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Review

Expression of polyketide biosynthesis and regulatory genes in heterologous streptomycetes

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SUMMARY

There are now several examples showing that hybrid secondary metabolites can be produced as a result of interspecies cloning of antibiotic biosynthesis genes in streptomycetes. This paper reviews examples of hybrid secondary metabolite production, and examines the underlying biochemical and regulatory principles leading to the formation of hybrid anthraquinones by recombinant anthracycline-producing streptomycetes carrying actinorhodin biosynthesis genes. An anthraquinone, aloesaponarin II, was produced by cloning the *actI*, *actIII*, *actIV*, and *actVII* genes (pANT12) of actinorhodin biosynthesis pathway from *Streptomyces coelicolor* in anthracycline producing streptomycetes. *Streptomyces galilaeus* strains 31133 and 31671, aclacinomycin and 2-hydroxyaklavinone producers, respectively, formed aloesaponarin II as their major polyketide product when transformed with pANT12. Subcloning experiments indicated that a 2.8-kb *Xho*I fragment containing only the *actI* and *actVII* loci was necessary for aloesaponarin II biosynthesis by *S. galilaeus* 31133. When *S. galilaeus* 31671 was transformed with the *actI*, *actVII*, and *actIV* genes, however, the recombinant strain produced two novel anthraquinones, desoxyerythrolaccin and 1-*O*-methyldesoxyerythrolaccin. When *S. galilaeus* 31671 was transformed with only the intact *actIII* gene (pANT45), aklavinone was formed exclusively. These experiments indicate a function for the *actIII* gene, which is the reduction of the keto group at C-9 from the carboxyl terminus of the assembled polyketide to the corresponding secondary alcohol. The effects of three regulatory loci, *dauG*, *dnrRI*, and *asaA*, on the production of natural and hybrid polyketides were also shown.

INTRODUCTION

Streptomycetes produce a wide variety of biologically active and economically significant compounds, including antibiotics, chemotherapeutic agents, ionophores, immuno-modulators, hydrolytic enzymes, and enzyme inhibitors. Antibiotics are members of a class of compounds called secondary metabolites, which are defined as dispensable molecules not required for growth or survival of the producing organisms. Enzymes that catalyse secondary metabolic reactions often display a markedly lower chemical specificity for substrates than do enzymes of primary metabolism [12]. Investigators have long made use of this fact to affect the formation of new secondary metabolites. Traditional methods used to form novel secondary metabolites include: (i) screening organisms to find ones producing new, structurally and functionally different antibiotics; (ii) mutation of microorganisms to produce new activities; (iii) fusions of protoplasts of two microorganisms, each producing a desired trait, followed by selection for recombinants which have combined desired traits; (iv) chemical synthesis of new compounds using structures produced in nature as templates for enhanced or more desirable activities; (v) chemical or biochemical modification of a backbone molecule produced by a microorganism; (vi) directed biosynthesis by biochemical modification of structures synthesized chemically; and (vii) mutasynthesis in which analogs of antibiotics formed by convergent pathways are produced by eliminating the endogenous synthesis of one of the precursors by mutation and replacing the missing building block by exogenously added analogs. These traditional approaches have served well and have led to the discovery of the current production-scale antibiotics. However, they need to be complemented by newer approaches, because, particularly in screening, there is a diminishing return on

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investment due to the rediscovery of known entities. One promising new approach is the generation of new hybrid metabolites via interspecies cloning of biosynthesis genes.

CLONING AND CHARACTERIZATION OF ANTIBIOTIC BIOSYNTHESIS GENES

To produce hybrid molecules via interspecies cloning of antibiotic biosynthesis genes, it is necessary to have at least one cluster of antibiotic biosynthesis genes, or individual genes from a cluster, that can be introduced into streptomycete strains producing similar structures. Antibiotic biosynthesis genes encoding β-lactams, aromatic polyketides, macrolides, aminoglycosides, and modified peptide antibiotics have all been cloned in the past 7–8 years [5]. Approximately two dozen clusters of genes have now been cloned that encode various antibiotic biosynthesis pathways, as recently reviewed by Chater [5]. Many of these have been cloned by selection for resistance genes and searching for contiguous biosynthesis genes [4,10,36,41], whereas others have been cloned by complementation of blocked mutants [11,25,28]. More recently, several antibiotic biosynthesis genes and gene clusters have been cloned by using heterologous probes, such as the *actI* (polyketide synthase) gene [24], to hybridize and isolate predicted genes encoding steps in the pathway of interest [24,35,39]. For some antibiotics such as β-lactams [23], antibiotic biosynthesis genes encoding known functions have been cloned using reverse genetics. In other cases such as daunomycin biosynthesis, large DNA fragments have been cloned that are known to encode a series of functions [32,39], but individual genes are still being identified and characterized [2,32,39].

One attraction of hybrid antibiotic experiments is that interspecies cloning can be initiated even in the absence of detailed biochemical and (or) genetic knowledge on the biosynthesis pathways involved, i.e., even before all the precursors, intermediates, enzymes, and genes have been characterized. This is the 'shot-gun' approach toward hybrid molecule formation which has resulted in the formation of the mederrhodins [15,31], dihydrogranatirhodin [15], and the norerythromycins [27]. Attempts to produce hybrid secondary metabolites can also contribute to the understanding of the biochemistry and molecular biology of the pathways involved [3,13,38]. As our knowledge on the gene structure and biochemistry of the pathways in question is better understood, the production of hybrid antibiotics can be refined and implemented in an increasingly specific and directed manner.

PROCEDURES FOR OBTAINING HYBRID METABOLITES

A typical protocol for obtaining hybrid metabolites is shown in Fig. 1. To produce hybrid metabolites, genes encoding a known antibiotic are ligated into appropriate cloning vectors and introduced by protoplast transformation into heterologous streptomycetes that produce molecules of the same general class. If the genes for some of the biosynthetic steps in the pathway have been mapped, this offers the potential of producing molecules by design, rather than by shot-gun type experiments.

To limit screening procedures, the presence and structural integrity of the recombinant plasmids in the transformants are usually confirmed before further carrying out fermentations. The transformants typically are screened by culturing in 20 ml of medium, which after 5 to 7 days of growth, is extracted using a variety of solvents. The potential hybrid metabolites are screened by TLC and HPLC, using extracts from the parental strains as nega-

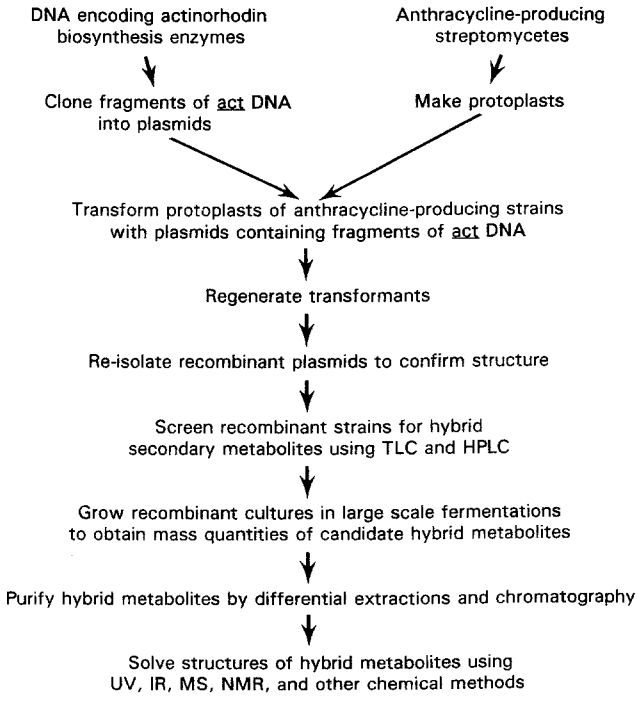

Fig. 1. Protocol for isolation and characterization of hybrid metabolites from streptomycetes. Abbreviations: *act*, actinorhodin genes; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; UV, ultraviolet spectroscopy; IR, infra-red spectroscopy; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.

tive controls (Fig. 1). New metabolites which were not present in the parental strains (host strain carrying just the vector without inserts and DNA donor strain) may be considered as candidate hybrid metabolites (Fig. 1). Cultures producing potential hybrid metabolites are scaled up the 10–50 l scale, as required, and the novel metabolites are extracted and purified by chromatography. The exact procedures depend entirely on the structures and properties of the new metabolites [3]. The assay used during the purification of the novel metabolites should relate back to the original methods in which they were observed, i.e. TLC or HPLC. Although this can be labor-intensive, it is necessary to avoid purifying metabolites normally produced in low quantities by the host organism. In most cases, the structures of the purified metabolites can be solved using mass spectroscopy, and ^{1}H- and ^{13}C-NMR.

HYBRID METABOLITE FORMATION IN STREPTOMYCETES

Mederrhodins and dihydrogranatirhodin

In the first examples of the production of hybrid antibiotics via interspecies cloning, plasmid pIJ2303, containing the entire actinorhodin biosynthesis gene cluster from *Streptomyces coelicolor* (Fig. 2) was introduced into the granaticin producer, *Streptomyces violaceoruber* Tü22, to produce a hybrid metabolite, dihydrogranatirhodin [15]. The hybrid metabolite was different from the parental molecules only in the stereochemistry of protons on one carbon (Fig. 3A).

When the *actVa* locus (pIJ2315; Fig. 2) of the actinorhodin gene cluster was introduced into the medermycin-producer, *Streptomyces* sp. AM-7161, two novel hybrid antibiotics, mederrhodins A and B, were formed [15,31]

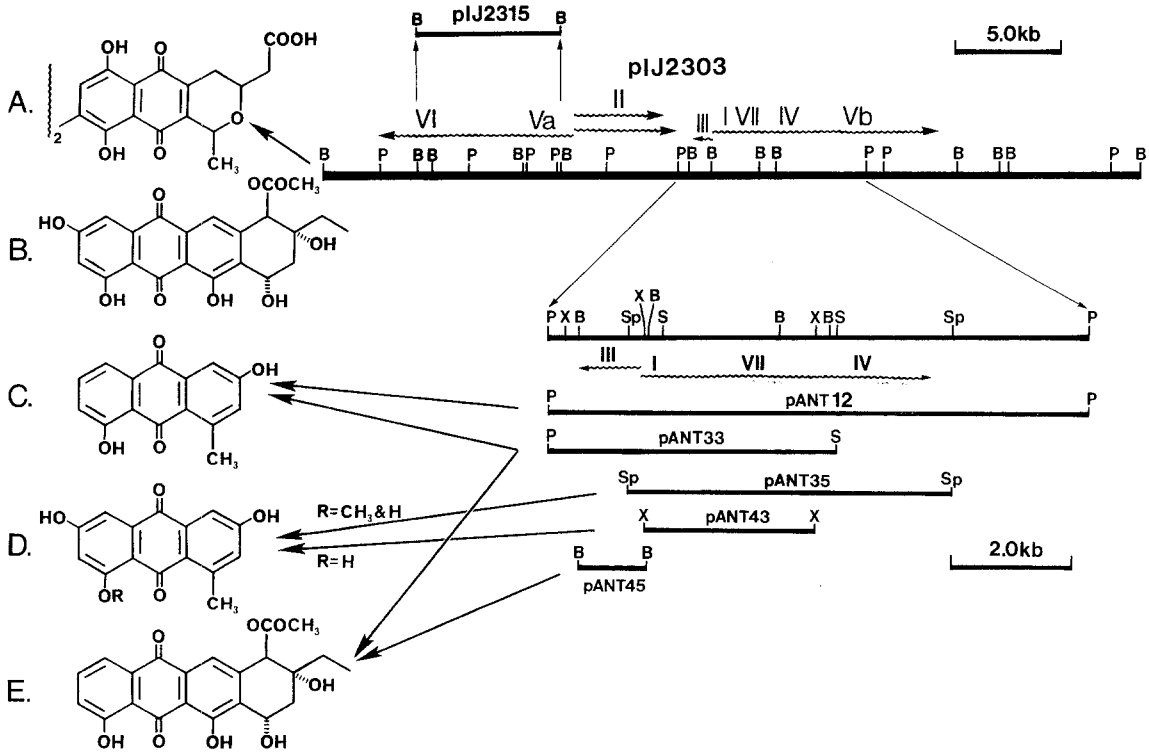

Fig. 2. Restriction map of the actinorhodin (*act*) gene cluster in plasmid pIJ2303, restriction maps of subclones derived from pIJ2303 (including pIJ2315 [15], and pANT12, pANT33, pANT35, pANT43, and pANT45 [3,38]), and compounds made by *S. galilaeus* strain ATCC 31671 transformed with plasmids containing subclones of pIJ2303. The transcriptional units of the *act* genes are shown by wavy lines, and an expanded restriction map of the 8.8 kbp *Pst*I fragment used to make pANT12 is also shown (data from [3]). Compounds and strain (clone) that produce each: A, actinorhodin {*S. galilaeus* 31671 (pIJ2303)}; B, 2-hydroxyaklavinone {*S. galilaeus* 31671}; C, aloesaponarin II {*S. galilaeus* 31671 (pANT12)}; D, desoxyerythrolaccin (R = H) and 1-*O*-methyldesoxyerythrolaccin (R = CH_3) {*S. galilaeus* 31671 (pANT35 or pANT43)}; E, aklavinone {*S. galilaeus* (pANT45)}.

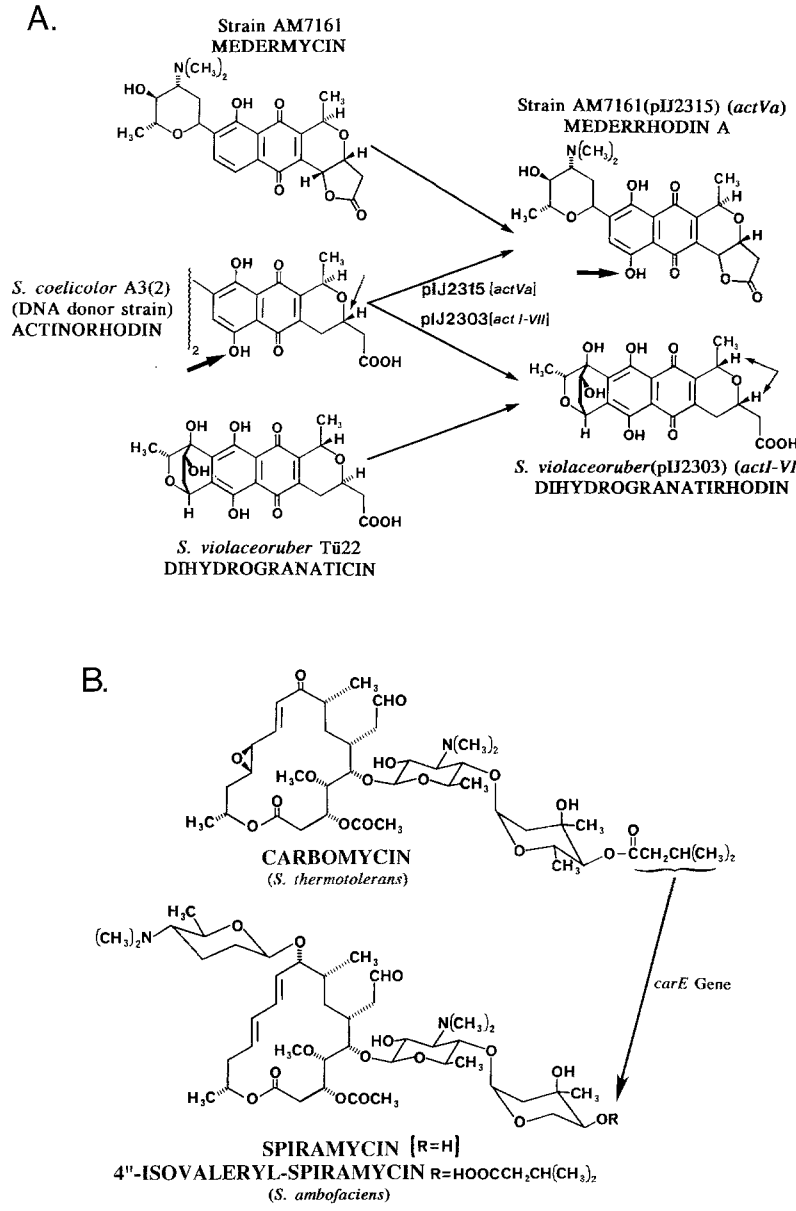

Fig. 3. Examples of hybrid antibiotic formation via interspecies cloning in streptomycetes carried out by other laboratories. **A.** Production of mederrhodin A by cloning the *actVa* gene locus from *S. coelicolor* A3(2) into the medermycin producing strain, AM7161 [15,31]. Notice the additional hydroxyl group (the addition of which is presumably encoded by *actVa*) pointed out by the large arrows. Also, production of dihydrogranatirhodin by cloning the entire *act* biosynthesis gene cluster (see Fig. 2) into the dihydrogranaticin producing strain, *S. violaceoruber* Tü22 [15]. In this case, notice the stereochemistry of the hydrogens pointed out by the small arrows. **B.** Cloning of the *carE* gene encoding the attachment of the isovaleryl sidechain on carbomycin from *S. thermotolerans* into the spiramycin producer, *S. ambofaciens*, results in the hybrid molecule, 4″-isovaleryl-spiramycin [10].

(Fig. 3A), which contained a hydroxyl group absent in medermycin (Fig. 3A). Since one of the predicted functions of the *actVa* gene includes a hydroxylation step, this was the first example where a hybrid metabolite had been produced by design. More recently, two hydroxylases have been purified from other pathways, the 6-deoxyerythronilide B hydroxylase, which is a P450-type monooxygenase [34], and the anhydrotetracycline oxygenase, which also appeared to be a monooxygenase (although the authors did not state whether this was a P450-type monooxygenase) [40]. In recent work, we have partially purified an NADPH- and oxygen-dependent

aklavinone 11-hydroxylase from the daunomycin biosynthesis pathway and have found it to consist of three components, a diaphorase, a possible iron-sulfur protein, and a non-P450-type monooxygenase (N.C. Connors and W.R. Strohl, unpublished data). The substrate specificities of these biosynthetic aromatic polyketide monooxygenases should offer new and interesting possibilities for directed hybrid metabolite formation.

Norerythromycins

A second group of hybrid antibiotics was produced by shot-gun cloning DNA from the oleandomycin-producing strain, *Streptomyces antibioticus*, into a blocked mutant of the erythromycin producer, *Saccharopolyspora erythraea* (formerly *Streptomyces erythraeus*). The hybrid antibiotics, norerythromycins A–D [27], were analogs of erythromycins A–D, respectively, lacking only the methyl group at C-2 of the macrolide ring [27]. To date, however, the mechanism for the formation of these demethylated analogs has not been published [21].

4″-Isovalerylspiramycin

A third example of hybrid antibiotic formation was recently described by Epp et al. [10]. The *carE* gene isolated from *Streptomyces thermotolerans*, which encodes the addition of an isovaleryl side chain on the macrolide carbomycin, was cloned into the spiramycin-producing strain, *S. ambofaciens*. The recombinant strain produced 4″-isovaleryl-spiramycin, a hybrid antibiotic (Fig. 3B). This experiment, along with the formation of mederrhodins by *Streptomyces* AM-7161 (pIJ2315) [15] and desoxyerythrolaccin by *S. galilaeus* 31671 [3,38], provides ample evidence that one can predict the formation of specific compounds produced via the interspecies cloning of secondary metabolite biosynthesis genes.

Formation of anthraquinones by interspecies cloning of actinorhodin DNA

The experiments described above involve subtle alterations of the host parental molecules via reactions encoded by the donor DNA which would be equivalent to those late in the biosynthesis pathways. An alternative approach would be to use genes encoding early steps in a pathway to determine if the host organism could alter molecules formed by the recombinant DNA [38]. In our attempts to form hybrid metabolites between actinorhodin- and anthracycline-producing streptomycetes, we subcloned from pIJ2303 an 8.8 *Pst*I fragment which contains *actI*, *actIII*, *actIV*, and *actVII* loci, encoding the early steps in actinorhodin biosynthesis, in the multicopy plasmid pIJ350 to construct pANT12 [3] (Fig. 2). Plasmid pANT12 conferred the ability to produce 1,7-dihydroxy-9-methylanthraquinone (aloesaponarin II; Fig. 2, compound C) on the aclacinomycin-producing strain, *S. galilaeus* ATCC 31133 [3]. We subsequently found that pANT12 also conferred aloesaponarin II biosynthesis on *S. peucetius* ATCC 29050, *S. parvulus*, *Streptomyces azureus* ATCC 14921, and *Streptomyces* C5, but not on *S. actuosus* ATCC 25421 (Table 1).

Aloesaponarin II was also produced as a shunt product of the actinorhodin biosynthesis pathway by class VI mutants (i.e., B22, B159) of *S. coelicolor*, strains from other mutant classes did not produce any detectable anthraquinones [3,38]. According to the deduced pathway for actinorhodin biosynthesis [3,13,38] and the predicted order of the various mutant classes [33], aloesaponarin II appeared to be produced by the action of the *actI*, *actIII*, *actIV*, and *actVII* loci gene products in the absence of an active *actVI* gene product, whether cloned in heterologous streptomycetes or in the *actVI* mutants of *S. coelicolor* [3]. Carbon-13 NMR studies showed that aloesaponarin II was formed from the successive condensation of 8 acetyl-CoA equivalents followed by decarboxylation [3], so it is evident that the backbone for aloesaponarin II is synthesized by the *actI* gene product, but is cyclized and aromatized in an altered fashion to produce a different compound than actinorhodin (Fig. 4).

Subcloning experiments demonstrated that only the *actI* and *actVII* loci (plasmid pANT43) were required for aloesaponarin II biosynthesis by *S. galilaeus* 31133 [3]; these two loci, however, did not support the synthesis of aloesaponarin II in *S. azureus*, leading us to propose that *S. galilaeus* 31133 provided the *actIII*- and *actIV*-equivalent activities [3]. Using this hypothesis as a basis, it is probable that the early reactions in anthracycline biosynthesis (i.e., those leading to the formation of aklanonic acid [8,38]) and actinorhodin biosynthesis [3] are very similar, as postulated in Fig. 4. From Fig. 4, it would be surmised that, upon further investigation, *actVII*- and *actIV*-like genes will be found in the daunomycin biosynthesis gene cluster isolated from *S. peucetius* by Hutchinson et al. [32,39].

Activity of the actIII gene product

S. galilaeus 31671, a mutant derived from *S. galilaeus* 31133 produces 2-hydroxyaklavinone [30] (Fig. 2; compound B). Introduction of the *actIII* gene (which encodes polyketide reductase of *S. coelicolor* [14]) into *S. galilaeus* 31671 resulted in the production of aklavinone (Fig. 2, compound E), indicating that this gene encodes a function which results in deoxygenation of C-2 of 2-hydroxyaklavinone [3]. Similarly, when *S. galilaeus* 31671 was transformed with pANT12 (*actI*, *actIII*, *actVII*, *actIV*) it produced aloesaponarin II (Fig. 2, compound C), but when it was transformed with pANT35

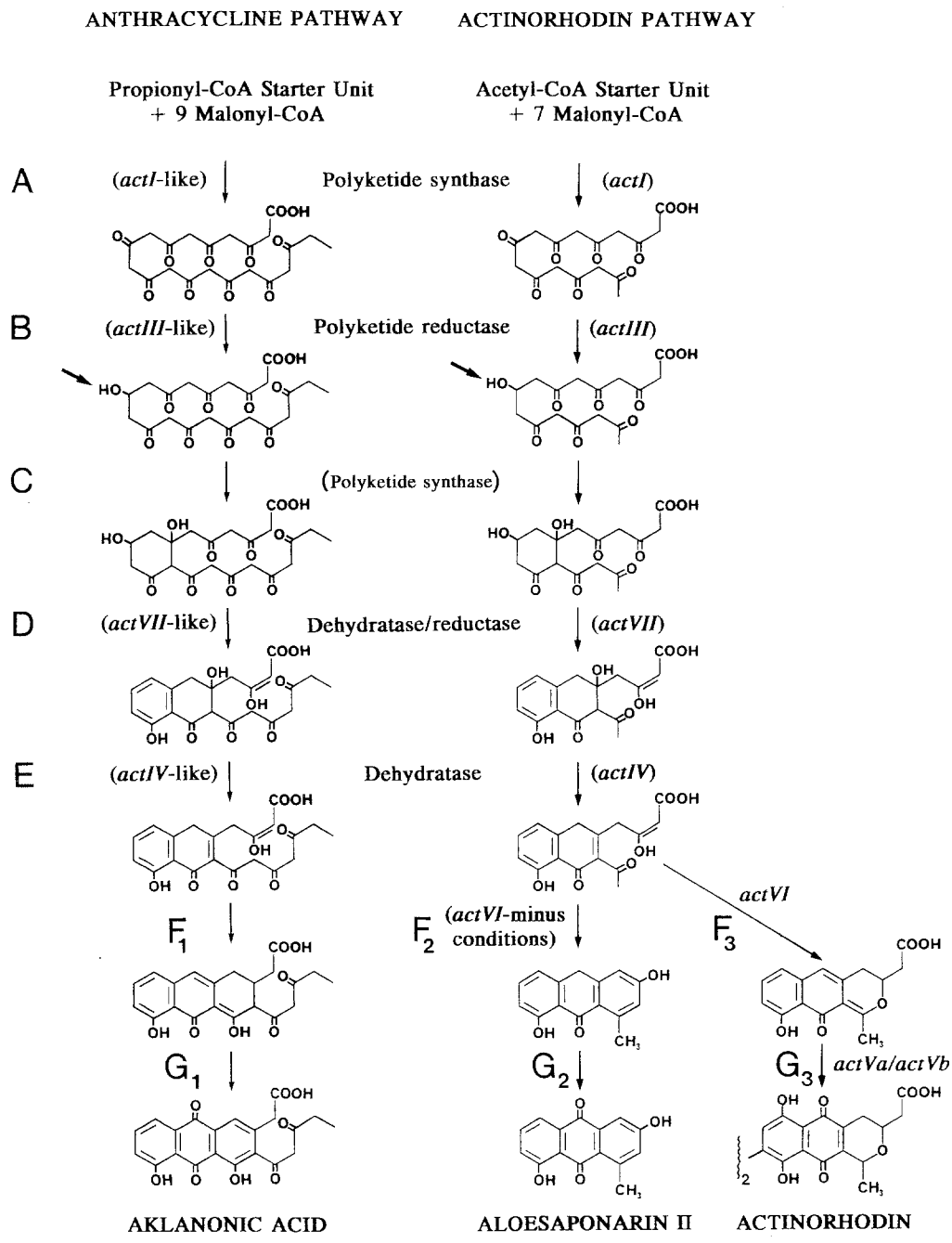

Fig. 4. Depiction of the hypothetical pathways from primary metabolite intermediates to aklanonic acid, a precursor in anthracycline biosynthesis in *S. galilaeus*, *S. peucetius*, and *Streptomyces* sp. C5 [8], and to actinorhodin and aloesaponarin II in *S. coelicolor* A3(2) and *S. galilaeus* (pANT12) [3], respectively. The similarities have been deduced based on the ability of *S. galilaeus* 31133 (pANT43) to produce aloesaponarin II. Plasmid pANT43 only carries the *actI* and *actVII* loci, indicating that *S. galilaeus* 31133 carries out the additional *actIII* and *actIV* functions also required for aloesaponarin II biosynthesis [3]. According to these hypothetical pathways, reactions B–E are carried out by essentially identical functions, whereas reaction A, generating the polyketide precursor, would be pathway-specific. Reactions F and G diverge, depending on the pathway, to produce different aromatic polyketide metabolites.

(*actI*, *actVII*, *actIV*), desoxyerythrolaccin (Fig. 2, compound D), containing an extra oxygen function, was formed. This also indicates that the *actIII* gene product is responsible for the formation of the deoxy-anthraquinone derivative.

On the other hand, when the wild-type aklavinone-producer, *S. galilaeus* 31133, was transformed with plasmids lacking the *actIII* gene, i.e., pANT35 (carrying the *actI*, *actVII*, and *actIV* loci) or pANT43 (*actI*, *actVII* loci), aloesaponarin II was the product formed [3]. This indicates that the *S. galilaeus* 31133 *actIII*-like gene (presumably encoding the polyketide reductase function for the aklavinone biosynthesis pathway) functions interchangeably with the *S. coelicolor actIII* gene [3].

Thus, the *actIII* gene product and its equivalent in *S. galilaeus* 31133 operate similarly in the biosynthesis of both aloesaponarin II and aklavinone (Fig. 5). Hallam et al. [14] proposed that the *actIII* gene encodes a reductase that reduces the β-keto group on the nascent polyketide chain once it has reached a chain length of ten carbons. Aloesaponarin II and aklavinone are produced from polyketide chains of different lengths, yet in both cases the keto group which is reduced to the corresponding secondary alcohol is at the ninth carbon from

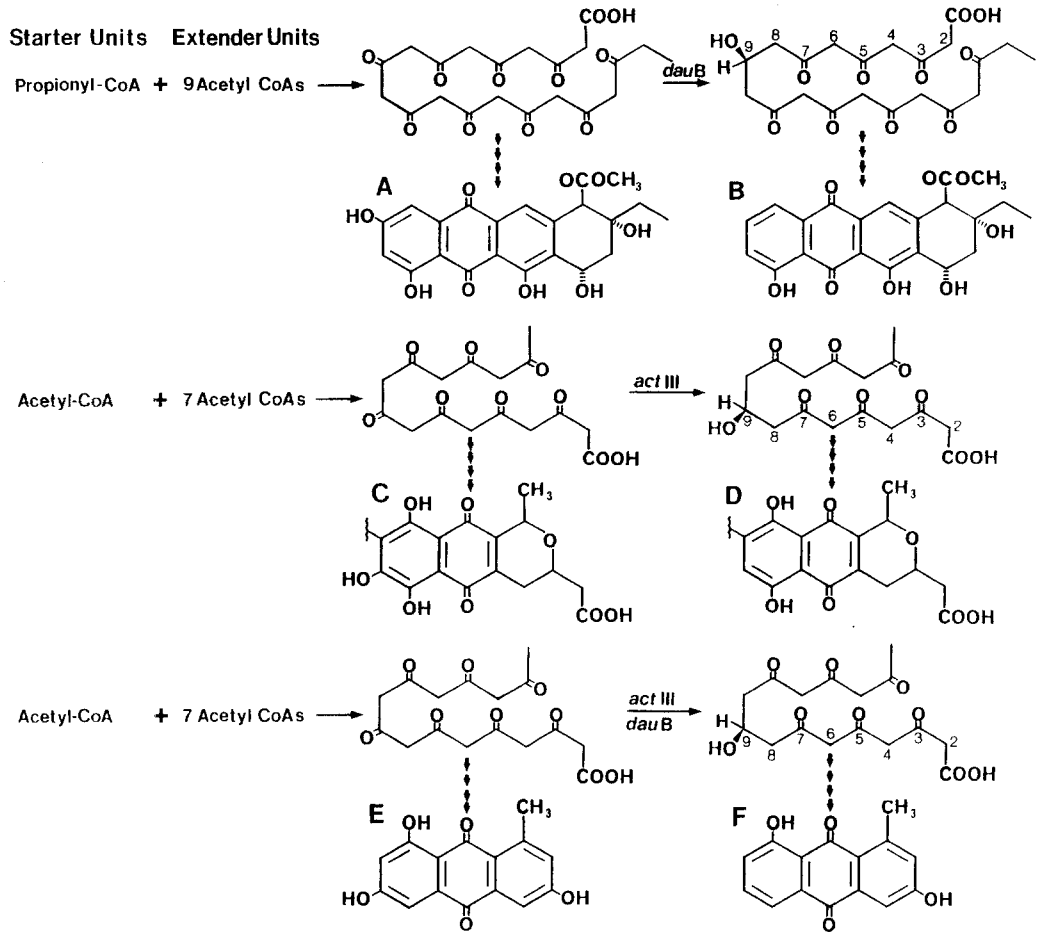

Fig. 5. Function of the *actIII* gene product, polyketide reductase, from *S. coelicolor* and its homolog (*actIII*-like gene product) from *S. galilaeus* 31133 and structures and theoretical polyketide precursors of polyketides formed as a result of cloning *act* genes in *S. galilaeus* strains 31133 and 31671. The proposed activity of the *actIII* and *actIII*-homologous gene products is shown in relation to the metabolites formed. If *actIII*, encoding polyketide reductase, is present, then the keto group at the ninth carbon from the carboxyl terminus of the polyketide chain in each case is reduced [3]. Upon ring closure and aromatization, a hydroxyl group is lost from that carbon and the resultant molecule is deoxygenated in that position. If a functional polyketide reductase is not present, then the resultant molecules contain hydroxyl groups in the respective positions [3]. Compounds: A, 2-hydroxyaklavinone; B, aklavinone; C, presumed hydroxy-actinorhodin monomer molecule (which has not been found); D, actinorhodin monomer; E, desoxyerythrolaccin; and F, aloesaponarin II.

the carboxyl terminus of the nascent polyketide chain (Fig. 5). Therefore, we postulated that both polyketide reductases operate by reducing the keto group which is located nine carbons from the carboxyl end of the assembled polyketide chain [3,38] (Fig. 5). Since the synthesis of a polyketide chain begins at the methyl terminus and proceeds to the carboxyl terminus [3,38], this hypothesis would suggest that the enzyme 'measures' the assembled polyketide and reduces the proper keto group prior to ring closure. Further investigations on the action of polyketide reductase on chains of various lengths should help to determine if this hypothesis is correct.

Formation of hybrid anthraquinones by S. galilaeus 31671

Since *S. galilaeus* ATCC 31671 produces 2-hydroxyaklavinone [3,30] (Fig. 2, compound B) and apparently lacks a functional polyketide reductase [3,38], we hypothesized that introduction of just the *actI*, *actVII*, and *actIV* loci into this strain would result in the formation of a hydroxy-derivative of aloesaponarin II. When transformed with pANT35, which carries the *actI*, *actVII* and *actIV* loci (Fig. 2), *S. galilaeus* 31671 produced two identifiable compounds, desoxyerythrolaccin (Fig. 2, compound D, R = H), which is 3-hydroxyaloesaponarin II, and 1-*O*-methyldesoxyerythrolaccin (Fig. 2, compound D, R = CH_3). The 1-*O*-methyldesoxyerythrolaccin was also contaminated with either a 3-*O*-methyl ether or a 7-*O*-methyl ether derivative [3]. These compounds were not found in extracts of any *S. coelicolor act* mutants [3]. When *S. galilaeus* 31671 was transformed with pANT43 (containing only the *actI* and *actVII* loci), desoxyerythrolaccin was the only major compound produced [3] (Fig. 2).

REGULATION OF HYBRID METABOLITE FORMATION

As mentioned above, pANT12 (*actI*, *actIII*, *actVII*, and *actIV* loci) conferred the ability to produce aloesaponarin II on all of the anthracycline-producing strains tested (Table 1). Additionally, several blocked mutants of *Streptomyces* C5 produced aloesaponarin II when transformed with pANT12, indicating that the nonfunctional steps in those mutant strains were not required

TABLE 1

Polyketide production by recombinant streptomycetes transformed with plasmids containing actinorhodin biosynthesis loci

Streptomyces strain	Class	Antibiotic normally produced	Plasmid[a]	Major polyketide produced
C5	parental type	daunomycin	pANT12	Aloesaponarin II
SC5-39	*dauC* mutant	aklanonic acid	pANT12	Aloesaponarin II
SC5-138	*dauD* mutant	AAME[b]	pANT12	Aloesaponarin II
SC5-24	*dauE* mutant	maggiemycin	pANT12	Aloesaponarin II
SC5-58	*dauE*/*dauF* mutant	aklaviketone	pANT12	Aloesaponarin II
SC5-38	*dauG* mutant	none	pANT12	none
SC5-71	*dauG* mutant	none	pANT12	none
SC5-72	*dauG* mutant	none	pANT12	none
SC5-96	*dauG* mutant	none	pANT12	none
S. galilaeus 31133	wild-type	aclacinomycin A	pANT12	Aloesaponarin II
S. galilaeus 31671	*dauB* (PKR^-)	2-hydroxyaklavinone	pANT33	Aloesaponarin II
S. galilaeus 31671	*dauB* (PKR^-)	2-hydroxyaklavinone	pANT35	DES; MDES
S. galilaeus 31671	*dauB* (PKR^-)	2-hydroxyaklavinone	pANT43	DES
S. galilaeus 31671	*dauB* (PKR^-)	2-hydroxyaklavinone	pANT45	Aklavinone
S. peucetius 29050	wild-type	daunomycin	pANT12	Aloesaponarin II
S. azureus 14921	wild-type	thiostrepton	pANT12	Aloesaponarin II
S. actuosus 25421	wild-type	nosiheptide	pANT12	none
S. parvulus 2266	wild-type	nonactin	pANT12	Aloesaponarin II

[a] Plasmid constructions (see also Fig. 2): pANT12: pIJ350 with 8.8 kb *Pst*I subclone from pIJ2303 that contains *actI*, *actIII*, *actVII*, and *actIV* loci; pANT33: pIJ941 with 4.7 kb *Pst*I-*Sst*I subclone from pANT12 that contains *actI*, *actIII*, and *actVII* loci; pANT35; pIJ702 with 5.3 kb *Sph*I subclone from pANT12 that contains *actI*, *actVII*, and *actIV* loci; pANT43: pIJ61 with a 2.8 kb *Xho*I fragment containing *actI* and *actVII* loci; pANT45: pIJ61 with a 1.1 kb *Bam*HI fragment from pANT12 containing only the *actIII* gene.

[b] Abbreviations: AAME, aklanonic acid methyl ester [8]; DES, desoxyerythrolaccin; MDES, 1-*O*-methyldesoxyerythrolaccin; PKR^-, polyketide reductase-minus.

for aloesaponarin II formation. A group of *Streptomyces* C5 mutants (*dauG*) has been isolated, however, that exhibit characteristics of anthracycline regulatory mutants, i.e., they do not produce detectable metabolites, they do not cross-feed to produce parental compounds with other blocked mutants, and they exhibit normal colony morphology and development [2]. When these *dauG* strains (SC5-38, SC5-71, SC5-72 and SC5-96) were transformed with pANT12, they did not produce aloesaponarin II or any other detectable anthraquinone (Table 1).

Hallam et al. [14] have demonstrated that transcription of the *actIII* gene is positively regulated by the *actII* gene product. When DNA from the anthracycline-producing strains was probed with pANT101 (6.0 kbp *Bam*HI fragment containing *actII*), hybridization was observed with *Streptomyces* sp. C5 and *S. peucetius* (data not shown). Moreover, Hutchinson et al. (personal communication) have shown that a regulatory gene, *dnrRI*, clustered with daunomycin biosynthesis genes in *S. peucetius*, has high homology with the *actII* gene from *S. coelicolor*. Thus, it is probable that the actinorhodin biosynthesis genes are expressed in the anthracycline-producing strains, at least in part due to activation by the apparently cross-functional *actII*-like homologs of the anthracycline-producing strains. This also suggested that the *dauG* mutants may be lacking the *actII*-homolog function. However, we have been unsuccessful in complementing the *dauG* mutants with either the *actII* gene from *S. coelicolor* or the *dnrRI* gene from *S. peucetius* (P.L. Bartel, Y. Li and W.R. Strohl, in preparation). This suggests that the *dauG* mutants lack a function that is required for *actII* (or *actII*-like) gene function in the regulatory paradigm. This may be a similar mutation to the *abs* mutations of *S. coelicolor* observed by Champness (cited by Chater [5]), or alternatively, it may be similar to the *afsB* mutation [18].

In related experiments, we also noted that the thiostrepton producer, *S. azureus* ATCC 14921, produced aloesaponarin II when transformed with plasmids containing the *actI*, *actIII*, *actVII* and *actIV* loci, whereas the nosiheptide-producing strain, *S. actuosus* ATCC 25421, did not (Table 1). Further experiments have shown that *S. azureus* contains DNA that hybridizes with probes representing the *actI* and *actII* genes, indicating that a silent pathway exists that probably allows expression of the actinorhodin genes in the strain. DNA isolated from *S. actuosus*, on the other hand, did not hybridize with either the *actI* or the *actII* probes, which is consistent with the lack of apparent expression of the *act* genes in this strain (data not shown).

REGULATORY GENES ENHANCING ACTINORHODIN PRODUCTION IN *S. lividans*

Regulatory genetic loci whose activity is required for the expression of various antibiotic biosynthesis genes fall into two general classes: those loci which are clustered with the antibiotic genes that they regulate (e.g., *actII* [14,26], *milbII* [20], *redD* [29], *mmy* [6], *brpA* [1], *strR* [9], and *dnrR1/dnrR2* [C.R. Hutchinson, personal communication]), and those loci which apparently may or may not be pleiotropic and are not necessarily clustered with antibiotic biosynthesis genes (e.g., *afsA* [19], *afsB* [18], *afsR* [37], *whiG* [7], *bldA* [22]). In two cases the structures of the regulatory loci have indicated the functions that those genes encode. The *whiG* gene [7], required for sporulation of *S. coelicolor* but not for antibiotic biosynthesis, encodes a sigma factor, σ^{whi} (M_r 31000), that is very similar in structure to the *Bacillus subtilis* motility sigma factor (σ^{28}; also known as σ^{D}). The *bldA* gene encodes leucyl-$tRNA_{UUA}$, a tRNA species that recognizes a very rare codon in streptomycete genes [22].

Data generated thus far indicate that most antibiotic biosynthesis genes are regulated by pathway-specific genes via activation mechanisms [5], as exemplified by the *actII* [14] and *redD* [29] genes. These pathway-specific regulatory genes may themselves also be regulated by activation mechanisms. An example of this is the overproduction of actinorhodin by *S. lividans* transformed with the pleiotropic gene, *afsB* [16]. It also has been shown that an *afsB*-minus mutant of *S. coelicolor* lacked transcripts for the *act* genes which were restorable by complementation with *afsB*, and extra copies of *afsB* stimulated the transcription of the *act* genes in *S. coelicolor* and *S. lividans* [17].

In collaboration with C.R. Hutchinson and his colleagues, we subcloned a 3.0-kbp *Bam*HI DNA fragment from pMHW333 (cluster IV daunomycin biosynthesis genes of *S. peucetius*) in pIJ702 and introduced this fragment into *S. lividans*. This DNA fragment, now known to contain the regulatory gene, *dnrRI*, caused *S. lividans* to overproduce actinorhodin (Bartel, unpublished data). Hutchinson et al. (personal communication) have found that *dnrRI* has high DNA homology with *actII* and *redD*, indicating that there appears to be a distinct class of activator genes that are clustered with the antibiotic biosynthesis genes which they activate.

We further used the concept of stimulation of actinorhodin production by *S. lividans* in attempts to isolate similar activator genes which might be used to overproduce the hybrid anthraquinone metabolites described earlier in this article. Recently we isolated a 984 bp *Sst*I DNA fragment from an, as yet, unidentified strain that caused the remarkable overproduction of actinorhodin in

S. lividans (Fig. 6). Moreover, the presence of this DNA fragment caused *S. lividans* to oversporulate within 3 days of inoculation. This DNA fragment contained one open reading frame of 468 bp which was followed by a small inverted repeat with a ΔG° of formation of -41 kcal/mol. The deduced gene product shows no apparent identity with the gene products of *actII*, *dnrRI*, *redD*, *afsA*, *afsB*, *whiG*, or *nshA* (Strohl, unpublished data). We are calling this gene *asaA* (activation of sporulation and actinorhodin). We used the 984-bp *Sst*I fragment to probe DNA isolated from other streptomycetes in efforts to discern its ubiquity. We found that *S. coelicolor* and *S. peucetius* have DNA fragments which strongly hybridized with *asaA* (Table 2), indicating that it may be a heretofore unknown regulatory gene common in several streptomycetes. Preliminary dot-blot experiments also showed that *asaA* at high copy number in *S. lividans* dramatically increased the transcriptional level of the *actII* (actinorhodin activator) gene (data not shown), suggesting that *asaA* may be an important activator of the pathway-specific antibiotic biosynthesis activator genes.

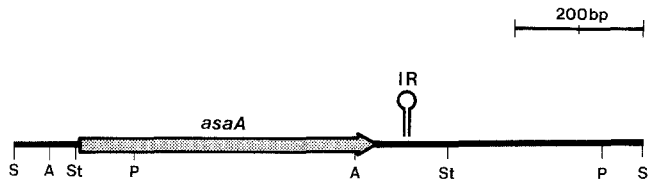

Fig. 6. Restriction map of the 984-bp fragment isolated from an as yet unidentified streptomycete that contains the *asaA* gene which stimulates sporulation and actinorhodin production in *S. lividans*. The open reading frame of 468 nt is followed by an inverted repeat which can form a stem–loop structure with a ΔG of formation of -41 kcal/mol. Abbreviations for restriction endonuclease sites: S, *Sst*I; A, *Aat*II; St, *Sty*I, P, *Pvu*II.

EPILOGUE

Our experiments [3,38], and those of other investigators [10,15,27,31] have clearly shown that hybrid metabolites can be formed by interspecies cloning in streptomycetes. As more is learned about the chemistry of antibiotic biosynthesis pathway, the substrate specificity of enzymes catalysing reactions in those pathways, and the genes which encode the enzymes and regulatory circuits, more examples of hybrid metabolite formation are certain to occur. The most promising alterations which may be relatively easy to effect on secondary metabolites are those which make relatively simple modification on the previously produced nascent polyketide chains, including oxidation, reduction, methylation, and glycosylation reactions. On the other hand, as we learn more about polyketide synthases and similar antibiotic synthesizing enzyme systems, it may eventually be possible to produce hybrid metabolites by altering the way in which the initial precursors are polymerized to form the nascent molecules.

Beyond the scope of enzyme substrate specificity, the major perceived limitation to producing hybrid metabolites may be in expressing certain heterologous genes in strains not containing the proper activator genes. At this

TABLE 2

Presence of DNA fragments in *Streptomyces* spp. that hybridize with *asaA* gene

Source of DNA	Strength of hybridization	Size of DNA fragment(s) (kbp) hybridizing with *asaA* probe[a]	
		*Sst*I-digested	*Bam*HI-digested
S. insignis	strong	1.0	4.5
S. coelicolor	strong	1.2	6.1
S. peucetius	strong	5.2	ca. 10
Streptomyces C5	weak	7.6, 9.5	9.5
S. griseus	weak	n.d.	ca. 12
S. steffisburgensis	weak	1.2	n.d.
S. punecius	weak	n.d.	3.5, ca. 11
S. californicus	none	n.a.	n.a.
Escherichia coli	none	n.a.	n.a.
Pseudomonas aeruginosa	none	n.a.	n.a.

[a] Abbreviations: n.d., not done; n.a., not applicable.

time, however, this problem has not surfaced as a major obstacle, mostly because the activator genes (e.g., *actII*, *dnrRI* and its apparent homologs) appear to have high degrees of similarity and therefore appear to function broadly in the streptomycetes. Moreover, if this problem does occur, it is expected that expression vectors will be available to overcome problems of transcriptional activation.

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