Ectopic expression of the *Streptomyces coelicolor* *whiE* genes for polyketide spore pigment synthesis and their interaction with the *act* genes for actinorhodin biosynthesis

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The *whiE* gene cluster of *Streptomyces coelicolor* is normally expressed shortly before sporulation in the aerial mycelium, leading to production of the grey polyketide spore pigment. By placing the *whiE* genes under the control of the thiostrepton-inducible *tipA* promoter, they were artificially expressed on plasmids or in the chromosome during vegetative growth in a strain deleted for the *act* genes, which control biosynthesis of the polyketide antibiotic actinorhodin. Certain combinations of *whiE*-ORF I–VII led to production of mycelial pigments; these were exported into the medium when *whiE*-ORF I was absent, but poorly in its presence. Combined with comparative sequence data, the results allowed deductions to be made, or confirmed, about the normal roles of the eight known genes, *whiE*-ORF I–VIII, as follows: *whiE*-ORF III, IV, V encode the three components (ketosynthase, chain length factor and acyl carrier protein) of the *whiE* 'minimal' polyketide synthase (PKS) needed for assembly of the carbon chain of the spore pigment precursor; *whiE*-ORF II, VI, VII are likely to be involved in cyclizations of the nascent carbon chain; *whiE*-ORF VIII controls a late step in the spore pigment biosynthetic pathway, probably a hydroxylation; and *whiE*-ORF I may encode a protein needed for correct targeting or retention of spore pigment at an appropriate cellular location. In other experiments, genes encoding components of the *act*-PKS and *whiE*-PKS were artificially co-expressed. Each of the three *whiE* minimal PKS subunit genes could complement lesions in the corresponding *act*-PKS genes to produce actinorhodin or related mycelial pigments, and each of the three *act* minimal PKS genes could complement lesions in the *whiE* minimal PKS genes to cause spore pigmentation. Thus the two sets of PKS subunits, which are encoded by genes that have presumably diverged from a common ancestor, are still capable of biochemical 'cross-talk', but this is normally prevented because the gene sets are expressed in different 'tissues' of the differentiated *Streptomyces* colony. Ectopic expression of sets of *whiE*-PKS genes presumed to be sufficient to assemble a carbon chain caused inhibition of early growth of the strains, perhaps by causing interference with fatty acid biosynthesis; this yielded circumstantial evidence that the *whiE*-PKS gene products can also interact with those of the fatty acid synthase(s) of the organism.

Keywords: antibiotic biosynthesis, polyketides, spore pigment, *Streptomyces coelicolor*, *whiE* genes

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Abbreviations: ACP, acyl carrier protein; CLF, chain length factor; FAS, fatty acid synthase; KS, ketosynthase; PKS, polyketide synthase.

INTRODUCTION

Spore colour in *Streptomyces coelicolor* A3(2) (Davis & Chater, 1990) and *Streptomyces halstedii* (Blanco *et al.*, 1992, 1993) apparently depends on the presence of one or more polycyclic aromatic polyketides. Attempts to purify the spore pigments have failed (Brian, 1992), perhaps because they are covalently bound to macromolecular spore components. Deductions about the chemical nature of the pigments rest instead on the discovery that mutations abolishing or modifying spore pigmentation reside in a cluster of genes in each species – named *whiE* and *sch* respectively – that resemble, in arrangement and sequence, the sets of genes encoding the subunits of the so-called type II polyketide synthases (PKSs) for *Streptomyces* aromatic antibiotics, including those for actinorhodin in *S. coelicolor* itself (Fig. 1). These PKS genes resemble in turn genes for fatty acid synthases (FASs). Indeed, there is now ample evidence not only that the biochemistry of fatty acid and polyketide synthesis, in which carbon chains are formed by the successive condensation of small acyl building units, are mechanistically related (O'Hagan, 1991), but that the genes have a common evolutionary origin (Hopwood & Sherman, 1990; Hopwood & Khosla, 1992; Katz & Donadio, 1993; Smith, 1994). Amongst the actinorhodin-like PKSs, the basis of PKS 'programming' is currently being elucidated (McDaniel *et al.*, 1993a, 1994; Shen & Hutchinson, 1993). Programming refers to the mechanism by which each different PKS controls the appropriate carbon chain length, degree and regiospecificity of keto-group reduction, and cyclization pattern of its product. It turns out that three subunits of the enzyme, which constitute the 'minimal' PKS and are the ketosynthase (KS), chain length factor (CLF) and acyl carrier protein (ACP), are responsible for building the carbon chain and largely for establishing its first critical cyclization. These subunits are encoded by *actI*-ORF1, -ORF2 and -ORF3, respectively, in the actinorhodin cluster and by their homologues in the other gene clusters (Fig. 1). Additional subunits of the PKS are responsible for further cyclizations of the nascent carbon chain (McDaniel *et al.*, 1994). Some, but not all, PKSs also include a keto-reductase (KR), which reduces a specific keto-group.

The known extent of the *whiE* cluster of *S. coelicolor* consists of an operon of seven genes (*whiE*-ORF1–VII) and at least one divergently transcribed gene, *whiE*-ORFVIII (Davis & Chater, 1990; Fig. 1). Normally the *whiE* genes are transcribed just before sporulation in the aerial mycelium (G. H. Kelemen, personal communication). In order to express the genes at other times, and so to gain insight into their roles in spore pigment biosynthesis, we inserted the thiostrepton-inducible *tipA* promoter (Murakami *et al.*, 1989) into the *whiE* cluster either on plasmids or in the chromosome. By adding thiostrepton to the medium we could then observe production of *whiE*-derived pigments during vegetative growth. Study of mutants in which various of the *whiE* genes had been deleted allowed deductions to be made about the roles of individual genes in pigmentation. This was conveniently done in a strain from which the entire

Fig. 1. Organization of genes for aromatic PKS components from *Streptomyces* spp.: actinorhodin (*act*) from *S. coelicolor* (Fernández-Moreno *et al.*, 1992), granaticin (*gra*) from *S. violaceoruber* (Sherman *et al.*, 1989), frenolicin (*fren*) from *S. roseofulvus* (Bibb *et al.*, 1994), griseusin (*gris*) from *S. griseus* (Yu *et al.*, 1994), oxytetracycline (*otc*) from *S. rimosus* (Kim *et al.*, 1994a), tetracenomycin (*tcm*) from *S. glaucescens* (Bibb *et al.*, 1989), the *whiE*-encoded spore pigment from *S. coelicolor* (Davis & Chater, 1990; Blanco *et al.*, 1993) and the *sch*-encoded spore pigment from *S. halstedii* (Blanco *et al.*, 1992, 1993). The arrows depict the relative sizes of the genes and are shaded to represent their functions. The symbol // indicates that one or more genes lie between those shown.

set of *act* genes had been deleted so that the effects of expressing the *whiE* genes could be observed in the absence of possible interactions with the products of genes encoding the *act*-PKS.

Other experiments were designed to study the functional relationships between the *act*- and *whiE*-PKS genes, capitalizing on the presence, in the same streptomycete host, of the two sets of related genes that are expressed at different stages in the life-cycle of the organism to produce antibiotic and spore pigments respectively. Building on earlier findings that 'hybrid' PKSs can arise by complementation between subunits of synthases from two different streptomycetes producing structurally related aromatic polyketide antibiotics (Sherman *et al.*, 1992; Khosla *et al.*, 1993), genes for subunits of the *act*- and *whiE*-PKSs were artificially co-expressed. It was found that each of the three subunits of the *whiE* minimal PKS could complement lesions in the *act*-PKS to produce actinorhodin (and perhaps related compounds). Conversely, the corresponding *act*-PKS subunits could complement mutations in the *whiE* locus to restore spore pigmentation. These results, building on those of Kim *et*

al. (1994b), introduce a favourable system for the further investigation of differential gene expression during the development of a complex prokaryote (Chater, 1993).

METHODS

Bacterial strains and plasmids. The *Escherichia coli* hosts (Sambrook *et al.*, 1989) were DH5 α for the manipulation of plasmid DNA and JM101 for the preparation of phagemid DNA. The non-methylating (*dam dcm hsdM*) strain ET12567 (MacNeil *et al.*, 1992) was used to obtain DNA for transformation of *S. coelicolor*. *S. coelicolor* strains are described in Table 1, and plasmids in Table 2.

Propagation of organisms and general genetic and recombinant DNA techniques. Handling of *E. coli* strains and general recombinant DNA methods were as described by Sambrook *et al.* (1989). For *Streptomyces* the methods and media described by Hopwood *et al.* (1985) were used. For studying the pigmentation of the colonies or medium, R2 agar was found to be the most suitable. For analysis of spore colour, cultures were grown either on minimal medium (MM) with mannitol replacing glucose as carbon source and supplemented with 'tiger milk', or on SFM (per litre: 20 g mannitol, 20 g soya flour, 20 g agar), both of which encourage sporulation. Where relevant, thio-strepton was added at 5 $\mu\text{g ml}^{-1}$ to induce the *tipA* promoter(s).

Construction of the *ptipA* divergent promoter cassette. A fragment carrying *ptipA*, with the λt_0 transcriptional terminator upstream of it, was excised as an *Sst*II (blunt-ended)–*Eco*RI fragment from pIJ6017 and ligated into pAK113, which also carries *ptipA* (Murakami *et al.*, 1989), cut with *Sma*I and *Eco*RI to yield pIJ4267 (Fig. 2). The resulting *ptipA* divergent promoter cassette was then excised as a *Pst*I–*Eco*RI fragment and ligated into pDH5, digested with the same enzymes, to yield pIJ4268. The promoter cassette was further excised as a convenient *Sph*I fragment for creating pIJ4274 and pIJ4275 (see Results). [For the record, pIJ6017 carries a variant of *ptipA*, *ptipA**, with an extra G inserted 64 bases upstream of the

transcriptional start site (Takano, 1993), whereas pAK113 carries the wild-type *ptipA*; in some situations (H. Wang, personal communication) *ptipA** has been reported to act as a stronger promoter but this was not significant in the present work. In pIJ4274, *ptipA** was responsible for transcription of *whiE*-ORFI–VII.]

RESULTS

Construction of *S. coelicolor* strains deleted for the *whiE* gene cluster

A prerequisite for studies of the biochemical functions of the various *whiE* genes was a set of strains containing defined deletions in the *whiE* locus. A primary deletion of the known *whiE*-ORFI–VII genes was engineered using a marker-replacement ('suicide') vector, pIJ4263 (Fig. 3). On this plasmid, the hygromycin resistance gene (*hyg*) of *Streptomyces hygroscopicus* (Malpartida *et al.*, 1983) replaced the *Sph*I fragment that includes the seven *whiE* genes on pIJ2156 sequenced by Davis & Chater (1990), leaving ~1.1 kb of DNA from the *whiE* locus on either side of *hyg*. The starting strain for transformation by pIJ4263 was *S. coelicolor* CH999 (McDaniel *et al.*, 1993a), which carries an engineered replacement of the *act* gene cluster in the chromosome with the *ermE* gene (Δact). It also contains a mutation in the *red* gene cluster controlling biosynthesis of the red prodigiosine pigments. Southern hybridization of transformants of CH999 by pIJ4263 demonstrated that they arose by the expected single crossover, either upstream or downstream of *whiE*-ORFI–VII, in approximately equal numbers. Both classes of recombinants had normal sporulation and grey-pigmented spores. One of the strains was propagated on a medium lacking thio-strepton but containing hygromycin to yield the thio-strepton-sensitive $\Delta act \Delta whiE$ strain YU105. Southern

Table 1. *S. coelicolor* A3(2) derivatives

Strain	Genotype*	Reference
B60	<i>hisA1 uraA1 strA1 act-160 (actI-ORFI) SCP2⁺</i>	Rudd & Hopwood (1979)
B78	<i>hisA1 uraA1 strA1 act-178 (actI-ORF2) SCP2⁺</i>	
C107	<i>whiE107 SCP1⁺ SCP2⁺</i>	Chater (1972)
C124	<i>whiE124 SCP1⁺ SCP2⁺</i>	
CH1	<i>proA1 argA1 redE60</i>	Khosla <i>et al.</i> (1992)
CH5	<i>proA1 argA1 redE60 actI-ORF3/actVII uncoupled</i>	
CH6	<i>proA1 argA1 redE60 actI-ORF3 (mis-sense)</i>	
CH12	<i>proA1 argA1 redE60 actI-ORF3 (frame-shift)</i>	
CH13	<i>proA1 argA1 redE60 actI-ORF3 (deletion)</i>	McDaniel <i>et al.</i> (1993a)
CH999	<i>proA1 argA1 redE60 Δact (ermE)</i>	
YU105	<i>proA1 argA1 redE60 Δact (ermE) $\Delta whiE$ (hyg)</i>	This work
YU106	<i>proA1 argA1 redE60 $\Delta whiE$ (hyg)</i>	
YU107	<i>proA1 argA1 redE60 Δact (ermE) <i>whiE</i></i>	
YU108	<i>proA1 argA1 redE60 Δact (ermE) <i>whiE</i></i>	
YU114	<i>proA1 argA1 redE60 Δact (ermE) <i>whiE</i></i>	
YU116	<i>proA1 argA1 redE60 Δact (ermE) <i>whiE</i></i>	
YU127	<i>proA1 argA1 redE60 actI-ORF3 (deletion) <i>whiE</i></i>	

*All strains are SCP1⁺ SCP2⁺ except where indicated.

Table 2. Plasmids

Plasmid	Relevant characteristics (resistance markers)	Reference or Figure
pAK113	Derivatives of pUC13 carrying <i>ptipA</i>	Murakami <i>et al.</i> (1989)
pDH5	<i>E. coli</i> vector with phage f1 replication origin for isolation of single-stranded DNA (<i>bla</i> <i>tsr</i>)	Hillemann <i>et al.</i> (1991)
pGM160	<i>E. coli</i> - <i>Streptomyces</i> shuttle vector, temperature-sensitive for replication in <i>Streptomyces</i> (<i>bla</i> <i>tsr</i>)	Muth <i>et al.</i> (1989)
pIJ68	Derivative of pIJ699 containing <i>actII</i> -ORF4 (<i>tsr</i>)	Passantino <i>et al.</i> (1991)
pIJ680	Multicopy <i>Streptomyces</i> vector (<i>aph</i> , <i>tsr</i>)	Hopwood <i>et al.</i> (1985)
pIJ699	Multicopy <i>Streptomyces</i> vector (<i>tsr</i>)	Kieser & Melton (1988)
pIJ922	Low-copy-number SCP2* <i>Streptomyces</i> vector (<i>tsr</i>)	Lydiat <i>et al.</i> (1985)
pIJ2156	Derivative of pIJ922 containing a partial <i>Sau3A1</i> fragment of 7.9 kb carrying <i>whiE</i> -ORFI-VII (<i>tsr</i>)	Davis & Chater (1990)
pIJ4256	Derivative of pDH5 containing an 8.9 kb <i>BglII</i> - <i>EcoRI</i> fragment carrying the <i>whiE</i> -ORFI-VII genes from pIJ2156 and SCP2* vector sequences downstream of them (<i>bla</i> <i>tsr</i>)	—
pIJ4257	Derivative of pGM160 containing an 8.9 kb <i>HindIII</i> - <i>EcoRI</i> fragment carrying <i>whiE</i> -ORFI-VII and the SCP2* vector sequences from pIJ4256 (<i>bla</i> <i>tsr</i>)	Figs 2 and 4
pIJ4263	Derivative of pIJ4256 with the 5.75 kb <i>SphI</i> fragment carrying <i>whiE</i> -ORFI-VII replaced by a 1.7 kb <i>SphI</i> <i>hyg</i> -containing fragment from pIJ5607 (<i>bla</i> <i>tsr</i>)	Fig. 3
pIJ4267	Derivative of pAK113 containing a 0.65 kb <i>SstII</i> - <i>EcoRI</i> fragment from pIJ6017 (<i>bla</i>)	Fig. 2
pIJ4268	Derivative of pDH5 containing a 0.8 kb <i>PstI</i> - <i>EcoRI</i> fragment from pIJ6017 (<i>bla</i> <i>tsr</i>)	
pIJ4274	Derivative of pIJ4257 containing a 0.8 kb <i>SphI</i> fragment from pIJ4268 carrying the <i>ptipA</i> divergent promoter pair from pIJ6017 (<i>bla</i> <i>tsr</i>)	Fig. 4
pIJ4275	As pIJ4274, but with the <i>SphI</i> fragment inserted in the opposite orientation (<i>bla</i> <i>tsr</i>)	
pIJ4276	Derivative of pIJ4274 with the 1.32 kb <i>SphI</i> - <i>BamHI</i> fragment including most of <i>whiE</i> -ORFI deleted (<i>bla</i> <i>tsr</i>)	
pIJ4277	Derivative of pIJ4274 with the 1.73 kb <i>BamHI</i> - <i>XhoI</i> fragment including <i>whiE</i> -ORFI and part of -ORFII deleted (<i>bla</i> <i>tsr</i>)	
pIJ4278	Derivative of pIJ4274 in which the <i>XhoI</i> site was cut and self-ligated after end-filling with Klenow enzyme to introduce four additional (TCGA) base pairs into <i>whiE</i> -ORFII (<i>bla</i> <i>tsr</i>)	
pIJ4279	Derivative of pIJ4274 with the 730 bp <i>BstXI</i> fragment of <i>whiE</i> -ORFIII deleted by digestion with <i>BstXI</i> and ligation after blunt-ending (<i>bla</i> <i>tsr</i>)	
pIJ4280	Derivative of pIJ4274 obtained by partial digestion with <i>SnoI</i> and self-ligation after end-filling with Klenow enzyme to introduce four additional (TGCA) base pairs into <i>whiE</i> -ORFIV (<i>bla</i> <i>tsr</i>)	
pIJ4281	Derivative of pIJ4274 obtained by partial digestion with <i>SnoI</i> and self-ligation after end-filling with Klenow enzyme to introduce four additional (TGCA) base pairs into <i>whiE</i> -ORFV (<i>bla</i> <i>tsr</i>)	
pIJ4284	Derivative of pIJ4274 obtained by digestion with <i>BglII</i> and self-ligation after end-filling with Klenow enzyme to introduce four additional (TGCA) base pairs into <i>whiE</i> -ORFVIII (<i>bla</i> <i>tsr</i>)	
pIJ4286	Derivative of pIJ4274 with the 1.23 kb <i>SnoI</i> fragment overlapping <i>whiE</i> -ORFIV/V deleted (<i>bla</i> <i>tsr</i>)	
pIJ4287	Derivative of pIJ4274 with the 0.95 kb <i>SnoI</i> fragment overlapping <i>whiE</i> -ORFIII/IV deleted (<i>bla</i> <i>tsr</i>)	
pIJ4288	Derivative of pIJ4274 with the 2.18 kb <i>SnoI</i> double fragment overlapping <i>whiE</i> -ORFIII-V deleted (<i>bla</i> <i>tsr</i>)	
pIJ4289	Derivative of pIJ4276 with the 2.2 kb <i>NruI</i> - <i>EcoRI</i> fragment including <i>whiE</i> -ORFVII and part of -ORFVI deleted (<i>bla</i> <i>tsr</i>)	
pIJ4290	Derivative of pIJ4277 with the 2.2 kb <i>NruI</i> - <i>EcoRI</i> fragment including <i>whiE</i> -ORFVII and part of -ORFVI deleted (<i>bla</i> <i>tsr</i>)	
pIJ4291	Derivative of pIJ4274 with the 2.2 kb <i>NruI</i> - <i>EcoRI</i> fragment including <i>whiE</i> -ORFVII and part of -ORFVI deleted (<i>bla</i> <i>tsr</i>)	
pIJ5607	Derivative of pUC18 containing a 1.7 kb <i>hyg</i> cassette (<i>bla</i>)	C. Khosla (personal communication)
pIJ6017	Derivative of pIJ680 containing <i>ptipA</i> *, λ _{tr} and <i>fd</i> transcriptional terminators and multicloning site (<i>tsr</i> , <i>kan</i>)	Takano (1993)
pSG5	Natural plasmid from <i>Streptomyces ghanaensis</i> ; parent of pGM160	Muth <i>et al.</i> (1989)
pUC13/18	High-copy-number <i>E. coli</i> cloning vectors (<i>bla</i>)	Yanisch-Perron <i>et al.</i> (1985)

hybridization confirmed that this strain arose by the expected second crossover, which replaced *whiE*-ORFI-VII on the chromosome by *hyg*. YU105 sporulated as well as the *whiE*⁺ CH999 but lacked spore pigmentation; like CH999, YU105 produced no actinorhodin or any pigmented precursor of it.

In order to construct an *act*⁺ Δ *whiE* recombinant, a protoplast fusion was made between YU105 and CH1, the *act*⁺ progenitor of CH999 (Khosla *et al.*, 1992). After protoplast regeneration, followed by selection for hygromycin-resistant colonies (thereby ensuring inheritance of the *whiE* deletion), they were screened for blue

Fig. 2. Construction of the bidirectional *ptipA* divergent promoter cassette (small arrows in rectangles) and its cloning into the *whiE* cluster. Only selected restriction sites are shown. The *SphI* sites on pIJ4268 are from the polylinker flanking the promoter cassette; the asymmetric *KpnI* site served to determine its orientation in pIJ4274 and pIJ4275.

pigment (actinorhodin) production. The resulting colonies were checked for lincomycin sensitivity by replica plating (the *ermE* marker that replaces the *act* cluster in CH999 and YU105 confers lincomycin resistance). This yielded the *act*⁺ Δ *whiE* strain YU106. Southern hybridization confirmed that YU106 had the expected genomic arrangement at the *whiE* and *act* loci.

To confirm that neither YU105 nor YU106 had suffered any mutation, apart from deletion of *whiE*-ORFI-VII, that might have had potential to affect spore pigmentation, pIJ4257 (a ColE1-pSG5 bifunctional replicon carrying the *whiE* gene cluster fragment from pIJ2156) was introduced into the chromosome of both strains by recombination, whereupon normal grey-pigmented spores were produced. (These strains were readily obtained after a period of growth at 39 °C, making use of the temperature-sensitive replication properties of the pSG5 replicon.)

Induced expression of the *whiE* genes

Expression of the *whiE* genes on plasmids. The thiostrepton-inducible *ptipA* was used to express the *whiE* genes ectopically. The *whiE* gene cluster includes a gene (ORFVIII) transcribed divergently from the *whiE*-ORFI-VII operon (Fig. 1). Therefore two copies of *ptipA* were engineered back-to-back as a cassette bounded by convenient restriction sites (see Methods). pIJ4274 and

Fig. 3. Strategy for replacement of the *whiE* gene cluster with the *hyg* gene. At the top is shown a restriction map of the *whiE* region of the *S. coelicolor* CH999 chromosome and, aligned with it, the suicide vector pIJ4263 containing the *hyg* gene replacing seven *whiE* ORFs and with ~1.1 kb of additional DNA on each side (black boxes). The vector, derived from pDH5, is equipped with a phage f1 origin of replication so that single-stranded DNA can readily be generated in *E. coli* for efficient transformation of *Streptomyces* strains (Hillemann et al., 1991; Yu et al., 1994), even when they contain restriction systems. After a first crossover in region (a) or (b), colonies resistant to both thiostrepton (conferred by *tsr*) and hygromycin (conferred by *hyg*) were selected. YU105, which was recovered by passaging these doubly resistant strains on a medium lacking thiostrepton, was the result of double crossing-over. B, *Bam*HI restriction site; S, *Sph*I restriction site.

pIJ4275 were made by inserting the *ptipA* cassette, in opposite orientations, into the *SphI* site upstream of the *whiE*-ORFI-VII operon on pIJ4257 (Fig. 2). (For expression of the *whiE* genes on these plasmids, only one member of the pair of *tipA* promoters is relevant, since the divergent *whiE*-ORFVIII is truncated, but both members of the pair of promoters were needed for later experiments: see below under **Induced expression of *whiE* genes in the chromosome.**)

The mycelium of young transformants of YU105 (Δ *act* Δ *whiE*) by both plasmids was yellow-green in the presence of thiostrepton; a small amount of yellow-green pigment appeared in the medium, but only after prolonged incubation (~1 week). The mycelium and medium were colourless in the absence of thiostrepton, proving that

Fig. 4. Organization of ORFs in *whiE* expression clones and phenotypes conferred by each plasmid on host strains. The restriction sites used for clone construction are indicated for pIJ4257, shown at the top. The rectangle to the left is the N-terminal part of *whiE*-ORFVIII and the one to the right is the 1.1 kb unsequenced DNA segment located downstream of *whiE*-ORFVII in the original *whiE* clone pIJ2156 (Davis & Chater, 1990). The symbol $\diamond$ indicates the divergent *ptpA* promoter pair cassette, and the asterisk in certain ORFs indicates the presence of a frame-shift mutation.

pigment production depended on expression of *whiE*-ORFI-VII. Unexpectedly, spores harvested from the yellow-green cultures grown in the presence of thiostrepton failed to grow on new plates containing the antibiotic. The cultures could be maintained either by successive propagation of mycelium from thiostrepton-containing plates, or by plating spores without thiostrepton and transferring the resulting mycelium to thiostrepton-containing medium. The inhibition by thiostrepton affected spore germination or very early growth of the cultures because, although complete inhibition occurred when thiostrepton was added up to 24 h after spore plating, the antibiotic did not affect growth after colonies were visible. Attempts to grow the strains in liquid medium (TSB) with thiostrepton resulted in

granular mycelial clumps in the first 3–4 d. Later, the clumps mostly lysed and the broth became deeper yellow-green. The significance of these results is discussed below.

In order to explore the functions of each *whiE* ORF, a series of deletions and frame-shift mutations were made on pIJ4274, as described in Table 2 and Fig. 4. Each plasmid was introduced into both YU105 ($\Delta act \Delta whiE$) and CH999 ($\Delta act whiE^+$) and the effects on spore pigmentation, colony pigmentation and the growth inhibition described above were examined in the presence of thiostrepton. The results are summarized in Fig. 4.

The spore pigmentation phenotypes of YU105 derivatives carrying each of the plasmids showed that each of *whiE*-ORFI-V, and at least one of ORFVI and VII, are

necessary for spore pigmentation, since this was abolished by all of the lesions in these ORFs (separate deletions of ORFVI and VII were not made). Unexpectedly, eight plasmids (pIJ4278–4281, pIJ4286–4288 and pIJ4291) even caused *S. coelicolor* CH999 to lose its normal spore pigmentation. This could be reversed by isolating strains in which the *whiE*-containing fragment had become integrated at single copy number by recombination into the relevant region of the *whiE* locus in the chromosome, again making use of the temperature-sensitive replication properties of the pSG5 replicon. Two features are shared by the eight clones that caused loss of spore pigmentation in CH999: a plasmid-borne *whiE* promoter for ORFI–VII, which was not inactivated by insertion of the *ptipA* cassette because the *SphI* site is about 100 bp upstream of the transcription start site (Brian, 1992), and a lesion in at least one of these seven ORFs on the plasmid. We speculate that a specific factor, such as an activator of the *whiE* promoter, is involved in regulation of spore pigment production. Introduction into CH999 of multiple copies (about 50) of the *whiE* promoter on these plasmids would, on this hypothesis, cause the endogenous activator to be sequestered on mutant copies of the plasmid-borne *whiE* operon, so that the chromosomal *whiE* promoter would not be activated.

Mutations involving any of *whiE*-ORFs III–VII abolished production of the yellow-green colony pigment induced by the complete set of ORFs I–VII on pIJ4274/5 (Fig. 4). In contrast, inactivation of ORFI, ORFII or both (pIJ4276, pIJ4278 or pIJ4277) modified the pigmentation phenotype rather than abolishing it. Inactivation of ORFI alone dramatically *increased* pigmentation in the medium, giving rise to a dark green colour. This suggested that the ORFI product may play a role in retention of spore pigment (or a precursor) at some appropriate intracellular or cell-surface site. In contrast, inactivation of ORFII alone changed the colour of pigmentation from a greenish to a yellowish hue. Thus ORFII is likely to control a step in spore pigment biosynthesis. Again, deletion of ORFI in the presence of the ORFII mutation (pIJ4277 compared with pIJ4278) *increased* extracellular pigmentation: YU105/pIJ4278 made a small amount of yellow pigment in the substrate mycelium, whereas YU105/pIJ4277 produced copious yellow-orange pigmentation in the medium.

The finding of a dark-green pigment in the medium of YU105/pIJ4276 (lacking *whiE*-ORFI) echoes the findings of Horinouchi & Beppu (1985), who reported production of a diffusible 'brown' pigment when expression of what is now known to be an incomplete set of *whiE* genes on their pARC1 plasmid (Davis & Chater, 1990) was activated in *Streptomyces lividans*; pARC1 would have lacked *whiE*-ORFI.

Certain of the expression clones carrying *whiE* deletions displayed the growth inhibition previously associated with the presence of the complete set of *whiE* ORFs I–VII on pIJ4274/5. The inhibition clearly involved ectopic expression of the *whiE* genes induced by thiostrepton, rather than activation of the *tipA* promoter itself or a

direct effect of thiostrepton, because other clones carrying the same promoter and challenged with an equal amount of thiostrepton did not show the same effect. A clue to the cause of the inhibition was given by its complete correlation with the presence on the plasmid of a subset of three of the *whiE* genes, ORFIII–V (Fig. 4). Studies of the expression in CH999 of sets of genes encoding the PKSs for the various aromatic antibiotics (Fig. 1) (McDaniel *et al.*, 1993a, 1994) have established that a 'minimal' PKS consisting of just three subunits, a ketosynthase (KS), chain length factor (CLF) and acyl carrier protein (ACP), is competent to synthesize the basic carbon backbone of the polyketide product. *whiE*-ORFIII, IV and V are the homologues of the genes encoding these three subunits. Thus the inhibition of early growth is associated with the potential to make a polyketide backbone. Since biosynthesis of the carbon chains of aromatic polyketides is biochemically related to that of fatty acids, induction of high levels of the *whiE* minimal PKS at an inappropriate stage during growth might lead to exhaustion of precursors, auxiliary enzymes and/or cofactors required for fatty acid metabolism. Alternatively, the metabolic products of the *whiE*-PKS might be toxic to the host or be processed into novel fatty acids that could disturb cell physiology.

Induced expression of the *whiE* genes in the chromosome.

To study the effects of induced expression of the *whiE* ORFs in their normal chromosomal location, some of the DNA fragments used for induced expression of the *whiE* ORFs on plasmids (Fig. 4) were inserted into the chromosome at the *whiE* locus. To do so, derivatives of YU105 ($\Delta act \Delta whiE$) containing each plasmid were grown at 39 °C with selection for thiostrepton resistance (encoded by the *tsr* gene on the vector), and the resulting colonies were propagated on a medium lacking both thiostrepton and hygromycin (*hyg* replaces *whiE*-ORFI–VII in YU105) to recover the appropriate $Tsr^S Hyg^S$ double crossover product. Southern hybridization confirmed that the *hyg*-containing fragment of YU105 had indeed been replaced in these strains (YU107, YU108, YU114, YU116) by the *whiE* genes from the relevant plasmid. pGM160 was then introduced into each strain as an autonomous plasmid to render it thiostrepton resistant so that the pair of *tipA* promoters inserted into the chromosomes of the recombinant strains could be induced. The phenotypes of the recombinants in respect of colony and spore pigmentation were then observed (Table 3).

In the absence of thiostrepton, YU107 had the same phenotype as CH999 ($\Delta act whiE^+$): an absence of pigment in the substrate mycelium and medium and the normal grey spore colour. This confirmed that the naturally developmentally regulated *whiE* promoters were not affected by insertion of the *ptipA* cassette. In the presence of thiostrepton, a yellow-green mycelial pigment, indistinguishable from that induced in YU105/pIJ4274, was seen. For YU108, lacking *whiE*-ORFI, induction of *whiE* expression by addition of thiostrepton caused abundant dark green pigment to appear in the medium of young cultures; this behaviour was the same as for

Table 3. Phenotypes of *Δact* strains with sets of chromosomal *whiE* genes expressed by *ptipA* induction

Strain	Corresponding plasmid*	<i>whiE</i> ORFs present	Culture pigmentation		Spore pigmentation
			Mycelium	Medium	
YU107	pIJ4274	I–VIII	Yellow-green	–	Grey (wild-type)
YU108	pIJ4276	II–VIII		Dark green	Colourless
YU116	pIJ4277	III–VIII		Light yellow	Colourless
YU114	pIJ4284	I–VII	Yellow-green	–	Greenish

*See Fig. 4.

Table 4. Complementation of three different *actI*-ORF3 (ACP) mutants by *whiE*-ORFV

Plasmids used for complementation	CH999 (<i>Δact</i> control)	Culture pigmentation induced in <i>actI</i> -ORF3 (ACP) mutants		
		CH6 (mis-sense)	CH12 (frame-shift)	CH13 (deletion)
pIJ4277 (<i>whiE</i> -ORFIII–VII)	Yellow-orange	Brown	Purple	Blue
pIJ4287 (<i>whiE</i> -ORFI, II, V, VI, VII)	Colourless	Brown	Purple	Blue-purple

YU105/pIJ4276. In comparable fashion, YU116, lacking both *whiE*-ORFI and -ORFII, secreted a light-yellow pigment resembling that made by YU105/4277 into thio-strepton-containing medium from the early stages of growth. The spores of YU108 and YU116 were unpigmented. These results confirmed the conclusions, from YU105/pIJ4276 and YU105/pIJ4277 respectively, that *whiE*-ORFI is required for spore pigmentation, and when expressed ectopically in the vegetative mycelium along with the products of *whiE*-ORFII–VII it leads to retention of the resulting spore pigment-related metabolites in or on the mycelium. A role for *whiE*-ORFVIII in normal spore pigmentation was demonstrated for the first time by the spore colour of the ORFVIII mutant YU114. This was greenish, compared with the normal grey colour of *whiE*⁺ strains.

Co-operation between components of the *act* and *whiE* minimal PKS subunits induced by ectopic expression

Complementation of *act*-PKS mutations by the corresponding *whiE*-PKS genes. Attempts were made to complement mutations in each of the three *act* minimal PKS genes by the corresponding *whiE* genes. The first experiments involved lesions in *actI*-ORF3, encoding the *act*-ACP. Since none of the 12 'classical' *actI* mutants of Rudd & Hopwood (1979) mapped in ORF3 (Fernández-Moreno *et al.*, 1992; Sherman *et al.*, 1992), three constructed null

mutants (CH6, CH12 and CH13) (Khosla *et al.*, 1992) were used. The three strains were transformed with two of the expression plasmids (Fig. 4) carrying the ACP-encoding *whiE*-ORFV, either as the only member of the minimal *whiE*-PKS gene set (pIJ4287) or as part of the trio of such genes that includes the KS and CLF genes (pIJ4277). The results (Table 4) clearly demonstrated formation of a functional 'hybrid' PKS in each of the six combinations, since all strains produced diffusible pigment in the presence but not in the absence of thio-strepton.

Interestingly, the amount of pigment induced by both plasmids in CH6 was considerably less than in CH12 and CH13. A possible explanation lies in the difference between the ACP defects in the three *actI*-ORF3 mutants. CH6 carries a mis-sense mutation that changes serine-42 (the site of attachment of the 4'-phosphopantetheine prosthetic group) of the ACP to alanine; CH12 contains a frame-shift mutation near the 5' end of the ACP gene; in CH13 a precise deletion has removed the whole of the gene. In CH6 the mutant *act*-ACP could probably associate with the other two *act*-PKS subunits to produce an inactive PKS, lacking the acyl carrier function because of a failure to attach the 4'-phosphopantetheine prosthetic group. The functional *whiE*-ACP component, supplied from the expression of *whiE*-ORFV on the plasmids, might not compete fully with the non-functional *act*-ACP to associate with the other two *act*-PKS components, and

Table 5. Complementation of *actI*-ORF1(KS) and *actI*-ORF2 (CLF) mutants by *whiE*-ORFIII/IV

<i>act</i> mutants	Culture pigmentation induced by plasmids carrying various <i>whiE</i> -PKS genes (encoding the PKS subunits in parentheses)					
	pIJ4277 (KS, CLF, ACP)	pIJ4279 (CLF, ACP)	pIJ4280 (KS, ACP)	pIJ4281 (KS, CLF)	pIJ4286 (KS)	pIJ4287 (ACP)
B60 (<i>act</i> -KS mutant)	–	–	Purple	Dark green	Blue	–
B78 (<i>act</i> -CLF mutant)	–	Dark green	–	Dark green	–	–

–, Same phenotype as host strain without plasmid.

so the proportion of functional ‘hybrid’ PKS would be small. In contrast, the frame-shift and deletion mutants (CH12 and CH13) would not contain complete *act*-ACP protein. This hypothesis is also compatible with the different performances of pIJ4277 and pIJ4287. As well as providing a functional *whiE*-ACP, pIJ4277 contains the genes encoding the *whiE*-KS and *whiE*-CLF, whereas pIJ4287 encodes only the *whiE*-ACP. pIJ4277 induced much less diffusible pigment than pIJ4287, consistent with the idea that the *whiE*-ACP encoded by pIJ4277 was efficiently sequestered by the *whiE*-KS and *whiE*-CLF, making it less available to interact productively with the corresponding *act*-PKS components.

As a further test of complementation of an *act*-ACP lesion by *whiE*-ORFV, this time at single copy number in the chromosome, the wild-type *whiE* cluster of CH13 was replaced by the ‘mutant’ *whiE* cluster on pIJ4277 by double crossing-over. To do so, CH13/pIJ4277 was transferred to 39 °C and selection was made for a thiostrepton-sensitive recombinant that failed to produce blue pigment and had colourless spores. Southern blotting confirmed that this strain, YU127, had the expected deletion of ORFI and II at the chromosomal *whiE* locus. One or other of two plasmids carrying *tsr* was then introduced into YU127: these were pGM160 and pIJ68 (Passantino *et al.*, 1991). pIJ68 is a multi-copy plasmid that carries the activator for the actinorhodin gene cluster, *actII*-ORF4 (Fernández-Moreno *et al.*, 1991). When the resulting strains were grown in the presence of thiostrepton, blue diffusible pigment was produced, which was especially abundant in YU127/pIJ68.

To test for complementation of a lesion in the *act*-KS, the mutant was B60 (Rudd & Hopwood, 1979), with a C (frame-shift) deletion in the 5′ region of *actI*-ORFI, and for the *act*-CLF it was B78, with a GC (frame-shift) deletion in the 5′ region of *actI*-ORF2 (Sherman *et al.*, 1992). Both mutants are *red*⁺, so they produce the red prodigiosine pigments. They were transformed with a set of six plasmids carrying various combinations of *whiE*-ORFIII–V (KS, CLF, ACP) under the control of *ptipA*. Marked pigmentation was induced in B60 by the three plasmids carrying the *whiE*-KS gene (pIJ4280, pIJ4281 and pIJ4286) and in B78 by the two plasmids carrying the *whiE*-CLF gene (pIJ4279 and pIJ4281) (Table 5). The lack

of any pigmentation induced by pIJ4277 in B60 or B78 was surprising because this plasmid carries both the *whiE*-KS and -CLF genes and caused yellow-orange pigment to be induced in the Δact strain YU105 (see above); probably such pigmentation was masked by the *red*-encoded pigments. As in the case of the *actI*-ORF3 (ACP) results, we can invoke sequestration of homologous PKS subunits to explain the much lower level of pigmentation (if any) in transformants carrying pIJ4277 (which carries the trio of genes for the *whiE* minimal PKS) than in transformants carrying the plasmids encoding only one or two of the *whiE*-PKS subunits, which caused much more intense pigmentation.

Complementation of *whiE*-PKS mutations by the corresponding *act*-PKS genes. The results described above indicated that each of the components of the *whiE* minimal PKS can relieve blocks in the actinorhodin biosynthetic pathway caused by lesions in the corresponding *act* minimal PKS components. To test the possibility of ‘reciprocal’ complementation, in which *act*-PKS components could compensate for *whiE*-PKS lesions to produce spore pigment, use was again made of six of the expression plasmids carrying one, two or all three of the *whiE* minimal PKS genes (Fig. 4), which were introduced into YU106 (*act*⁺ $\Delta whiE$). pIJ4274 was the ‘*whiE*⁺’ control plasmid carrying *whiE*-ORFI–VII, while YU105 was the $\Delta act \Delta whiE$ control host. The results are in Table 6.

As expected, the spore pigmentation of both YU105 and YU106 was restored to the normal grey colour by pIJ4274. All six of the *whiE* mutant plasmids induced a pale grey spore phenotype in YU106. That this was caused by complementation of the lesions in *whiE*-ORFIII–V by the corresponding *act* minimal PKS genes was shown by the total absence of spore pigmentation induced by these plasmids in the Δact strain YU105. Moreover, all spore pigmentation induced by the various plasmids in YU106 depended on the presence of thiostrepton in the medium, confirming that it was due to expression of the plasmid-encoded *whiE* genes. Interestingly, all the plasmids caused some delay in production of blue diffusible pigment (actinorhodin) by YU106, consistent with the possible sequestration of *act*-PKS subunits by the presence of ectopically expressed *whiE* minimal PKS components in the substrate mycelium.

Table 6. Spore pigmentation induced by plasmids carrying sets of *whiE* genes in mutants lacking *whiE*-ORFI-VII in the chromosome

Plasmid*	<i>whiE</i> genes on plasmids	Spore pigment induced in strain†	
		YU105 ($\Delta act \Delta whiE$)	YU106 ($act^+ \Delta whiE$)
pIJ4274	I-VII	+	+
pIJ4279	I, II, IV, V, VI, VII	—	±
pIJ4280	I, II, III, V, VI, VII	—	±
pIJ4281	I, II, III, IV, VI, VII	—	±
pIJ4286	I, II, III, VI, VII	—	±
pIJ4287	I, II, V, VI, VII	—	±
pIJ4288	I, II, VI, VII	—	±

*See Fig. 4.

†+, Full spore pigmentation (grey); ±, partial spore pigmentation (pale grey); —, no spore pigmentation (white).

DISCUSSION

The results reported here have further demonstrated the power of the colony and spore pigmentation phenotypes of *S. coelicolor* for genetic analysis of the *act* (Rudd & Hopwood, 1979; Malpartida & Hopwood, 1984; Sherman *et al.*, 1992; Khosla *et al.*, 1992, 1993), and *whiE* genes (Hopwood *et al.*, 1970; Chater, 1972; Davis & Chater, 1990). They have yielded new information about the roles of the various *whiE* genes in controlling spore pigmentation and about the possibilities for biochemical 'cross-talk' between the *act* and *whiE* gene products when the normal regulation of the genes is changed.

Roles of the *whiE* ORFs

The *whiE* DNA sequenced by Davis & Chater (1990) complemented the two available *whiE* mutants (C107 and C124; Chater, 1972), but their locations within the *whiE* cluster are unknown. Therefore the demonstration that at least seven of the eight known *whiE*-ORFs (i.e. with the possible exception of either ORFVI or ORFVII) are essential for normal spore pigmentation is novel. Information on the roles of the various ORFs is as follows.

The *whiE*-ORFI gene product does not resemble known proteins (Davis & Chater, 1990). Ectopic expression of the gene in the substrate mycelium has provided circumstantial evidence that this protein plays a role in retaining, or targeting, the spore pigment to an appropriate site in or on the spore. This came from the finding that in the absence of *whiE*-ORFI copious pigmentation appeared in the medium, whereas the pigment remained with the mycelium when *whiE*-ORFI was present.

The other seven known *whiE* genes probably all control steps in the biosynthetic pathway for spore pigment. *whiE*-ORFVIII almost certainly encodes a 'tailoring' enzyme for a late step in the pathway because its disruption resulted in a change in spore colour from grey to greenish.

This echoes an early observation that mutants with greenish spores could readily be isolated after mutagenesis of wild-type *S. coelicolor* and that they mapped to a chromosomal location compatible with that of the later mapped *whiE* mutations (D. A. Hopwood & H. M. Kieser, unpublished). Furthermore, the greenish spore pigment phenotype of YU114 (carrying a *whiE*-ORFVIII mutation) may explain the observation that inserting extra copies of the DNA fragment carrying *whiE*-ORFI-VII into wild-type *S. coelicolor*, *S. lividans* or *Streptomyces parvulus* caused the spore colour to change from grey to greenish (Davis & Chater, 1990). Perhaps this resulted in a comparative excess of the *whiE*-ORFVIII substrate, mimicking the effect of blocking the reaction catalysed by *whiE*-ORFVIII. *whiE*-ORFVIII resembles *S. halstedii* *sch*-ORFC, and both gene products in turn resemble FAD-linked hydroxylases (Blanco *et al.*, 1993). Mutation of *sch*-ORFC changed the spore colour of *S. halstedii* from green to lilac (Blanco *et al.*, 1993). Perhaps the different spore colours in *S. coelicolor* and *S. halstedii* reflect slight differences in one or more tailoring steps in the biosynthesis of the two spore pigments, since their carbon backbones are probably similar (Yu, 1995). However, the finding that expression of *whiE*-ORFVIII did not change the mycelial pigmentation (YU114 compared with YU107) might suggest that the tailoring step catalysed by the *whiE*-ORFVIII product cannot occur on the incompletely elaborated pathway intermediates or shunt products induced by ectopic expression of *whiE*-ORFII-VII, which are presumed to encode early biosynthetic steps (see below). There may well be further steps in the pathway controlled by unidentified genes acting between the metabolite produced by the *whiE*-ORFVII product and the substrate for the *whiE*-ORFVIII product. Alternatively, this might have reflected a lack of expression of the chromosomal copy of *whiE*-ORFVIII in these experiments.

The roles of *whiE*-ORFIII-V can readily be rationalized because of the resemblance of their protein products to

the KS, CLF and ACP of other minimal PKSs (Davis & Chater, 1990; McDaniel *et al.*, 1994), even though their ectopic expression alone gave rise to no obvious pigmentation. Their use to complement mutations in the corresponding genes for the *act* minimal PKS (see below) confirms their roles.

The *whiE*-ORFVI gene product resembles the N-terminal half of a family of related gene products in the PKS gene clusters for aromatic polyketide antibiotics. These other genes, many of which show evidence of an ancestral internal duplication to give rise to the C-terminal halves of the gene products (Bibb *et al.*, 1994), are implicated in early cyclization steps of aromatic polyketides (Zhang *et al.*, 1990; Sherman *et al.*, 1991), probably by catalysing aromatization of the first carbocyclic ring (McDaniel *et al.*, 1994). Similarly, both *whiE*-ORFII and *whiE*-ORFVII resemble genes encoding presumed cyclases acting in tetracenomycin biosynthesis after the product of the *whiE*-ORFVI homologue; these are *tcmJ* and *tcmI* respectively (Summers *et al.*, 1992, 1993; McDaniel *et al.*, 1995). Our results strongly support an enzymic role for the *whiE*-ORFII product in the biosynthetic pathway to spore pigment because of the replacement of a yellow-orange shunt product by a dark green compound when ectopic expression of *whiE*-ORFII was added to that of *whiE*-ORFIII–VII.

Taking all these observations together, we predict a likely order of action of the *whiE* gene products in the biosynthesis of the spore pigment as (III + IV + V) → VI → II → VII → → → VIII.

Interactions between *act* and *whiE* expression

Following the discovery that lesions in the *act*-PKS could be complemented by genes encoding subunits of other aromatic PKSs (Sherman *et al.*, 1992; Khosla *et al.*, 1992, 1993), Kim *et al.* (1994b) found that an *actI*-ORFI (KS) null mutant could produce blue pigment (presumably actinorhodin) when the corresponding *whiE* gene (*whiE*-ORFIII) was introduced into it on a plasmid vector, but that an *actI*-ORF2 (CLF) mutation was not complemented by its *whiE* homologue. In the experiments of Kim *et al.* (1994b), expression of the introduced *whiE*-CLF gene relied on readthrough from the *tsr* vector promoter, which may not have been expressed at a high level, even though this was sufficient for complementation of the *act* mutation by the corresponding granaticin CLF gene. In our experiments, ectopic expression of the *whiE*-CLF gene did cause the same *actI*-CLF mutant to produce pigment, perhaps because of stronger expression of the *whiE* gene by induction of *ptipA*.

We have now shown that deficiencies of *any* of the three components of the minimal *act*-PKS – KS, CLF or ACP – can be complemented by the corresponding *whiE*-PKS subunits to produce diffusible mycelial pigments. These are likely to have been actinorhodin and/or intermediates or shunt products in its biosynthesis, except when the CLF subunit came from the *whiE*-PKS. This deduction is plausible because exchanging the KS or ACP components of various aromatic PKSs has not so far altered the

‘programming’ of the synthase (McDaniel *et al.*, 1993a, b). In contrast, complementation of an *act*-CLF mutant by the *whi*-CLF subunit may well have given rise to one or more novel metabolites (as is hinted at by the greenish colour of cultures in which the *whiE*-CLF could have been active: Table 5), because the carbon chain length of the spore pigment is longer than the 16 carbons of actinorhodin (Yu, 1995). Unfortunately, chemical analysis of these cultures did not reveal a clear picture, probably because of the instability of the metabolites (Yu, 1995). The programming capability of the *whiE* minimal PKS is currently under study in the expression system (McDaniel *et al.*, 1993a) that has proved so successful for other aromatic PKSs (Yu, 1995). Interestingly, the results also provided circumstantial evidence that homologous combinations of minimal PKS subunits may form more effectively than ‘hybrid’ combinations, implying some degree of protein–protein interaction in the type II aromatic PKS, which was already suggested by the failure of some other KS/CLF hybrids to work effectively (McDaniel *et al.*, 1993a, b; Kim *et al.*, 1994b).

In reciprocal fashion, any of the three *act* minimal PKS subunits could complement lesions in the corresponding *whiE*-PKS subunits to produce spore pigment. Again, the possibility remains that this differed from the normal spore pigment when the *act*-PKS was the source of the CLF, but information is lacking on this point. It was perhaps unexpected that the presence of thiostrepton in the agar medium could lead to efficient enough induction of *ptipA* and resulting expression of the *act* minimal PKS genes to complement lesions in the *whiE*-PKS genes in an appropriate location, but this turned out not to be a problem.

Clearly, a differential spatial localization of expression of the two sets of PKS genes during normal development of the *S. coelicolor* colony is implied by the findings of Kim *et al.* (1994b) and substantiated by our results. This would prevent the potential for biochemical co-operation between the *act*- and *whiE*-PKS subunits from being realized; otherwise, mutations in the *act*- or *whiE*-PKS genes would not abolish actinorhodin production or spore pigmentation respectively. The same reasoning implies that the fatty acid synthase(s) (FAS) of the organism also is normally not involved in productive ‘cross-talk’ with the *act*- and/or *whiE*-PKS. Whether this too depends on different spatial and/or temporal expression of the FAS and PKS genes, or whether the FAS gene products are biochemically incapable of interacting productively with those of the *act*- and/or *whiE*-PKS genes, is not known, but can now be addressed because putative *S. coelicolor* FAS genes are becoming available (Revill *et al.*, 1995). Already it is clear that one enzyme that may well interact with all minimal FAS and PKS complexes, malonyl CoA: ACP acyltransferase, is likely to be shared by the FAS and at least the *act*-PKS (Revill *et al.*, 1995). A possible indication of a potential for ‘cross-talk’ between the FAS and *whiE*-PKS is the observation of early growth inhibition when all three *whiE* minimal PKS subunits were ectopically expressed in the substrate mycelium, but it remains to be seen whether this reflects

an interaction between the synthases to produce abnormal, potentially harmful, fatty acids, or whether the interference is only at the level of competition for substrates or cofactors.

ACKNOWLEDGEMENTS

We thank Peter Revill for help and many useful discussions during the course of this work. We also thank Peter Revill, Tobias Kieser, Mark Buttner, Keith Chater and Mervyn Bibb for useful comments on the manuscript. The research was supported by grants-in-aid from the BBSRC and the John Innes Foundation.

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Received 10 March 1995; revised 6 July 1995; accepted 12 July 1995.