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A Study of Iterative Type II Polyketide Synthases, Using Bacterial Genes Cloned from Soil DNA: a Means To Access and Use Genes from Uncultured Microorganisms

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To examine as randomly as possible the role of the β-ketoacyl and acyl carrier protein (ACP) components of bacterial type II polyketide synthases (PKSs), homologs of the chain-length-factor (CLF) genes were cloned from the environmental community of microorganisms. With PCR primers derived from conserved regions of known ketosynthase (KS_α) and ACP genes specifying the formation of 16- to 24-carbon polyketides, two CLF (KS_β) genes were cloned from unclassified streptomycetes isolated from the soil, and two were cloned from soil DNA without the prior isolation of the parent microorganism. The sequence and deduced product of each gene were distinct from those of known KS_β genes and, by phylogenetic analysis, belonged to antibiotic-producing PKS gene clusters. Hybrid PKS gene cassettes were constructed with each novel KS_β gene substituted for the *actI-ORF2* or *tcmL* KS_β subunit genes, along with the respective *actI-ORF1* or *tcmK* KS_α, *tcmM* ACP, and *tcmN* cyclase genes, and were found to produce an octaketide or decaketide product characteristic of the ones known to be made by the heterologous KS_α gene partner. Since substantially less than 1% of the microorganisms present in soil are thought to be cultivatable by standard methods, this work demonstrates a potential way to gain access to a more extensive range of microbial molecular diversity and to biosynthetic pathways whose products can be tested for biological applications.

The polyketide metabolites are a large group of structurally complex and diverse natural products, many of which are clinically valuable antibiotics or chemotherapeutic agents or have other useful pharmacological activities (48). Polyketides are synthesized by polyketide synthases (PKSs) which, like the related fatty acid synthases, are multifunctional enzyme assemblies that catalyze repeated decarboxylative condensations between enzyme-bound acylthioesters (52). The structural diversity of polyketides is a result of the different numbers and types of acyl units involved, coupled with various possibilities for enzyme-mediated reduction, dehydration, cyclization, and aromatization of the initial β-ketoacyl condensation products (52). Manipulation of the sequence or specificity of the PKS enzymes can lead to the production of novel secondary metabolites (28, 29, 33). The key to further exploitation of this approach to molecular diversity lies in an increased understanding of the genetics and biochemistry of the various PKS systems.

Recent progress in the cloning and sequencing of microbial PKS genes has led to the identification of at least two architecturally different types of PKSs (28, 33). Modular type I PKSs, represented by erythromycin A (11, 17), avermectin (41), rapamycin (58), and soraphen (57), are discrete multifunctional enzymes sporting numerous unique active site domains. The domains are grouped into distinct modules that in turn control the sequential addition of acylthioester units to the growing polyketide chain and the subsequent modification of the respective condensation products. In contrast, the type II PKSs, such as those responsible for the biosynthesis of the aromatic polyketides actinorhodin (ACT) (19) and tetracenomycin (TCM) (5, 67, 68), are composed of three to seven separate mono- or bifunctional proteins, the active sites of which are used iteratively for the assembly and early modification of the polyketide chain. Interestingly, fungal PKSs, although architecturally similar to modular PKSs, also appear to use their active sites repeatedly, much like the fatty acid synthases of higher eukaryotes, and can produce aromatic compounds (3, 9).

All of the bacterial type II PKSs contain a set of three genes that encode the so-called minimal PKS. It includes two β-ketoacyl synthase subunits; KS_α, which catalyzes the condensation between the acylthioester species and chain length factor (42) (we and others [37, 54] prefer the KS_β designation for this gene, albeit without any evidence that the two KS proteins form a heterodimer); and an acyl carrier protein (ACP) that acts as an anchor for the polyketide chain during the various biochemical manipulations. Although the minimal PKS alone is sufficient to produce the basic carbon skeleton of the polyketide chain (21, 43, 44), additional enzymes, such as cyclases (60, 61) (or their equivalent, aromatases [43]) and ketoreductases, are required to fold and cyclize the poly-β-ketoacyl chain into a (poly)cyclic structure. The challenge in generating novel polyketides is to create a variety of combinations of a minimal PKS, a ketoreductase, and an aromatase or a cyclase to assemble and process nascent polyketides of various chain lengths into a range of novel metabolites.

McDaniel et al. (42, 45) have created such a combinatorial library of aromatic polyketides and proposed a set of tentative rules for polyketide design. These rules are based on studies of

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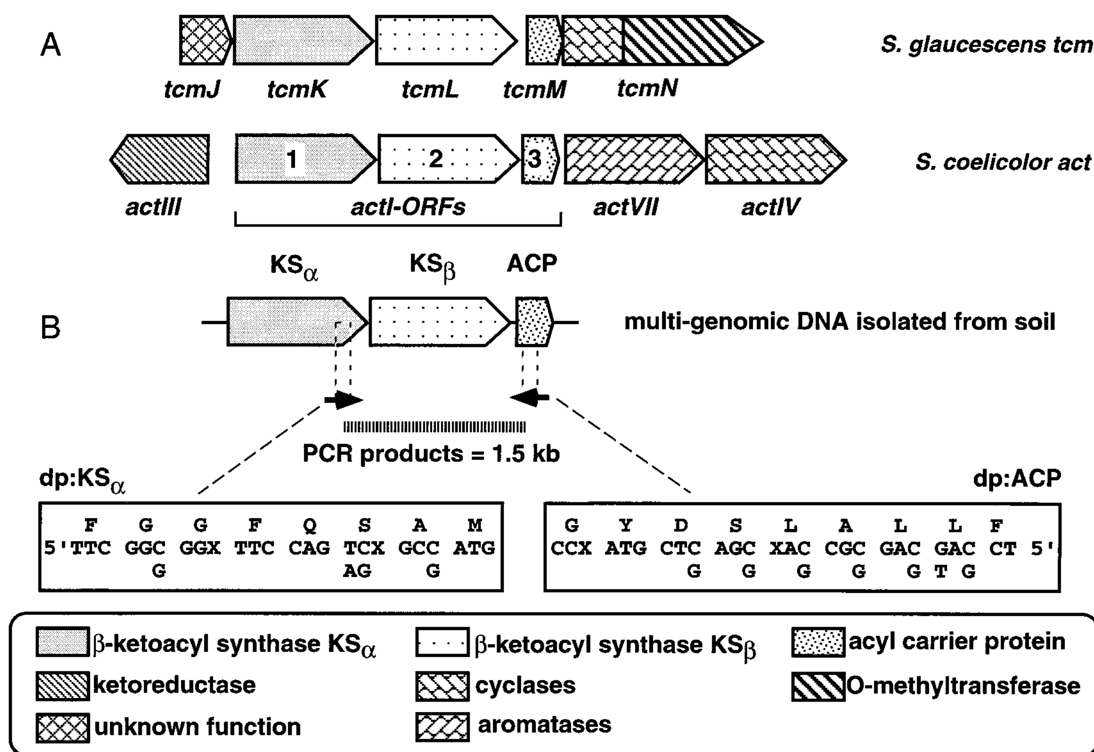

FIG. 1. (A) Conservation of the minimal PKS gene organization exemplified by portions of the *tcm* and *act* PKS gene clusters. (B) General strategy used to obtain novel full-length KS_β genes from soil and uncharacterized *Streptomyces* strains. The sequence of the degenerate PCR primers, dp:KS_α and dp:ACP, are shown in boxes; X, deoxyinosine; the lower line indicates the nucleotide mixture used at degenerate positions of certain codons.

a few PKSs that produce chain having between 16 and 24 carbons and that originate from well-defined gene clusters for the biosynthesis of ACT, TCM C, griseusin (73), frenolicin (6), and an uncharacterized spore pigment (13), although aromatic polyketide metabolites with as few as 8 carbons (4, 22) and up to 30 carbons (10) have been isolated from bacteria.

Recently, McDaniel et al. (42) and Shen et al. (60) showed that genes encoding the KS_α subunit from the TCM pathway, which makes a 20-carbon decaketide, can be functional when combined with the KS_β gene from the ACT pathway, which produces a 16-carbon octaketide. Prior to this finding, the only heterologous $KS_{\alpha\beta}$ pair reported to work was an in *trans act* KS_α::*gra* KS_β combination (63), in which both genes came from PKS pathways that make very similar octaketides. According to Shen et al. (60), when the *tcmL* gene was replaced with the *actI-ORF2* KS_β gene in a PKS gene expression system consisting of the *tcmJKLMN* genes (Fig. 1A), trace amounts of a 16-carbon polyketide, UWM1, were observed in addition to the main product, TCM F2, in *Streptomyces lividans* 1326 (26) or *Streptomyces glaucescens* WMH1077 (14, 50). In contrast, McDaniel et al. (42) had reported earlier that the same $KS_{\alpha\beta}$ combination expressed in *Streptomyces coelicolor* CH999 together with the ActIII ketoreductase, the ActVII aromatase, and the ActIV second-ring cyclase (Fig. 1A) produced the 16-carbon compounds aloesaponarin II and DMAC. UWM1 and the 20-carbon polyketide RM20 were the respective major products when the *tcmK–tcmL* gene pair was replaced by the *actI-ORF1* and *actI-ORF2* genes in the *tcm* expression system (60) or when the *actI-ORF1–actI-ORF2* gene pair was replaced by the *tcmK* and *tcmL* genes in *act* expression cassettes (42). (RM77, an oxidized form of UWM1, was produced in *S. coelicolor* CH999 by the *tcmJ*, *tcmK*, *actI-ORF*, *actI-ORF2*, *tcmM*, and *tcmN* genes [46].) Replacing the *tcmK* KS_α gene with the *actI-ORF1* gene resulted in the production of an inactive hybrid PKS. These results suggested that the interaction between KS_α and KS_β subunits determines the chain length and that only homologous $KS_{\alpha\beta}$ gene pairs define a specific chain length independently from the genetic environment.

Since the lack of the KS_α or KS_β (or ACP) gene in general results in an inactive minimal PKS, the function of neither KS gene can be studied alone. Therefore, we decided to expand our investigation of how the size of the poly-β-ketone product of the minimal PKS is determined by testing heterologous $KS_{\alpha\beta}$ pairs involving four different KS_β genes of unknown origin, obtained from soil or uncharacterized microbes by a PCR with primers corresponding to conserved regions in the KS_α and ACP genes that normally flank the KS_β genes. Using the *tcmJ*-$KS_{\alpha\beta}$-*tcmM* and *tcmN* PKS expression system described previously (60), we demonstrate that this approach produces functional minimal PKSs whose products largely are characteristic of the KS_α partner. Furthermore, our approach also illustrates one way to access and use the immense pool of biosynthetic genes from unculturable microorganisms in the soil, which has significant implications for microbial biotechnology.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. glaucescens* WMH1077^T, *S. lividans* 1326, and the plasmids pWHM3 (70), pWHM860 (47), pWHM752 (60), and pWHM769 (60) are described elsewhere. The *ermE$_p$** promoter was obtained as described earlier (47, 60). The TA PCR cloning vector was purchased from Invitrogen (San Diego, Calif.). The *Escherichia coli* strains used were DH5α and −DH10. The plasmids used in this study are described in Table 1.

Culture conditions, chemicals, and other materials. R2YE (27) and R2YENG (51) liquid media (pH 6.0) and agar plates were used for the production of

TABLE 1. Plasmids used for polyketide biosynthesis

Plasmid	KS_α	KS_β	ACP	Cyclase	Compound(s)		Source or reference
					Expected	Observed	
pWEC860							This work
pWEC752	*tcm*	*act*	*tcm*	*tcm*	TCM F2	TCM F2, UWM1	This work
pWEC769	*act*	*act*	*tcm*	*tcm*	UWM1	UWM1, SEK4, SEK4b	This work
pWHM765	*act*	*tcm*	*tcm*	*tcm*			60
pWEC753	*tcm*	U1	*tcm*	*tcm*	TCM F2	TCM F2, UWM1	This work
pWEC754	*tcm*	U2	*tcm*	*tcm*	TCM F2	UWM1	This work
pWEC755	*tcm*	S1	*tcm*	*tcm*	TCM F2	TCM F2, UWM1	This work
pWEC756	*tcm*	S2	*tcm*	*tcm*	TCM F2	TCM F2, UWM1	This work
pWEC770	*act*	U1	*tcm*	*tcm*	UWM1	UWM1, SEK4, SEK4b	This work
pWEC771	*act*	U2	*tcm*	*tcm*	UWM1	UWM1, SEK4, SEK4b	This work
pWEC772	*act*	S1	*tcm*	*tcm*	UWM1	UWM1, SEK4, SEK4b	This work
pWEC773	*act*	S2	*tcm*	*tcm*	UWM1	UWM1, SEK4, SEK4b	This work

secondary metabolites. *S. glaucescens* spores were isolated after growth on HT medium (Difco, Detroit, Mich.) (50), and *S. lividans* spores were isolated after growth on R2YE agar. Both strains were grown on CRM medium (8) for the preparation of protoplasts, and the protoplasts were regenerated on R2YE agar. Thiostrepton concentrations of 50 μg/ml on solid media and 10 μg/ml in liquid media were used for selective growth of all *Streptomyces* spp. Recombinant *E. coli* DH5α and −DH10 were grown in LB or TB medium (56) containing 200 μg of ampicillin per ml. Thiostrepton was obtained from Sal Lucania at the Squibb Institute for Medical Research (Princeton, N.J.), and all chemicals used in this study were analytical grade. Silica gel (Kieselgel 60; 0.040 to 0.063 μM) and thin-layer chromatography (TLC) plates (precoated silica gel 60 F_{254} glass plate [0.25 mm]) were obtained from E. Merck AG (Darmstadt, Germany). Restriction enzymes, DNA ligase, and other molecular biology materials were purchased from standard commercial sources.

PCR conditions for amplification of DNA isolated from unknown sources. The PCR conditions for the initial amplification of KS_β genes from different sources were as follows. A total reaction volume of 50 μl contained 50 ng of DNA, 0.5 μg of each PCR primer, 5% dimethyl sulfoxide, and a final concentration of 1.25 mM $MgCl_2$. *Taq* polymerase and reaction buffer were from Life Technologies (Gaithersburg, Md.). The cycle conditions started with a "touch down" sequence, which lowered the annealing temperature from 65 to 58°C over the course of 8 cycles, and were then maintained at 58°C for a total of 35 cycles. Cycle steps were as follows: denaturation (94°C; 45 s), annealing (65°C→58°C; 1 min), and extension (72°C; 2 min).

Plasmid construction and tailoring of PCR products. pWHM860 was modified by cutting it with *Hin*dIII, creating blunt ends, and religating the treated plasmid to yield pWEC860. The expression cassettes from pWHM752 and pWHM769 were obtained by digesting the plasmids with *Eco*RI and *Nsi*I. Similarly, pWEC860 was cut with *Eco*RI and *Pst*I, and the predigested DNA fragments were ligated to yield plasmids pWEC752 and pWEC769. These were then digested with *Hin*dIII and partially with *Bsp*HI to replace the *actI-ORF2* gene with the identically digested U1-, U2-, S1-, and S2-KS_β genes, obtained after a second round of PCR with specific primers and standard PCR conditions to introduce the *Bsp*HI and *Hin*dIII sites; these steps yielded plasmids pWEC753 to pWEC756 and pWEC770 to pWEC773 (U1-KS_β: forward primer [fp], 5′-GCC GGA ATT CAT GAT CCC GGT CGC GGT CA-3′, and reverse primer [rp], 5′-GCC AAT GCA TAA GCT TCA CCG CCC GGC ACG CAC CGC-3′; U2-KS_β: fp, 5′-GCC GGA ATT CAT GAG CGC GAG GTT CCT GG-3′, and rp, 5′-GCC AAT GCA TAA GCT TCA GTC GAC GGC GCG CAC CAC-3′; S1-KS_β: fp, 5′-GCC GGA ATT CAT GAG CAT CAG GAC GGT GG-3′, and rp, 5′-GCC AAT GCA TAA GCT TCA GTC GCG TGG TGC ACG CAC-3′; S2-KS_β: fp, 5′-GCC GGA ATT CAT GAC GAG CGA GCT GCT CG-3′, and rp, 5′-GCC AAT GCA TAA GCT TCA GTT CGC CGC GGT CAC GAC-3′ [underlined sequences in fp are the introduced *Eco*RI and *Bsp*HI restriction sites; underlined sequences in rp are the introduced *Nsi*I and *Hin*dIII restriction sites).

Chromatography of culture extracts. For TLC analysis, 1 to 5 ml of a recombinant strain culture was mixed with 0.1 volume of a 1 M KH_2PO_4 solution and extracted with 0.25 volume of ethyl acetate. A 60- to 80-μl aliquot of the extract was applied to a TLC plate, developed in $CHCl_3$-CH_3OH-acetic acid (80:20:2.5, vol/vol/vol), and visualized by long- and shortwave (366 and 254 nm, respectively) UV light. High-pressure liquid chromatography (HPLC) analysis was performed with 25-ml aliquots of recombinant strain cultures (4 days) mixed with 2.5 ml of a 1 M KH_2PO_4 solution and extracted with 5 ml of ethyl acetate. A 1-ml aliquot of the extract was concentrated in vacuo to dryness and redissolved in 100 μl of ethyl acetate, and 20 μl thereof was analyzed on a Waters Nova-Pak C_{18} column (3.9 by 150 mm). The column was developed with a linear gradient of CH_3CN-H_2O-H_3PO_4 from 0:100:0.1 to 30:70:0.1 over 10 min at a flow rate of 2 ml/min. The chromatogram was recorded at 254 nm with a Waters 960 photodiode array detector and Millenium 2010 chromatography manager software, version 2.15 (Waters Corp., Milford, Mass.).

Purification, modification, and NMR spectroscopy of secondary metabolites. One liter of culture broth was mixed with 14 g of KH_2PO_4, and the mycelia were subsequently removed by filtration. The filtrate was divided into two portions, and each was extracted five times with 500 ml of ethyl acetate. Similarly, the mycelia were extracted five times with 50 ml of acetone. The combined organic layers were evaporated to dryness, redissolved in 5 ml of a mixture of $CHCl_3$-CH_3OH (85:15, vol/vol), and subjected to chromatography on a silica gel column (35 by 3 cm) with the same solvent mixture as that used for dissolution. The collected fractions were analyzed by TLC, and positive fractions were evaporated to dryness, redissolved in methanol, and further purified on Sephadex LH20-CH_3OH to yield 9.7 mg of SEK4 and SEK4b (ratio, 9:5) and 5.0 mg of UWM1. To prove the identities of UWM1, SEK4, and SEK4b by comparison, 5.0 mg of UWM1 was dissolved in 5 ml of methanol, acidified with 1 ml of 1 N HCl, and kept overnight. After evaporation of the solvent and purification on Sephadex LH20, 4.3 mg (80%) of UWM2 could be obtained. The resulting nuclear mag-

TABLE 2. Comparison of the four KS_β genes to their homologs

Name	Gene		Gene product (amino acids)	% Similarity[a] to:			
	bp	% GC content		*act*-KS_α	*tcm*-KS_α	*act*-KS_β	*tcm*-KS_β
actI-ORF2	1,227	74.1	408	28.7	30.6	100	58.6
tcmL	1,233	75.0	410	29.4	32.2	58.6	100
U1-KS_β	1,212	73.8	403	30.1	32.8	61.8	61.9
U2-KS_β	1,224	72.9	407	30.3	31.8	56.7	55.1
S1-KS_β	1,221	72.7	406	30.8	32.5	59.5	60.8
S2-KS_β	1,238	75.1	415	30.3	34.0	58.6	58.6

[a] Percent similarity was calculated as the number of matching residues (consensus length) divided by the sum of mismatching residues, matching residues (consensus length), and gapped residues (gap length) × 100.

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         1                                                                                                     100
S1.pep}  .......... .MSirtVVTG lGIAAPNGLG ieEYWSATLA GrgaIGPlTR FDASsYPSRL AGEIrGFtAA EHLPGRLLPQ TDRMTqLALV sAgWALdDAG
U1.pep}  .......... ..mipVaVTG mGVAAPNGLG aaDYWaATrg GKSGIGrITR FDpSsYPaRL AGEIPGFEAA EHLPGRLLPQ TDRvTrLsLa AADWALADAG
U2.pep}  .......... .MSArflVTG iGVAAPsGLG veDFWSvTri GKnaIGPVTR FDASaYPSRL AGEIhGFEpk EHLPGRLvPQ TDRvTqLALV AADcAFADAG
S2.pep}  mtsellerta vrSAtaVfTG iGVtAPNGLG taaWWqATvA GeSGIrPVsR FDASgYPStL AGEVPGFDAe EHiPsRLLsQ TDhMTrLALt AAkeALeDsG
AcOrf2}  .......... ...msVlITG vGVvAPNGLG lapYWSAvLd GrhGlGPVTR FDvSrYPatL AGqIddFhAp DHiPGRLLPQ TDpsTrLALt AADWALqDAk
TcmL}    .........m sapApVVVTG lGIvAPNGtG teEYWaATLA GKSGIdvIqR FDphgYPvRv gGEVlaFDAA aHLPGRLLPQ TDRMTqhALV AAEWALADAG
Consen   ---------- -MSA-VVVTG -GVAAPNGLG ---YWSATLA GKSGIGPVTR FDAS-YPSRL AGEIPGF-AA EHLPGRLLPQ TDRMT-LALV AADWALADAG
         101                                                                                                   200
S1.pep}  VvPDeLpaYD MGVITAshAG GFEFGQnELk aLWSKGgKYV SAYQSFAWFY AVNsGQISIR nGMRGPSGVv VSDQAGGLDA LAQARRQiRK GT.PLIVsGa
U1.pep}  VEPEsFDplD MGVVTAghAG GFEFGQgELQ KLWaKGsqFV SAYQSFAWFY AVNsGQISIR HGMkGPnGVv VSDQAGGLDA LAQARRlvRK GT.PLIVcGa
U2.pep}  IEPgtiDpYa MGVVTAAgAG GFEFaenELr KLWSeGaKrV SAYQSFAWFY AVNsGQISIR nGLRGPaGVv ISDQAGGLDA LAQARRQlRK Gs.kLIaTGG
S2.pep}  aDPaeMpqYs aGaVTAAsAG GFEFGQRELQ aLWSKGgqYV SAYQSYAWFY AVNtGQISIR HGLRGPSGVl VtEQAGGLEA vAQARRQlRK Gs.kLIVTGG
AcOrf2}  aDPEsLtdYD MGVVTAnacG GFDFthREFr KLWSeGpKsV SvYeSFAWFY AVNtGQISIR HGMRGPSsal VaEQAGGLDA LghARRtiRr GT.PLVVsGG
TcmL}    lEPEkqDeYg LGVlTAAgAG GFEFGQREMQ KLWgtGperV SAYQSFAWFY AVNtGQISIR HGMRGhSsVf VtEQAGGLDA aAhAaRllRK GTlntalTGG
Consen   VEPE-LD-YD MGVVTAA-AG GFEFGQRELQ KLWSKG-KYV SAYQSFAWFY AVN-GQISIR HGMRGPSGV- VS-QAGGLDA LAQARRQ-RK GT-PLIVTGG
         201                                                                                                   300
S1.pep}  VDASlCtWGW VAQ..LAGGR LSrsdDPghA YvPFDDaAvG hVPGEGGAlL ILEEAEhARs RGARrIYGEi tGhAsTFDPp PwSGRgPavq RvIeeALaDA
U1.pep}  VDASiCPWGW VAQ..LAGGR MSDsdEPaRA YLPFDrdArG YlPGEGGAiL IMEpAaAARa RGA.kVYGEi sGYgaTFDPp PGSGsgstLR tAIrvALdDA
U2.pep}  fDApiCslGW asQprtgGlm FhErTEPeRA YLPFEDaAAG YVPGEGGAmL ILEDeDsARD RGARTVYGEf AGYgaTLDPk PGSGRePGLR RAIdvALtDA
S2.pep}  VDgavCPWGW tAQ..LAGGR MSpvaDPaRA FLPFDseAsG YVaGEGGAiL VLEDAEAARE RGAR.IYGrl sGYAaTFDPa PGrGgePGLR RAaeLALtEA
AcOrf2}  VDsaldPWGW VsQ..iAsGR iStaTDPdRA YLPFDErAAG YVPGEGGAiL VLEDsaAAea RGrhdaYGEl AGCAsTFDPa PGSGRpaGLe RAIrLALnDA
TcmL}    cEASlCPWGl VAQ..ipsGf LSEaTDPhdA YLPFDarAAG YVPGEGGAmL VaErADsARE RdAaTVYGri AGhAsTFDar PGtGRptGpa RAIrLALeEA
Consen   VDAS-CPWGW VAQ--LAGGR -SE-TDP-RA YLPFDD-AAG YVPGEGGA-L -LEDA-AARE RGARTVYGE- AGYA-TFDP- PGSGR-PGLR RAI-LAL-DA
          301                                                                                                  400
S1.pep}  GtvPDEVDVV FADaAalPEL DRiEAaAITk VFGPhaVPVT aPKTMTGRLY SGaAPLDVAa AcLaiRDGlI PPTIhs.SlS grYeiDLVtg aPRTAPVRTA
U1.pep}  GvAPgDVDaV FADgAGVPEL DRAEAEAITd VFGsggVPVT vPKTMTGRLY SGaAPLDVAc ALLamqaGVI PPTVhI.dPc PeYGlDLVLh qaRpAtVRTA
U2.pep}  achPaEVEVV FADgAatPrL DReEAEAITa VFGPraVPVT vPKTMTGRin SGGAPiDVvs AvLSmREGlI PPTtnV.elS daYdlDLVav rPRTAsVRTA
S2.pep}  GlsasDVDVV FADasGVPEL DRqEeaAlTa lFGPrgVPVT aPKTMTGRLs aGGAsLDlAa ALLSiRDaVI PPTVnVTSPv aadalDLVt. eaRrgPVRTA
AcOrf2}  GtgPEDVDVV FADgAGVPEL DaAEArAIgr VFGregVPVT vPKTtTGRLY SGGgPLDVvt ALMSlREGVI aPTagVTSvp reYGiDLVLg ePRstapRTA
TcmL}    rvAPEDVDVV YADaAGVPaL DRAEAEAlae VFGPgaVPVT aPKTMTGRLY aGGAaLDVAt ALLSiRDcVV PPTVgtgaPa PglGiDLVLh qPRelrVdTA
Consen   G-APEDVDVV FAD-AGVPEL DRAEAEAIT- VFGP--VPVT -PKTMTGRLY SGGAPLDVA- ALLS-RDGVI PPTV-VTSPS P-YG-DLVL- -PRTAPVRTA
          401                    424
S1.pep}  LVvARGhGGF NSAvVVRApR d*..
U1.pep}  LVLARGhGGF NSAmaVRAgR *...
U2.pep}  LVLARGrGGF NSAvVVRAvd *...
S2.pep}  LVLARGtGGF NaAaVVtAan *...
AcOrf2}  LVLARGrwGF NSAaVlRrfa ptp*
TcmL}    LVvARGmGGF NSAlVVRrhg *...
Consen   LVLARG-GGF NSA-VVRA-R ----
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FIG. 2. Comparison of the amino acid sequences of the deduced products of the KS_{β} genes. The six proteins were compared by the PILEUP method with default values (16). AcOrf2, ActI-Orf2; Consen, consensus sequence, in which the capital letters indicate that at least two residues are identical in a vertical column; *, translational stop site.

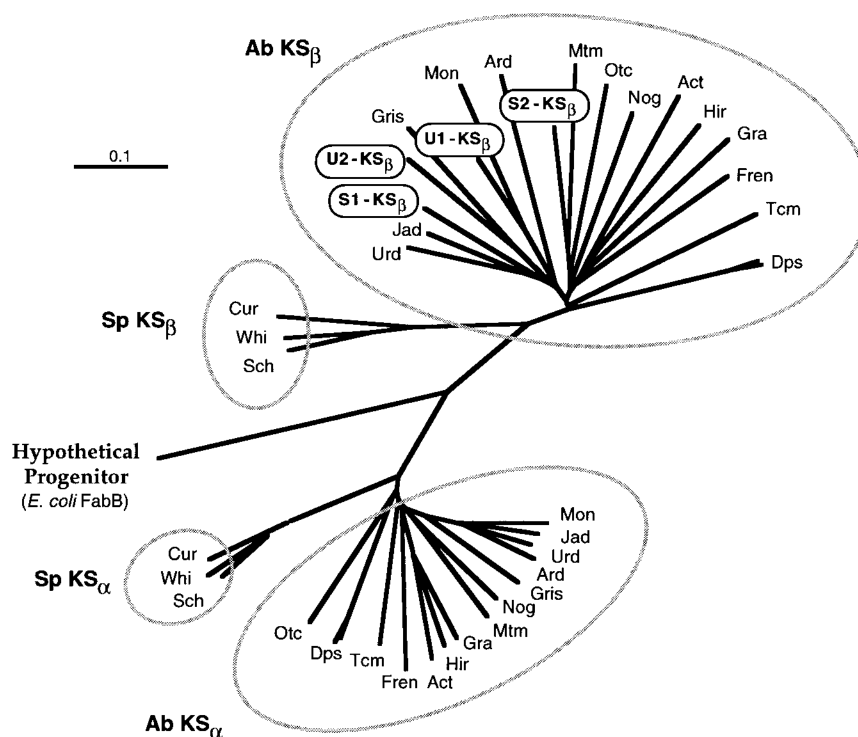

FIG. 3. Phylogeny of amino acid sequences of β-ketoacyl synthase KS_α and KS_β gene products from 18 actinomycete PKS gene clusters, constructed by the neighbor-joining method described by Saiton and Nei (55) with the *E. coli* FabB sequence (34) for an outlier. Sources of data are as follows: Gris, griseusin biosynthesis (73); Ard, PKS with unknown function from the ardicin producer *Kibdelosporangium aridum* (53); Mon, PKS with unknown function from the monensin producer *S. cinnamonensis* (1); Tcm, TCM C $KS_{\alpha\beta}$ from *S. glaucescens* (5); Otc, oxytetracycline biosynthesis in *S. rimosus* (36); Gra, granaticin $KS_{\alpha\beta}$ from *S. violaceoruber* (62); Hir, PKS of unknown function from *Saccharopolyspora hirsuta* (39); Act, ACT biosynthesis in *S. coelicolor* (19); Fren, frenolicin $KS_{\alpha\beta}$ from *S. roseofulvus* (6); Dps, daunorubicin biosynthesis in *S. peucetius* (23) and *Streptomyces* sp. strain C5 (71); Nog, nogalamycin biosynthesis in *S. nogalater* (72); Mtm, mithramycin $KS_{\alpha\beta}$ from *S. argillaceus* (40); Jad, jadomycin biosynthesis in *S. venezuelae* (24); Urd, urdamycin $KS_{\alpha\beta}$ from *S. fradiae* Tü2717 (15); Whi, spore pigment biosynthesis in *S. coelicolor* (13); Sch, spore pigment biosynthesis in *S. halstedii* (7); and Cur, possible spore pigment biosynthesis in *S. curacoi* (4). Ab and Sp, antibiotic-type or spore pigment-type KS_α and KS_β, respectively.

netic resonance (NMR) spectra of UWM1, UWM2, SEK4, and SEK4b were determined with a Bruker AM300 spectrometer; spectra were referenced internally to the solvent and directly compared to the literature (21, 43, 60).

RESULTS

Cloning of full-length KS_β genes from uncharacterized *Streptomyces* strains and soil. To amplify full-length polyketide KS_β genes from diverse DNA sources, advantage was taken of the conserved organization (KS_α-KS_β-ACP) of the genes representing the minimal PKS in most type II PKS gene clusters so far investigated (Fig. 1A). As a consequence, the existing approach was limited to detecting KS_β genes from unknown gene clusters adhering to this pattern. Consensus sequences flanking known KS_β genes were used to design two degenerate oligodeoxynucleotide primers (dp:KS_α and dp:ACP) for subsequent PCRs. The sequence of dp:KS_α was based on a conserved region at the C termini of the known KS_α subunits encoded by the upstream genes, and the sequence of dp:ACP was based on the active-site sequences of ACPs produced by the downstream genes (Fig. 1B). The data set comprised the proteins encoded by the *actI-ORF1* (19), *tcmK* (5), and *whiE-ORF3* (13) genes so as to encompass type II PKSs that produce bacterial polyketides in the currently known range of 16- to 24-carbon chain lengths. The sizes of amplified DNA fragments were expected to be approximately 1.5 kb. In principal, this method could be used to clone the adjacent KS_α genes from a given cluster as well, but the portion of the N terminus upstream of the conserved region closest to the 5′ end of the gene would then have to be recreated with an artificial, unknown sequence whose functional effect could not be assessed.

To test the specificities of the designed primers, two otherwise uncharacterized *Streptomyces* strains (68A and 71B), isolated from soil and previously shown to contain type II PKS genes (59), were chosen for the initial PCR experiments. In both cases, the reactions yielded amplified DNA fragments of about 1.5 kb (U1 in 68A and U2 in 71B), which were cloned into the TA PCR cloning vector. After sequencing, their sizes were determined to be 1,482 bp (U1) and 1,538 bp (U2). Open reading frame (ORF) analysis of the two sequences by the CODONPREFERENCE method (16) revealed the presence of a set of three ORFs each, corresponding to the 3′ ends of putative KS_α subunit genes (50 to 60 bp), possible full-length KS_β genes (~1.2 kb), and the first halves of potential ACP genes (~100 bp). In each sequence, the first and second ORFs were linked by a stop-start codon overlap typical of $KS_{\alpha\beta}$ gene pair junctions and a possible indication of tight coexpression through translational coupling, and the two KS_β genes were separated from the downstream ACP genes by a short spacer, as expected (5, 13, 19).

The same approach was then used on DNA isolated from soil (Morris et al. have independently conceived of and used a similar approach in their studies of actinomycetes systematics [49]). The soil sample was obtained from a forest area near Vancouver, British Columbia, Canada, and multigenomic

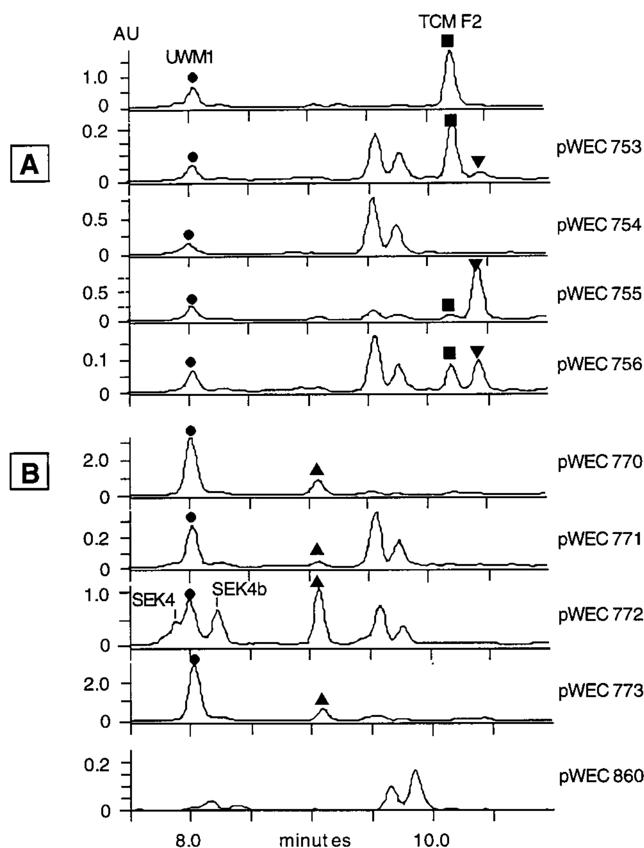

FIG. 4. HPLC analysis given as UV absorbances (254 nm) (AU) of the in vivo biosynthesis of UWM1 (●) and/or TCM F2 (■) by the pWEC752 derivatives pWEC753 to pWEC756 in *S. lividans* 1326 (A) and SEK4 and SEK4b by the pWEC769 derivatives pWEC770 to pWEC773 (B). Identities of peaks exhibiting the same R_f values as the UWM1 and TCM F2 standards were established by comparing their UV spectra with those of standard substances by use of a photodiode array detector. Spectral analysis of peaks marked with ▼ showed no relationship to UWM1 or TCM F2, while peaks indicated by ▲ had spectra clearly similar but not identical to those of SEK4 and SEK4b.

DNA was prepared according to the protocol of Holben et al. (25). Since most of the fungal mycelium was removed during the extraction of microbial cells, the DNA preparation was expected to contain genomic DNA only from the bacterial fraction of the soil sample. Again, the PCR produced predominantly 1.5-kb DNA fragments. After gel purification, cloning, and sequencing of DNA from 20 randomly selected transformants containing the 1.5-kb fragments, two exhibited similarity to known KS_β gene products. The nucleotide sequences of the resulting S1 and S2 fragments revealed the presence of three ORFs each, as described above. Sequence analysis of the other clones selected did not reveal any deduced proteins that matched any known sequence(s) in the databases (GenBank, December 1995; data not shown). This result may reflect true genetic diversity or the more prosaic outcome of mispriming and the ensuing possibility for spurious amplification of DNA due to the expected large heterogeneity of the DNA sample. Either possibility is supported by the somewhat broad band of amplified DNA obtained from the PCR (data not shown).

Sequencing and phylogenetic analysis identify the unknown KS_β genes as part of antibiotic-producing biosynthetic pathways. The four unknown, putative KS_β genes had G+C contents over 70%, typical for the coding regions of actinomycete genes (Table 2). This outcome was anticipated in view of the basis for primer design and the common occurrence of actinomycetes in the soil. Results of database searches established that the deduced products of all four ORFs were similar to known KS_β gene products from type II PKSs. The four deduced protein sequences were compared with the sequences of the ActI-Orf2 and TcmL proteins by PILEUP analysis (16) (Fig. 2). The deduced sequences of the four KS_β homologs have about 30% similarity to ActI-Orf1 and TcmK but have about 58 to 61% similarity to ActI-Orf2 and TcmL (Table 2).

Two classes of type II PKSs have been isolated from *Streptomyces* strains: those involved in the production of antibiotics, such as ACT (19), and those responsible for spore pigment formation (e.g., the WhiE spore pigment in *S. coelicolor* [13]). Consequently, a KS_β gene of unknown origin could be involved in the production of an antibiotic-type or a spore pigment-type aromatic polyketide. A phylogenetic tree (55) constructed from a set of 18 different $KS_{\alpha\beta}$ pairs demonstrated that it is not only possible to separate these pairs into two classes but also to distinguish between the α and β subunits, as noted by others using essentially the same method (33, 73). Four groups representing the two classes of type II PKSs were observed (Fig. 3); they comprised the antibiotic $KS_{\alpha\beta}$ and the spore pigment $KS_{\alpha\beta}$ PKS subunits. Parsimony analysis (18) of the tree showed that the four groups are reproducible throughout the 1,000 replicate bootstrap analyses of the trees tested by the program (18). Since the four newly cloned KS_β homologs clearly fell into the class of antibiotic KS_β subunits (Fig. 3), they are likely to be part of type II PKS gene clusters producing aromatic polyketide antibiotics.

Aromatic octa- and decaketides are synthesized by hybrid type II PKS expression cassettes containing the isolated KS_β genes. To obtain additional information about the nature of the four KS_β genes, we expressed them within the context of two previously published hybrid PKS expression cassettes (obtained from pWHM752 and pWHM769 [60]) by replacing the intrinsic KS_β genes of those constructs (Table 1). We expected that if any of the four KS_β genes was derived from a decaketide-producing type II PKS gene cluster, its product would participate with the other PKS subunits jointly expressed from the corresponding pWEC752 derivatives (pWEC753 to pWEC756; Table 1) to produce TCM F2, thereby revealing the nature of its parent PKS. Similarly, KS_β genes isolated from octaketide-producing PKS gene clusters were expected to functionally replace the *actI-ORF2* KS_β gene in pWEC769 to produce UWM1 (pWEC770 to pWEC773; Table 1). KS_β genes from tetradecaketide-producing PKS gene clusters might have been inactive, on the basis of the fact that the *whiE* minimal PKS genes do not produce a definable product in *S. coelicolor* CH999 (35). This was the outcome, in fact, when *tcmK* and *actI-ORF2* in pWEC752 were replaced with their respective *whiE-ORF3* and *whiE-ORF4* homologs. On the other hand, "mismatched" $KS_{\alpha\beta}$ pairs were anticipated to result in either no production or in low-level production of more than one major compound (60).

Expression experiments were carried out with either *S. lividans* 1326 or *S. glaucescens* WMH1077^T as hosts. The respective transformant strains were grown in liquid cultures. All pWEC769 derivatives expressing the S1-, S2-, U1-, and U2-KS_β genes (pWEC770 to pWEC773) produced a UV-fluorescent yellow compound and two UV-dark compounds with R_f values identical to those of UWM1 (60) and SEK4 and SEK4b (21, 43), respectively, when tested by TLC against UWM1, SEK4, and SEK4b standards or by HPLC (Fig. 4B and 5). Purification of these compounds from a 1-liter culture of *S. lividans*(pWEC773) (including derivatization of the UV-yellow compound) and subsequent NMR spectral analysis proved

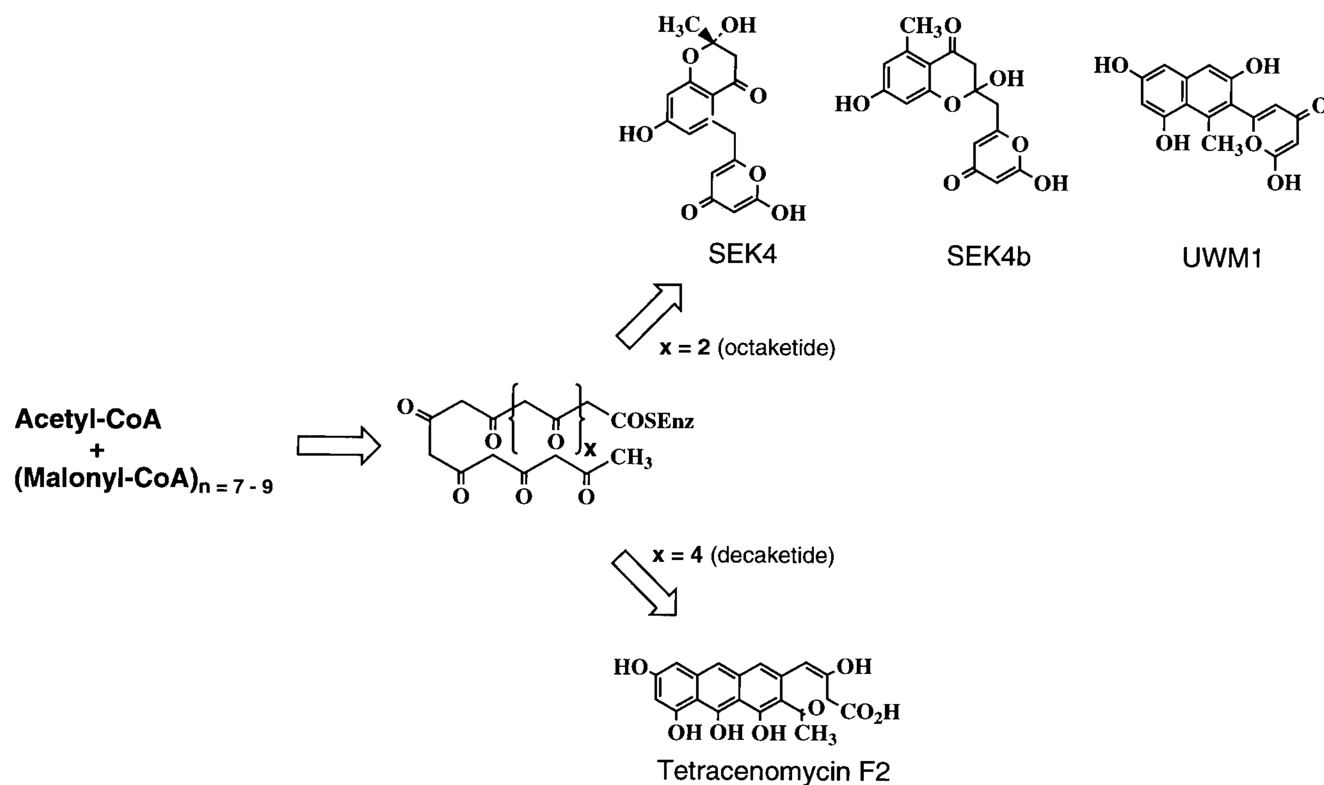

FIG. 5. Products made by the heterologous PKS gene cassettes listed in Table 1. CoA, coenzyme A; COSEnz, the thioester by which the polyketide is attached to the ACP.

their identity to the expected products (Table 1). SEK4 and SEK4b are the major compounds formed when octaketide-producing minimal PKS gene cassettes (the ACT minimal PKS [21, 43, 44]) are expressed without additional cyclase genes. Therefore, the production of SEK4 and SEK4b from the expression of pWEC770 to pWEC773 reflects an imperfect interaction between the minimal PKSs and the cyclase enzyme in these hybrid PKS systems; the relative amounts of UWM1 produced (Fig. 4A) showed that the magnitude of the imperfection varied considerably among the four KS_β genes cloned. Without each of the companion KS_α genes to enable an accurate assessment of the chain length specificity, we do not know if these results mean that the S1-, S2-, U1-, and U2-KS_β genes are derived from octaketide-producing PKS gene clusters. Metabolite production from the expression of the pWEC752 derivatives (pWEC753 to pWEC756) was generally low or occasionally undetectable, consistent with the expected behavior of mismatched $KS_{\alpha\beta}$ pairs (60). The metabolite patterns produced from the expression of pWEC753, pWEC755, and pWEC756 in *S. lividans* proved to be identical and were shown to contain TCM F2 and UWM1 as the major components by comparative HPLC spectral analysis, whereas metabolite production from the expression of pWEC754 was either undetectable or showed minute amounts of UWM1 (Fig. 4A and 5). Two other compounds were formed but were not purified due to the small amounts produced. Their UV spectra (data not shown) suggested that one (Fig. 4B) is related to SEK4 and SEK4b but that the other one is unlike TCM F2 or UWM1 (Fig. 4A). We presume that both of these compounds are novel.

DISCUSSION

We devised a method to probe multigenomic DNA samples prepared from bacteria in soil (or, in principle, other sources, such as marine bacteria) for type II PKS gene clusters using PCR and were able to obtain four full-length KS_β subunit genes. Functional analysis of the gene products revealed that each of the four novel KS_β subunits formed complexes with the ActI-Orf1 KS_α subunit that were quantitatively more effective in producing the octaketides UWM1, SEK4, and SEK4b than the heterologous complexes resulting from the S1-, S2-, and U1-KS_β enzymes matched with the TcmK KS_α subunit. The latter PKSs all showed the same pattern of low-level octaketide (UWM1) and decaketide (TCM F2) production, suggesting that the heterologous KS_β subunits occasionally caused the minimal PKS to miscount the number of times the chain-extending malonate reacted with the growing $CH_3(COCH_2)_xCOSEnz$ intermediate (Fig. 5). This did not appear to happen with the heterologous ActI-ORF1–KS_β pairs, since octaketides were by far the main products. In the case of pWEC754, the results may reflect an even less effective cooperation of the TcmK–U2-KS_β subunits than of the other three TcmK-KS_β pairs, yielding metabolite production levels sometimes too low to detect.

One conclusion to be drawn from these results could be that the majority of polyketide-producing bacteria in the soil sample represented by the four KS_β genes did indeed manufacture octaketides. We cannot assess the validity of this conclusion, since it is impossible to know whether the PCR method used truly reflects the diversity of bacterial genomes present in the soil sample or the percentage that contain type II PKS genes. On the other hand, we cannot rule out the possibility that,

despite the degree of degeneration in the original set of PCR primers (Fig. 1), they still exhibited a higher specificity for octaketides, since it is unclear what particular DNA sequence features would distinguish octaketide PKS genes from those of other chain-length specificities. Regardless, since the hybrid PKSs largely still formed the product characteristic of the respective KS_{α} gene (Table 1 and Fig. 4 and 5), the results suggest that the KS_{α} gene has more influence over chain length than the KS_{β} (chain-length-factor) gene. However, it is important to note that these two genes are not the only factors that determine chain length of the nascent poly-β-ketone intermediate (Fig. 5); the presence of heterologous polyketide cyclase or ketoreductase genes in iterative bacterial PKSs also can alter the size of the polyketide produced, from a mixture of 16- and 18-carbon chains to 16- or 18-carbon chains only (38) and from to 20- to 18-carbon chains (74).

Despite the possible limitations of the PCR primer choice and expression system, our approach enabled the rapid identification of novel biosynthetic genes from environmental samples without isolation of the parent organisms. This method has been used expeditiously by Morris et al. to classify actinomycetes in soil samples (49). In addition, we were able to obtain preliminary data about the function of the gene products and therefore about the specificities of the underlying biosynthetic pathways. The two G+C-rich genes that were cloned directly from the soil may have come from actinomycetes, which are ubiquitous soil bacteria and often culturable, yet biosynthetic genes could also be obtained from other bacterial families as well as fungi and expressed by suitable variations of our approach. Since minimal type II PKS genes are particularly suited to exhibiting variations in the product formed as a function of KS subunits (few other secondary metabolism enzymes would display much plasticity in substrate utilization), this approach could be extended to accessing full-length $KS_{\alpha\beta}$ gene pairs as well as to tailoring enzymes and final products of such biosynthetic pathways by use of cloned genes as specific probes to clone the rest of the gene cluster from cosmid or BAC (64) libraries of the multigenomic DNA sample.

With further refinements, we expect that fundamentally different biosynthetic pathways will be obtained by the cloning and expression of secondary metabolism genes in surrogate hosts. This is an important goal, since one of the major bottlenecks of conventional natural product discovery processes is that the various strategies depend on screening microbial products (enzymes and secondary metabolites) from cultured microorganisms. Since it has been shown that microorganisms cultured from soil samples or other sources by standard methods usually represent less than 1% of the available microbial diversity (2, 69), the discovery of novel microbial secondary metabolites is restricted to this small proportion. Consequently, an approach that overcomes this limitation is likely to have general utility in harvesting a broader spectrum of molecular microbial diversity for pharmaceutical and industrial purposes. Hopefully, the approach described here, which was developed to enable a broader investigation of the type II PKSs, will complement other, newly emerging approaches to antibiotic discovery (28). These include several genome projects involving known pathogens (20) to provide additional targets for conventional screening programs and de novo drug design, new strategies in combinatorial biology (12, 31, 32, 45, 65, 66) to yield novel scaffolding for decorative (bio-)chemistry, and attempts to access the immense marine biodiversity (30).

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