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-ORFII-VII led to production of mycelial pigments; these were exported into the medium when whiE-ORFI 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-ORFII-VIII, as follows: whiE-ORFIII, 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-ORFII, VI, VII are likely to be involved in cyclizations of the nascent carbon chain; whiE-ORFVIII controls a late step in the spore pigment biosynthetic pathway, probably a hydroxylation; and whiE-ORFI 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 \( \text{whiE} \) and \( \text{sch} \) 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 ketoreduction, 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 \( \text{actI-ORFI} \), \( \text{-ORF2} \) and \( \text{-ORF3} \), respectively, in the actinorhodin cluster and by their homologues in the other gene clusters (Fig. 1). Additional subunits of the PKSs are responsible for further cyclizations of the nascent carbon chain (McDaniel et al., 1994). Some, but not all, PKSs also include a ketoreductase (KR), which reduces a specific keto-group.

The known extent of the \( \text{whiE} \) cluster of \( S. \) coelicolor consists of an operon of seven genes (\( \text{whiE-ORFI-VII} \)) and at least one divergently transcribed gene, \( \text{whiE-ORFVIII} \) (Davis & Chater, 1990; Fig. 1). Normally the \( \text{whiE} \) 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 to gain insight into their roles in spore pigment biosynthesis, we inserted the thiostrepton-inducible \( \text{tipA} \) promoter (Murakami et al., 1989) into the \( \text{whiE} \) cluster either on plasmids or in the chromosome. By adding thiostrepton to the medium we could then observe production of \( \text{whiE} \)-derived pigments during vegetative growth. Study of mutants in which various of the \( \text{whiE} \) 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 set of \( \text{act} \) genes had been deleted so that the effects of expressing the \( \text{whiE} \) genes could be observed in the absence of possible interactions with the products of genes encoding the \( \text{act} \)-PKS.

Other experiments were designed to study the functional relationships between the \( \text{act} \)- and \( \text{whiE} \)-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 \( \text{act} \)- and \( \text{whiE} \)-PKSs were artificially co-expressed. It was found that each of the three subunits of the \( \text{whiE} \) minimal PKS could complement lesions in the \( \text{act} \)-PKS to produce actinorhodin (and perhaps related compounds). Conversely, the corresponding \( \text{act} \)-PKS subunits could complement mutations in the \( \text{whiE} \) 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 DH5α 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, thioestrepton was added at 5 µg ml⁻¹ to induce the tspA promoter(s).

Construction of the tspA divergent promoter cassette. A fragment carrying tspA, with the tspα transcriptional terminator upstream of it, was excised as an StuI (blunt-ended)–EcoRI fragment from pJ6017 and ligated into pAK113, which also carries tspA (Murakami et al., 1989), cut with SmaI and EcoRI to yield pJ4267 (Fig. 2). The resulting tspA divergent promoter cassette was then excised as a PstI–EcoRI fragment and ligated into pDH5, digested with the same enzymes, to yield pJ4268. The promoter cassette was further excised as a convenient Spal fragment for creating pJ4274 and pJ4275 (see Results). [For the record, pJ6017 carries a variant of tspA, tspA*, with an extra G inserted 64 bases upstream of the transcriptional start site (Takano, 1993), whereas pAK113 carries the wild-type tspA; in some situations (H. Wang, personal communication) tspA* has been reported to act as a stronger promoter but this was not significant in the present work. In pJ4274, tspA* was responsible for transcription of wbiE-ORFI-VII.]

RESULTS

Construction of S. coelicolor strains deleted for the wbiE gene cluster

A prerequisite for studies of the biochemical functions of the various wbiE genes was a set of strains containing defined deletions in the wbiE locus. A primary deletion of the known wbiE-ORFI–VII genes was engineered using a marker-replacement (‘suicide’) vector, pIJ4263 (Fig. 3). On this plasmid, the hygromycin resistance gene (byg) of Streptomyces hygroscopicus (Malpartida et al., 1983) replaced the Spal fragment that includes the seven wbiE genes on pIJ2156 sequenced by Davis & Chater (1990), leaving ~1:1 kb of DNA from the wbiE locus on either side of byg. 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 wbiE-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 thioestrepton but containing hygromycin to yield the thioestrepton-sensitive Δact ΔwbiE strain YU105.

Table 1. S. coelicolor A3(2) derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B60</td>
<td>hisA1 araA1 strA1 act-160 (actI-ORF1) SCP2+</td>
<td>Rudd &amp; Hopwood (1979)</td>
</tr>
<tr>
<td>C107</td>
<td>wbiE17 SCP1+ SCP2+</td>
<td></td>
</tr>
<tr>
<td>C124</td>
<td>wbiE124 SCP1+ SCP2+</td>
<td></td>
</tr>
<tr>
<td>CH1</td>
<td>proA1 argA1 red60</td>
<td>Khosla et al. (1992)</td>
</tr>
<tr>
<td>CH5</td>
<td>proA1 argA1 red60 actI-ORF3/actVII</td>
<td></td>
</tr>
<tr>
<td>CH6</td>
<td>proA1 argA1 red60 actI-ORF3 (mis-sense)</td>
<td></td>
</tr>
<tr>
<td>CH12</td>
<td>proA1 argA1 red60 actI-ORF3 (frame-shift)</td>
<td></td>
</tr>
<tr>
<td>CH13</td>
<td>proA1 argA1 red60 actI-ORF3 (deletion)</td>
<td></td>
</tr>
<tr>
<td>CH999</td>
<td>proA1 argA1 red60 Δact (ermE)</td>
<td>McDaniel et al. (1993a)</td>
</tr>
<tr>
<td>YU105</td>
<td>proA1 argA1 red60 Δact (ermE) ΔwbiE (byg)</td>
<td></td>
</tr>
<tr>
<td>YU106</td>
<td>proA1 argA1 red60 ΔwbiE (byg)</td>
<td>This work</td>
</tr>
<tr>
<td>YU107</td>
<td>proA1 argA1 red60 Δact (ermE) wbiE</td>
<td></td>
</tr>
<tr>
<td>YU108</td>
<td>proA1 argA1 red60 Δact (ermE) wbiE</td>
<td></td>
</tr>
<tr>
<td>YU114</td>
<td>proA1 argA1 red60 Δact (ermE) wbiE</td>
<td></td>
</tr>
<tr>
<td>YU116</td>
<td>proA1 argA1 red60 Δact (ermE) wbiE</td>
<td></td>
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<tr>
<td>YU127</td>
<td>proA1 argA1 red60 actI-ORF3 (deletion) wbiE</td>
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*All strains are SCP1- SCP2- except where indicated.
hybridization confirmed that this strain arose by the expected second crossover, which replaced \textit{whiE-ORFI–VII} on the chromosome by \textsc{byg}. \textit{YU105} sporulated as well as the \textit{whiE} \textit{CH999} but lacked spore pigmentation; like \textit{CH999}, \textit{YU105} produced no actinorhodin or any pigmented precursor of it.

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

<table>
<thead>
<tr>
<th>Table 2. Plasmids</th>
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<tr>
<td><strong>Plasmid</strong></td>
</tr>
<tr>
<td>pAK113</td>
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<tr>
<td>pDH5</td>
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<tr>
<td>pGM160</td>
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<tr>
<td>plJ68</td>
</tr>
<tr>
<td>plJ680</td>
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<tr>
<td>plJ699</td>
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<tr>
<td>plJ922</td>
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<tr>
<td>plJ4256</td>
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<td>plJ4257</td>
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<tr>
<td>plJ4263</td>
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<tr>
<td>plJ4267</td>
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<tr>
<td>plJ4268</td>
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<tr>
<td>plJ4274</td>
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<td>plJ4281</td>
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<td>plJ4286</td>
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<td>plJ4291</td>
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<tr>
<td>plJ5607</td>
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<tr>
<td>plJ6017</td>
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<tr>
<td>pSG5</td>
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<tr>
<td>pUC13/18</td>
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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+ ΔwbiE strain YU106. Southern hybridization confirmed that YU106 had the expected genomic arrangement at the wbiE and act loci.

To confirm that neither YU105 nor YU106 had suffered any mutation, apart from deletion of wbiE-ORFⅧ-VII, that might have had potential to affect spore pigmentation, plJ4257 (a ColE1-pSG5 bifunctional replicon carrying the wbiE gene cluster fragment from plJ2156) 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 wbiE genes

### Expression of the wbiE genes on plasmids.

The thioestrepton-inducible ptipA was used to express the wbiE genes ectopically. The wbiE gene cluster includes a gene (ORFⅧ-VII) transcribed divergently from the wbiE-ORFⅧ-VII operon (Fig. 1). Therefore two copies of ptipA were engineered back-to-back as a cassette bounded by convenient restriction sites (see Methods). plJ4274 and plJ4275 were made by inserting the ptipA cassette, in opposite orientations, into the SphI site upstream of the wbiE-ORFⅧ-VII operon on plJ4257 (Fig. 2). (For expression of the wbiE genes on these plasmids, only one member of the pair of tipA promoters is relevant, since the divergent wbiE-ORFⅧ is truncated, but both members of the pair of promoters were needed for later experiments: see below under *Induced expression of wbiE genes in the chromosome.*)

The mycelium of young transformants of YU105 (Δact ΔwbiE) by both plasmids was yellow-green in the presence of thioestrepton; 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 thioestrepton, proving that...
pigment production depended on expression of \textit{whiE-ORF}I–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 \textit{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 (\textit{△act △whiE}) and CH999 (\textit{△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 \textit{whiE-ORF}I–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 SpbI 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 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 extracellular pigment biosynthesis. Again, deletion of ORFII in the presence of the ORFIII 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 pARCl plasmid (Davis & Chater, 1990) was activated in Streptomyces lividans; pARCl 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 ttipA 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 (Δact ΔwhiE) containing each plasmid were grown at 39°C with selection for thiostrepton resistance (encoded by the ttr gene on the vector), and the resulting colonies were propagated on a medium lacking both thiostrepton and hygromycin (byg replaces whiE-ORFI–VII in YU105) to recover the appropriate TspE′HygR double crossover product. Southern hybridization confirmed that the byg-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 ttipA 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 (Δact 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

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

*See Fig. 4.

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

<table>
<thead>
<tr>
<th>Plasmids used for complementation</th>
<th>CH999 (act control)</th>
<th>Culture pigmentation induced in actI-ORF3 (ACP) mutants</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CH6 (mis-sense)</td>
</tr>
<tr>
<td>pIJ4277 (whiE-ORFIII–VII)</td>
<td>Yellow-orange</td>
<td>Brown</td>
</tr>
<tr>
<td>pIJ4287 (whiE-ORFI, II, V, VI, VII)</td>
<td>Colourless</td>
<td>Brown</td>
</tr>
</tbody>
</table>

YU105/pIJ4276. In comparable fashion, YU116, lacking both whiE-ORFI and -ORFII, secreted a light-yellow pigment resembling that made by YU105/4277 into thiostrepton-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 thiostrepton.

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
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 wbiE-ACP, pIJ4277 contains the genes encoding the wbiE-KS and wbiE-CLF, whereas pIJ4287 encodes only the wbiE-ACP. pIJ4247 induced much less diffusible pigment than pIJ4287, consistent with the idea that the wbiE-ACP encoded by pIJ4277 was efficiently sequestered by the wbiE-KS and wbiE-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 wbiE-ORFV, this time at single copy number in the chromosome, the wild-type wbiE cluster of CH13 was replaced by the ‘mutant’ wbiE 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 replacement of ORFI and II at the chromosomal wbiE 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-ORF1, 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 prodigine pigments. They were transformed with a set of six plasmids carrying various combinations of wbiE-ORFII–V (KS, CLF, ACP) under the control of ptpA. Marked pigmentation was induced in B60 by the three plasmids carrying the wbiE-KS gene (pIJ4280, pIJ4281 and pIJ4286) and in B78 by the two plasmids carrying the wbiE-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 wbiE-KS and -CLF genes and caused yellow-orange pigment to be induced in the act 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 wbiE minimal PKS) than in transformants carrying the plasmids encoding only one or two of the wbiE-PKS subunits, which caused much more intense pigmentation.

**Table 5. Complementation of actI-ORF1(KS) and actI-ORF2 (CLF) mutants by wbiE-PKS genes**

<table>
<thead>
<tr>
<th>act mutants</th>
<th>Culture pigmentation induced by plasmids carrying various wbiE-PKS genes (encoding the PKS subunits in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plIJ4277 (KS, CLF, ACP)</td>
</tr>
<tr>
<td>B60 (act-KS mutant)</td>
<td>–</td>
</tr>
<tr>
<td>B78 (act-CLF mutant)</td>
<td>–</td>
</tr>
</tbody>
</table>

–, Same phenotype as host strain without plasmid.
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-ORFVII 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 scb-ORFC, and both gene products in turn resemble FAD-linked hydroxylases (Blanco et al., 1993). Mutation of scb-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-ORFII–V can readily be rationalized because of the resemblance of their protein products to

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

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>whiE genes on plasmids</th>
<th>Spore pigmentation induced in strain†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>YU105 (ΔwhiE)</td>
</tr>
<tr>
<td>pJ4274</td>
<td>I–VII</td>
<td>+</td>
</tr>
<tr>
<td>pJ4279</td>
<td>I, II, IV, V, VI, VII</td>
<td>−</td>
</tr>
<tr>
<td>pJ4280</td>
<td>I, II, III, V, VI, VII</td>
<td>−</td>
</tr>
<tr>
<td>pJ4281</td>
<td>I, II, III, IV, VI, VII</td>
<td>−</td>
</tr>
<tr>
<td>pJ4286</td>
<td>II, III, VI, VII</td>
<td>−</td>
</tr>
<tr>
<td>pJ4287</td>
<td>I, II, V, VI, VII</td>
<td>−</td>
</tr>
<tr>
<td>pJ4288</td>
<td>I, II, VI, VII</td>
<td>−</td>
</tr>
</tbody>
</table>

*See Fig. 4.

†+, Full spore pigmentation (grey); ±, partial spore pigmentation (pale grey); −, no spore pigmentation (white).
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 wbiE-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 wbiE-ORFII and wbiE-ORFVII resemble genes encoding presumed cyclases acting in tetracenomycin biosynthesis after the product of the wbiE-ORFVI homologue; these are tcmI and tcmI respectively (Summers et al., 1992, 1993; McDaniel et al., 1995). Our results strongly support an enzymic role for the wbiE-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 wbiE-ORFII was added to that of wbiE-ORFIII–VII.

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

Interactions between act and wbiE 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 wbiE gene (wbiE-ORFIII) was introduced into it on a plasmid vector, but that an actI-ORF2 (CLF) mutation was not complemented by its wbiE homologue. In the experiments of Kim et al. (1994b), expression of the introduced wbiE-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 wbiE-CLF gene did cause the same actI-CLF mutant to produce pigment, perhaps because of stronger expression of the wbiE 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 wbiE-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 wbiE-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 wbi-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 wbiE-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 wbiE 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 wbiE-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 wbiE-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 wbiE-PKS subunits from being realized; otherwise, mutations in the act- or wbiE-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 wbiE-PKS. Whether this too depends on different spatial and/or temporal expression of the FAS and PKS genes, or whether the FAS genes are biochemically incapable of interacting productively with those of the act- and/or wbiE-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 wbiE-PKS is the observation of early growth inhibition when all three wbiE minimal PKS subunits were ectopically expressed in the substrate mycelium, but it remains to be seen whether this reflects
an interaction between the syntheses to produce abnormal, potentially harmful, fatty acids, or whether the interference is only at the level of competition for substrates or cofactors.

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