54] and the *actII* gene regulates expression of the *actIII* gene and perhaps other *act* genes in *S. coelicolor*.[20] Further information about how such genes function and about the more indirect controls on secondary metabolism in the *Streptomyces*[58,59] will surely lead to ways to construct high-producing recombinant strains that can be used in large-scale fermentations.

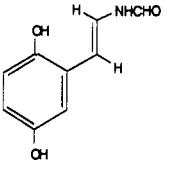
Another way to increase metabolite production is to identify the step in a pathway that is the "rate-limiting bottleneck" and to introduce additional copies of the gene encoding the associated enzyme so as to increase the titer of this enzyme. This should result in more of the final product unless the increased amount of the intermediate causes feedback inhibition of some step in the pathway. When an extra copy of the *Cephalosporium acremonium celEF* gene encoding the expandase/hydroxylase enzyme of cephalosporin C biosynthesis[60] was inserted into the fungal chromosome #3, the LU4-79-6 recombinant strain contained 55 ± 25% more expandase activity and produced 47 ± 10% more cephalosporin C than its parent in shake flask cultures.[61] An increase in production was also seen in pilot plant (150 L) fermentations but less than that observed in shake flask cultures. In another example, transformation of a tylosin-producing strain of *S. fradiae* with the *tylEDHFJ* genes, one of which (*tylE*) encodes the enzyme that converts macrocin to tylosin, partially overcame the bottleneck at this point in tylosin biosynthesis but feedback inhibition limited the amount by which tylosin production could be increased.[62] These pioneering examples presage the many others that will follow as the need arises.

Molecular Biology and Drug Design

The material discussed so far shows that knowledge about the molecular biology and biochemistry of metabolite production in microorganisms can be quite beneficial in drug discovery and production research. There is no doubt that further advances in our understanding of microbial secondary metabolism will lead to even more significant discoveries and stellar applications in this area, yet antibiotics have been and still are most often discovered by screening programs rather than by designing a molecule a priori to inhibit some indispensible process in the metabolism of susceptible organisms. While the antibiotic may be modified synthetically in attempts to improve its efficacy, such research has seldom depended on knowledge about the molecular biology of the target organism (this should not be a truism for the future, however!).

Among other areas where medicinal chemists and molecular biologists could collaborate fruitfully is in the discovery of compounds that can block cell proliferation. Antiproliferative drugs aimed at specific pathological conditions like cancer could be designed on the basis of what is known about the role of oncogenes and protooncogenes in tumor development and metastasis.[63] Specific inhibitors of the expression of oncogenes or the functions of their products could be developed empirically, or designed from empirical leads or detailed knowledge about how the expression of these genes is controlled or how their products function in controlling cellular growth and development.

A recent report about epidermal growth factor (EGF) receptor kinase inhibitors[64] is an excellent example of how such research can be done. Following the lead compound, erbstatin (**15**), a streptomycete metabolite discovered in 1986 by screening for naturally occurring inhibitors of tyrosine protein kinase,[65] Levitzki and co-workers[64] prepared a series of analogues of **15** and used three criteria to evaluate their potential as selective inhibitors of EGF receptor kinase: competition with the natural substrate of the kinase, crossover inhibition of the closely related insulin receptor kinase, and solubility in mildly hydrophobic solvents as an indication that the compounds could traverse the cell membrane. One of the most effective inhibitors they studied was **16** with a K_i of 0.85 μM and



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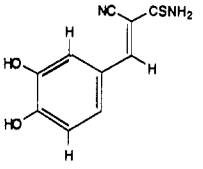

16

3 orders of magnitude less activity against the insulin receptor kinase. The most potent compounds effectively inhibited the EGF-dependent proliferation of A431 epidermoid carcinoma cells with little or no effect on the EGF-independent proliferation of these cells. These compounds are potential antitumor drugs because many

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oncogene products exhibit protein tyrosine kinase activity.[63]

Drugs that modulate gene transcription are already known, of course, since steroid hormone analogues and some antitumor drugs function in this manner. Other drugs of this type could be sought, with knowledge of how hormones or other physiological signals control eukaryotic gene expression. These new drugs could then be used to regulate the synthesis of important receptors or enzymes. For agents that directly affect gene transcription, sequence-specific DNA binding will be the most difficult aspect of development since, without this property, such drugs would be too toxic for use against non-life-threatening diseases. Synthetic agents with selective DNA binding ability are now being developed, as demonstrated by the work of Dervan[66] and Hurley[67] and their co-workers, so it is likely that this will soon lead to new drugs that control gene transcription.

Summary and Prognosis

The exciting developments reviewed show the positive value of the interplay between medicinal chemistry, biochemistry, and molecular biology to antibiotic discovery and production. Considerable progress is being made by the empirical approaches currently necessitated due to the complexity of the biological systems involved. Much more can be done by rational approaches whose importance will be more forcefully felt as our knowledge about how microorganisms make antibiotics matures. Genetically engineered microorganisms certainly will play an active role in the production of known metabolites, and when enough knowledge about the workings of antibiotic production genes is acquired, recombinant organisms will be designed to produce new biologically active secondary metabolites with potential drug use. It also is increasingly clear that the areas of drug discovery and development involving the study of enzyme inhibitors and drug–receptor interaction will greatly benefit from the ability to alter protein structures by genetic engineering.[68] Thus, even if a medicinal chemist has only enough knowledge about molecular biology to communicate intelligently with biologists and biochemists, this will open avenues for fruitful research otherwise hidden in the complexity of biological systems modification.

Acknowledgment. I am grateful for the enthusiastic efforts of John Anderson, Sandor Biro, Sophia Deli, Stefano Donadio, Janice Duncan, Mary Jo Donovan, Pat Guilfoile, Osamu Hara, Haruyasu Kinashi, Marlena Skarbek, Karl Maurer, Haideh Motamedi, Hiroshi Nakayama, Rosa Navarette, Naryan Punekar, Ali Shafiee, Toshio Takatsu, Jesus Vara, Mark Weber, Bill Wessel, Connie Wierman, Hirokazu Yamamoto, and especially Evelyn Wendt-Pienkowski, who have made the transition into molecular biology research possible in my laboratory. The unpublished research described here was supported by grants from the National Institutes of Health (GM 25799) and Xechem, Inc.

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Communications to the Editor

A Multisubstrate Adduct Inhibitor of a Purine Biosynthetic Enzyme with a Picomolar Dissociation Constant

Sir:

Glycinamide ribonucleotide transformylase (GAR TFase; EC 2.1.2.2) is a crucial, reduced folate requiring enzyme involved early in de novo purine biosynthesis, catalyzing the formyl transfer from (6*R*,α*S*)-10-formyl tetrahydrofolate, **3**, to glycinamide ribonucleotide (GAR, **2a**) shown in Figure 1.[1] It has thus attracted some interest as a target enzyme for rational drug design of antineoplastic agents.[2] Published data showed these agents to be modest inhibitors of GAR TFase, but they were not specific.[3,4] An in vivo test of 5,10-dideazatetrahydroaminopterin,[5-7] which demonstrated potency against solid tumors in mice, also provided indirect evidence that the site of action was GAR TFase. Interestingly, these types of tumors are resistant to methotrexate therapy.

A number of potent specific inhibitors of enzymes have been designed[8] with use of the concept of multisubstrate adduct inhibition (MAI).[9,10] Tying together both sub-

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