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The complex *whiJ* locus mediates environmentally sensitive repression of development of *Streptomyces coelicolor* A3(2)

José A. Aínsa · Nick Bird · N. Jamie Ryding · Kim C. Findlay · Keith F. Chater

Abstract A segment of DNA was isolated that complemented several poorly characterised sporulation-defective white-colony mutants of *Streptomyces coelicolor* A3(2) from an early collection (Hopwood et al., J Gen Microbiol 61: 397–408, 1970). Complementation was attributable to a gene, SCO4543, named *whiJ*, encoding a likely DNA-binding protein. Surprisingly, although some mutations in *whiJ* had a white colony phenotype, complete deletion of the wild-type or mutant gene gave a wild-type morphology. The *whiJ* gene is a member of a large paralogous set of *S. coelicolor* genes including *abaAorfA*, which regulates antibiotic production; and genes flanking *whiJ* are paralogues of other gene classes that are often associated with *whiJ*-like genes (Gehring et al., Proc Natl Acad Sci USA 97: 9642–9647, 2000). Thus, the small gene SCO4542 encodes a paralogue of the *abaAorfD* gene product, and SCO4544 encodes a paralogue of a family of likely anti-sigma factors (including the product of *abaAorfB*). Deletion of SCO4542 resulted in a medium-dependent bald- or white-colony phenotype, which could be completely suppressed by the simultaneous deletion of *whiJ*. A model is proposed in which WhiJ binds to operator sequences to repress developmental genes, with repression being released by interaction with the WhiJ-associated SCO4542 protein. It is suggested that this activity of SCO4542 protein is prevented by an unknown signal.

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Introduction

Streptomyces coelicolor A3(2) has been widely used as the model for this genus of antibiotic-producing, developmentally complex bacteria. Early studies of sporulation-deficient white colony mutants identified seven loci, *whiA, whiB, whiC, whiD, whiG, whiH* and

whiI, that appeared to be specifically required for sporulation of aerial hyphae in *S. coelicolor* (Hopwood et al. 1970; Chater 1972). All of these genes except *whiC* have since been cloned and sequenced, and found to encode proteins playing likely regulatory roles that are essential for normal sporulation (Chater and Chandra 2006; Flärdh and Buttner 2009). Later studies of further, often phenotypically less well-defined, white colony mutants revealed some further genes (Ryding et al. 1999), including one gene (*bldN*) for a sigma factor that is involved in both aerial growth and the sporulation of aerial hyphae (Bibb et al. 2000). In addition, reverse genetic approaches have revealed further developmentally significant genes, such as the *ssg* genes that appear to be organisers of cell-division (Noens et al. 2005; Traag and van Wezel 2008; Xu et al. 2009).

Among the initial collection of *whi* mutants, three with somewhat poorly defined oligosporogenous phenotypes (C53, C77, C193) were found to have mutations close to *strA* (Chater 1972; Chater and Merrick 1976). Because of the difficulties of scoring these phenotypes, it was difficult to establish whether the mutations in these strains mapped to a single locus, so only one mutation was allocated a gene identifier: *whiC193*. Further studies of strains carrying this mutation were cut short when it turned out that all of the stocks had died. Here we describe the isolation and characterisation of a DNA fragment, containing intact genes SCO4541-SCO4545, that complemented the *strA*-linked mutations in strains C53 and C77, leading to the recognition of the *whiJ* gene (SCO4543). Following our deposition of the DNA sequence of the complementing clone, work done in parallel elsewhere showed that a transposon insertion in SCO4543 gave a similar (but more severe) phenotype (Gehring et al. 2000). As noted by Gehring et al. (2000) and Chater and Chandra (2006), *whiJ* is a member of a large family of paralogous genes present in the *S. coelicolor* genome, and the genes flanking it are likewise members of paralogous families, with the three kinds of genes often being found clustered together. We here describe the further analysis of *whiJ* and flanking sequences both by targeted mutagenesis and by computer analysis of the genome sequences of several streptomycetes. The results indicated that WhiJ protein is a repressor of sporulation that responds to a signal transmitted via at least one of the adjacent genes.

Materials and methods

Bacterial strains and microbiological techniques

S. coelicolor A3(2) derivatives C51, C53, C77, C141, C151, C246, C248, C249 and C253 were all from the collection of mutagen-induced developmental mutants described by Hopwood et al. (1970). Derivatives of strain M145, the plasmid-free version of A3(2) whose genome sequence has been determined (Bentley et al. 2002), were also constructed in this work (Table 1). The general conditions for maintenance and growth of these strains, and the media YEME (Yeast extract, Malt extract medium), TSB (tryptone soy broth), SMMS (supplemented minimal medium), DNA (Difco Nutrient Agar), MM (minimal medium, with mannitol as carbon source), SFM (soy flour mannitol medium), R2 (a hypertonic medium) and R2YE (a yeast extract-enriched version of R2) were as in Kieser et al. (2000). For work done in *E. coli*, maintenance and growth on medium LB were as in Sambrook and Russell (2001). The general hosts for cloning in *E. coli* were DH5α (Sambrook and Russell 2001) and XL1-Blue (Stratagene), and the non-methylating strain ET12567 was used for the preparation of non-methylated DNA for introduction into *S. coelicolor* (McNeil 1988).

Phase contrast microscopy and scanning electron microscopy were as described by Ryding et al. (1998).

DNA procedures and genetic manipulation

Basic procedures for DNA work were as in Sambrook and Russell (2001) for *E. coli* and Kieser et al. (2000) for *Streptomyces*. A previously described conjugatable library of *Sau*IIIAI-partially digested fragments of DNA from *S. coelicolor* M145, cloned in a conjugation-proficient vector (pIJ698, which confers thiostrepton resistance), was maintained as an array of clone-containing *S. coelicolor* J1501 patches on master-plates (MM containing thiostrepton) (Ryding et al. 1998, 1999; Aínsa et al. 2000). The library was replicated to R2YE plates spread with spores and fragments of C53, a sporulation mutant. After incubation for 5 days, during which the plasmids containing cloned DNA were transferred by natural mating to the C53 recipient, the plates were further replicated to MM lacking the histidine and uracil required by J1501, but containing thiostrepton to

Table 1 Strains, plasmids and primers

Streptomyces strain, plasmid or primer	Details	Purpose
A3(2)	*Streptomyces coelicolor* A3(2) wild-type (prototrophic, SCP1$^+$, SCP2$^+$)	
C51, C53, C77, C89, C141, C151, C241, C246, C248, C249, C253	White colony mutants isolated after mutagenesis of A3(2) by Hopwood et al. (1970)	Potential *whiJ* mutants
M145	Plasmid-free derivative of A3(2) used for genome sequencing by Bentley et al. (2002)	Starting strain for mutagenesis of *whiJ* locus
J2452	*whiJ::hyg* derivative of M145 (*hyg* inserted at *Eco*RI site in *whiJ*)	Evaluating effect of disrupting *whiJ*
J2490	*whiJ::*Apr^R derivative of M145 (*whiJ* replaced by Apr^R cassette)	Evaluating effect of eliminating *whiJ*
J2491	SCO4542::Apr^R derivative of M145 (SCO4542 replaced by Apr^R cassette)	Evaluating effect of eliminating SCO4542
J2492	SCO4542-*whiJ*::Apr^R derivative of M145 (SCO4542-*whiJ* replaced by Apr^R cassette)	Evaluating effect of simultaneous elimination of SCO4542 and *whiJ*
J2493	SCO4542-SCO4544::Apr^R derivative of M145 (SCO4542-SCO4544 replaced by Apr^R cassette)	Evaluating effect of simultaneous elimination of SCO4542, *whiJ* and SCO4544
J2494	*whiJ*:: Apr^R derivative of C77 (*whiJ77* replaced by Apr^R cassette)	Evaluating effect of deleting mutant *whiJ* gene
pDH5	pUC19 derived *E. coli* vector containing *tsr* for selection in *Streptomyces* (Hillemann et al. 1991).	Introducing disrupted *whiJ* into *Streptomyces*
pIJ698	Conjugation-proficient cloning vector derived from plasmid SCP2* containing *tsr* for vector selection	Library of M145 DNA readily introduced into many different mutants
pIJ6205	pIJ698 containing 3.5-kb insert complementing *whiJ* mutants	Identification of *whiJ* locus
pIJ6415	pSET152 derivative containing a 1.1 kb *Bam*HI-*Dra*III DNA fragment from pIJ6205 containing *whiJ* gene (SCO4543)	Complementation of *whiJ* mutants
pIJ6411	pDH5 derivative containing the 3.8 kb DNA fragment from pIJ6205 with *whiJ* gene (SCO4543) disrupted by *hyg* cassette	Construction of J2452, a *whiJ* knock-out mutant
pIJ6416	pDH5 derivative containing a 2.3 kb *Bam*HI DNA fragment from pIJ6205, which includes complete *whiJ* gene (SCO4543)	Complementation of *whiJ* mutants
pIJ6409	pDH5 derivative containing a 1.6 kb *Xba*I-*Eco*RI DNA fragment from pIJ6205 with an incomplete *whiJ* gene (SCO4543)	Complementation of *whiJ* mutants
2StD4_13 spe F	CGGCGTCTGAATTTAGCGCACCCGAGCGTATCGCCGGTC AactagtTGTAGGCTGGAGCTGCTTC	Replacement of SCO4542 with Apr^R
2StD4_13 xba R	TCCACCACCTGGCGAAGGAATTGTGAGGTACGGGACG TGtctagaATTCCGGGGATCCGTCGACC	Replacement of SCO4542 with Apr^R
2StD4_14 spe F	TTCCGCCAGCGGGGGCGTACGGTCACGTC CCGTACCTCAactagtTGTAGGCTGGAGCTGCTTC	Replacement of SCO4543 with Apr^R
2StD4_14 xba R	TACAGGGACAGGTGAGTTGAGGCCCGGAGGA AACACGTGtctagaATTCCGGGGATCCGTCGACC	Replacement of SCO4543 with Apr^R

Table 1 continued

Streptomyces strain, plasmid or primer	Details	Purpose
2StD4_15 spe F	CACCTGCCTCCCCCTTCCGACGAACGGAGTTCCCTCATG actagtaTTTCCGGGGATCCGTCGACC	Replacement of SCO4544 with Apr[R]
2StD4_15 xba R	CATCGGCTCTCCCCTCCGTACGACGCGATCAACGAGCT AtctagaTGTAGGCTGGAGCTGCTTC	Replacement of SCO4544 with Apr[R]
2StD4_13 T2	gacaccgtgcttcgcgact	Amplification of SCO4542
2StD4_14 T1	GTGGACCTCTTGAGGCTCTG	Sequencing and amplification of SCO4543
2StD4_14 T2	caggcatgagggaactccgt	Sequencing and amplification of SCO4543
2StD4_15 T1	GGCCTCAACTCACCTGTCc	Amplification of SCO4544
2StD4_15 T2	ccgggaacggaacatgcag	Amplification of SCO4544

select recipients that had received DNA associated with pIJ698. Potential complementation was indicated when exconjugants were grey and sporulating. To confirm complementation, plasmid DNA isolated from the corresponding patch in the library was used in direct transformation of C53 protoplasts (Kieser et al. 2000). The conjugative vectors pSET152 (Bierman et al. 1992) and pDH5 (Hillemann et al. 1991) were used to introduce subcloned DNA fragments into *S. coelicolor* mutants to test complementation. For routine subcloning, pIJ2925 (Kieser et al. 2000), pDH5 (Hillemann et al. 1991) and pBluescript vectors (Stratagene) were used. Plasmids constructed in this work are listed in Table 1.

To disrupt SCO4543, the entire 3.8–kb complementing insert from pIJ6205 was excised using *Xba*I and introduced in the *Xba*I site of a derivative of vector pDH5 so that the *Eco*RI site internal to SCO4543 was unique. Then, an *Eco*RI fragment containing a hygromycin resistance cassette was inserted at the *Eco*RI site of SCO4543, resulting in plasmid pIJ6411. pIJ6411 was used to replace the wild-type allele in *S. coelicolor* M145, generating strain J2452. For complete gene deletion-replacement mutations, cosmid 2D4 from the library of Redenbach et al. (1996), which contains a DNA insert that includes the entire *whiJ* locus, was used in PCR-targeted mutagenesis with the apramycin-resistance cassette (Gust et al. 2003, 2004) and primers listed in Table 1. All mutants were confirmed by PCR.

DNA isolated from putative *whiJ* mutants was used as template in PCR sequencing reactions, using primers 2StD4_14 T1 and 2StD4_14 T2 (Table 1). The sequencing was carried out by the Genome Centre of the John Innes Centre.

In BLAST searches, different cut-off expect values were used to search for paralogues of proteins encoded by the three genes of the *whiJ* locus, based on the levels at which proteins clearly belonging to different classes began to be found.

Results

Complementation of several poorly characterised *whi* mutants by a single cloned DNA fragment

Among the white colony mutants of *S. coelicolor* A3(2) isolated by Hopwood et al. (1970 and
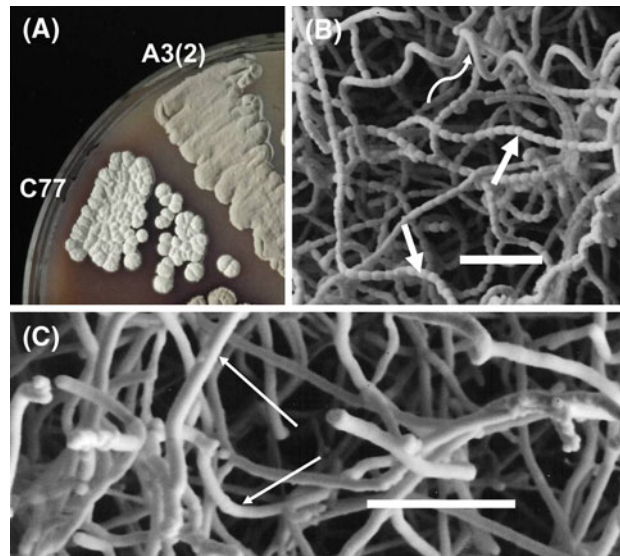
Fig. 1 Phenotype of C77, a typical *whiJ* mutant, after 5 days on SFM. **a** Petri-dish cultures of C77 and its parent strain, A3(2). C77 grows vigorously, and produces aerial mycelium, but does not develop the grey pigment typical of wild-type spores. **b** SEM of wild-type sporulating aerial mycelium. *Thick arrows*, well-developed long spore chains. *Thin wavy arrow*, spiral aerial hyphae at an early stage of sporulation. *Bar*, 10 μm. **c** SEM of C77. *Arrows*, straight aerial hyphae that have failed to sporulate. C77 sporulates so infrequently that spore chains are seldom seen by SEM, though a few spores are usually found by phase-contrast microscopy. Bar, 10 μm

unpublished) were many with a poorly defined oligosporogenous phenotype, such as C53 and C77 (Fig. 1). After conjugation of a library of DNA from *S. coelicolor* M145 (Ryding et al. 1998, 1999) with C53, three exconjugant patches were found to have grey aerial mycelium. The corresponding plasmid clones in the original library were isolated and used to transform C53 and C77. One of them (named pIJ6205) complemented both mutants. The other two complemented neither: possibly they contained inserts of DNA from close to the site of the mutation, which could mobilise nearby chromosomal DNA containing wild-type sequences corresponding to the mutation in C53. The 3.8-kb insert in pIJ6205 was sequenced (Accession number AF106004). Later, when the genome sequence of *S. coelicolor* M145 became available (Bentley et al. 2002), the sequence was found to correspond to genes SCO4541-4546 (Fig. 2; below we discuss a single nucleotide difference between the two deposited sequences).

In conjugation-mediated tests for its ability to complement a large number of other white mutants from the collection of Hopwood et al. (1970), pIJ6205 complemented several poorly characterised, mostly oligosporogenous, mutants (C51, C56, C89, C141, C151, C241, C246, C248, C249, C253). C53

and C77 had previously been found to have a *whi* mutation located just clockwise of *strA* (Chater and Merrick 1976). Since *strA* is presumed to encode the ribosomal protein RpsL (gene SCO4659, about 115 genes anticlockwise of SCO4542-4546), the genomic position of the complementing DNA was exactly as predicted from the earlier genetic linkage mapping. The locus corresponding to these mutations was named *whiJ*.

Identification of *whiJ* in the complementing DNA

The ability of smaller fragments from the original 3.8-kb clone to complement the *whiJ* mutation in C77 was tested (Fig. 2). A fragment in which the only intact ORF was SCO4543 (plasmid pIJ6415) complemented all the mutants that had been complemented by the initial 3.8-kb insert, except for C151.

To confirm that SCO4543 corresponded to *whiJ*, we constructed a mutant (J2452) with a hygromycin resistance gene inserted into the *Eco*RI site in SCO4543 (leaving codons 1–93 intact). Colonies of J2452 formed aerial mycelium, but remained white on prolonged incubation. Examination by light microscopy showed that the aerial mycelium mostly consisted of straight unbranched hyphae that failed to
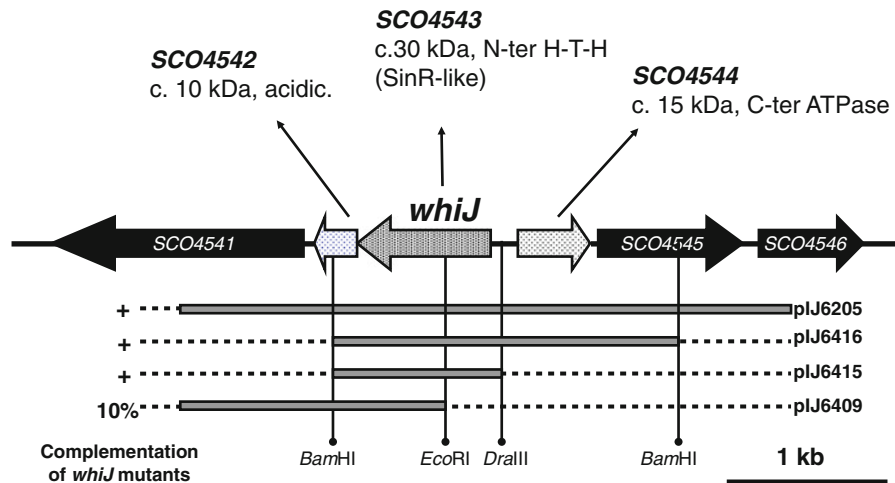
Fig. 2 The *whiJ* gene cluster. The plasmid pIJ6205, which complemented *whiJ* mutants, was isolated from a plasmid library of *S. coelicolor* M145 DNA. Sequencing revealed ORFs SCO4542, SCO4543, SCO4544, SCO4545, and incomplete SCO4541 and SCO4546. Complementation tests using the plasmid subclones shown (see also Table 1), followed by sequencing of mutant alleles and disruption of SCO4543 at the *Eco*RI site, confirmed that SCO4543 was *whiJ*

sporulate. This was similar to the phenotype of *whiJ* mutants (Fig. 1), including the transposon insertion mutant described by Gehring et al. (2000). The SCO4543-containing plasmid pIJ6415 complemented the SCO4543::*hyg* disruptant, showing that the white colony phenotype of the insertion mutant was not the result of polar effects on the expression of the downstream gene, SCO4542.

The ability of smaller fragments from the original 3.8-kb clone to complement the *whiJ* mutation in C77 was tested (Fig. 2). Complementation was observed with plasmids pIJ6416 (containing SCO4543 and SCO4544) and pIJ6415, in which the only intact ORF was SCO4543. Plasmid pIJ6415 further complemented all the other mutants that had been complemented by the initial 3.8-kb insert, except for C151. Sequencing of SCO4543 from C77 revealed one difference from the wild-type sequence in pIJ6205, at codon 104, resulting in a change from an acidic glutamate to a basic lysine residue, in agreement with the complementation of ca. 10% of C77 colonies that had received pIJ6409, an integrative plasmid carrying a truncated SCO4543 gene starting at codon 93 (Fig. 2). Sequencing also revealed SCO4543 mutations in C51 (M43I, G122K) and C248 (W88R, L100M). As mentioned above, the sequence of the 3.8-kb fragment differed at one base from that later determined for the equivalent sequence in the M145 genome (Bentley et al. 2002). This difference would

cause a Y215F difference in the WhiJ protein. Since the mutant alleles sequenced all encoded an F residue at this position, we presume that F is the correct residue in the wild-type, as indicated in the full genome sequence (Bentley et al. 2002).

Bioinformatic analysis of *whiJ* and the flanking genes

SCO4543, termed *whiJ* for the rest of the paper, is a member of a large family of paralogous genes present in the *S. coelicolor* chromosome (Table 2): 26 match *whiJ* in a protein-level BLAST search (expect value of 0.001), all gene products falling in the protein size range 258–306 aa. The genes flanking it are likewise members of paralogous families: there are 17 members of the SCO4542-like family, all in the size range 63–96 aa (the small size of these proteins means that some members of the family may be excluded by the cut-off expect value of 0.1); while SCO4544-related genes are strikingly abundant, with 48 members falling into two size classes: 31 of 120–237 aa, and 17 of 552–916 aa (cut-off expect value 0.1). Genes in the *whiJ* family are nearly always located either in between members of each of the other classes, or next to one or the other of them (Table 2) (Gehring et al. 2000; Chater and Chandra 2006).

The WhiJ protein, like all its paralogues, has a predicted N-terminal DNA-binding helix-turn-helix

Table 2 Gene clusters resembling *whiJ*

| **Paralogues of *whiJ* (SCO4543) (26)** |
| Between SCO4542-like and SCO4544-like genes (7) |
| SCO0704 SCO1979 SCO4543 SCO4678 SCO6129 SCO6236 SCO6629 |
| Next to SCO4542-like genes, not SCO4544-like genes (10) |
| SCO2246 SCO2253 SCO2381 SCO2513 (SCO2740) SCO4176 SCO4301 SCO4441 SCO5125 SCO6003 |
| Next to SCO4544-like genes, not SCO4542-like genes (5) |
| SCO1242 SCO2865 SCO3421 SCO4998 SCO7615 |
| Not flanked by SCO4542-like or SCO4544-like genes (4) |
| SCO2869 SCO3365 SCO6537 SCO7579 |
| |
| **SCO4542-like genes not adjacent to SCO4543-like or SCO4544-like genes (2)** |
| SCO2333 SCO5892 |
| |
| **SCO4544-like genes not adjacent to SCO4542-like or SCO4543-like genes (36)** |
| Size range 120–237 aa (19) |
| SCO0673 SCO0868 SCO1030 SCO3066 SCO3724 SCO3930 SCO3975 SCO4214 SCO4412 SCO4941 SCO5103 SCO5460 SCO5915 SCO5978 SCO6156 SCO6749 SCO7277 SCO7328 SCO7446 |
| Note: SCO0673 and 0868 are next to genes for anti-anti-sigmas, and 7277 is next to a *sigF*-like gene |
| Size range 552–916 aa (17) |
| SCO0542 SCO0676 SCO0767 SCO0751 SCO0946 SCO2560 SCO3284 SCO3796 SCO4120 SCO5040 SCO5104 SCO5747 SCO6484 SCO7009 SCO7158 SCO7220 SCO7354 |

domain (ending at residue 56) of the XRE/lambda C1 family. The downstream gene (SCO4542) encodes a 63-aa protein related to the c. 10 kDa acidic products of *abaA orfD*, one of a cluster of genes that influence secondary metabolism (Fernández-Moreno et al. 2002) and the developmental gene *bldB* (Pope et al. 1998; Eccleston et al. 2002), though the relationship to *bldB* was not detected at the cut-off expect value. The diverging upstream gene (SCO4544) encodes a protein with a C-terminal HATPase domain related to that of well-characterised anti-sigma factors, such as SpoIIAB of *Bacillus subtilis* (at expect values below the applied BLASTP cut-off of 0.1, *bona fide* anti-sigma factors of this class, and histidine protein kinases, were detected). Indeed, among the many SCO4544 paralogues present in *S. coelicolor*, one has a gene product that has been shown to interact with both a sigma factor and two anti-anti-sigma factors (Kim et al. 2008). SpoIIAB-like anti-sigmas antagonise members of the class of sigma factors such as σ^B and σ^F, in interactions that mediate stress-response and developmental gene expression (Helmann 1998; Mittenhuber 2002; Chen et al. 2003). The 17 larger members of the SCO4544 family all encode proteins

with long N-terminal extensions that include putative sensory domains of the PAS or GAF families, and putative phosphorylase domains related to SpoIIE and its paralogues in *B. subtilis*. In *B. subtilis*, SpoIIE functions to dephosphorylate (and thereby activate) an anti-anti-sigma factor. No member of this class of large (552–916-aa) SCO4544-family proteins is encoded by a gene adjacent to a *whiJ*-like gene.

Despite the white colony phenotype of known *whiJ* mutants, *whiJ* is not needed for sporulation

The *whiJ* alleles with white colony phenotypes that were characterised at the DNA sequence level [i.e, a constructed insertion mutant J2452; a transposon insertion mutant (Gehring et al. 2000); and the mutagen-induced *whiJ51*, −77, −248] retained an intact 5′-end region potentially sufficient to encode a DNA-binding fragment of WhiJ [with the caveat that one of two mutations in *whiJ51* results in a conservative change at codon 43 (M43I) that is also seen in several WhiJ paralogues]. To find out whether deletion of the entire gene gave a similar phenotype, we used PCR-targeted mutagenesis to replace the
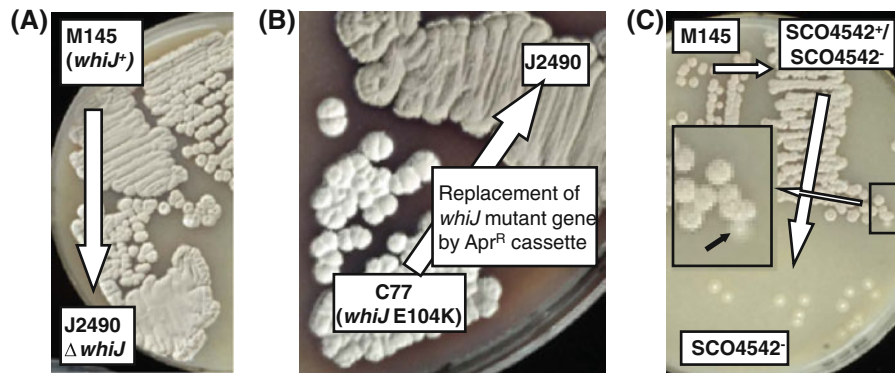
Fig. 3 Phenotypes of deletion mutants (SFM, 5 days). **a** Deletion of *whiJ* has no phenotypic effect. **b** Deletion of a point mutant allele of *whiJ* restores the wild-type phenotype. **c** Deletion of SCO4542 confers a bald phenotype. Inset, magnification showing segregation of a bald ΔSCO4542 colony from the heterogenote streakout

whole of SCO4543 with a cassette encoding apramycin resistance. To our surprise, the resulting mutant had grey, abundantly sporulating colonies that were indistinguishable from the wild-type (Fig. 3a), whether grown on minimal medium (MM), soy flour mannitol medium (SFM) or complex medium (R2YE). This suggested that the mutant alleles giving the white colony phenotype did indeed generate a partially functional WhiJ protein, capable of interfering with normal sporulation. This unexpected result led to the prediction that the introduction of the same deletion into a "standard" *whiJ* mutant, such as C77, would lead to the recovery of wild-type sporulation. This was confirmed (Fig. 3b).

From these results, a basic model was developed in which WhiJ normally binds to a cognate operator sequence close to the promoter of one or more genes whose function is essential for sporulation. Sporulation is thereby repressed by wild-type WhiJ, but can be relieved when an unknown checkpoint signal causes WhiJ to detach from operator sites. Mutant alleles giving a white phenotype would retain the ability to bind to operators and repress sporulation, but would have lost the ability to respond to the checkpoint signal. Although such alleles might be expected to be dominant to the wild-type protein, they were found to be recessive (see above). To explain this, we suppose that the mutant forms do not compete well with the wild-type protein in some way—for example, if (like many repressors) WhiJ forms oligomers, the mutant forms may fail to do so, and so be less efficient in DNA-binding; or they might form mixed oligomers with wild-type subunits to give repressors with wild-type responses.

Morphological deficiency of a SCO4542 mutant, and suppression of this phenotype by simultaneous deletion of *whiJ*

In view of the frequent association of gene pairs in the *whiJ* and SCO4542 families, it seemed possible that SCO4542 might also play a developmental role. We therefore constructed a cosmid containing a complete deletion of SCO4542, and used the mutated cosmid to replace the *whiJ* locus of the wild-type strain M145. In constructing the *Streptomyces* mutant, it was necessary first of all to introduce the mutated cosmid into the chromosome, thereby duplicating the entire chromosomal region contained in the cosmid. These partially diploid strains were selected from intergeneric conjugations by virtue of their resistance to kanamycin, conferred by the *neo* gene in the vector. Such exconjugants appeared to be morphologically normal. When they were re-plated on SFM medium selecting only for the apramycin resistance conferred by the disruption cassette, a mixture of colony morphologies was invariably seen: most wild-type, and a few bald (Fig. 3c). The latter colonies were kanamycin-sensitive, and were therefore presumptive haploid segregants with a single copy of the *whiJ* region of the chromosome carrying the SCO4542::Apr^R mutation. This was verified by PCR.

When the bald colonies were restreaked from SFM to Minimal Medium, a moderate amount of white aerial mycelium formed, and on the richer and hypertonic medium R2YE the colonies produced abundant aerial mycelium, which remained white and non-sporulating on prolonged incubation, while the substrate mycelium produced blue pigment (as did
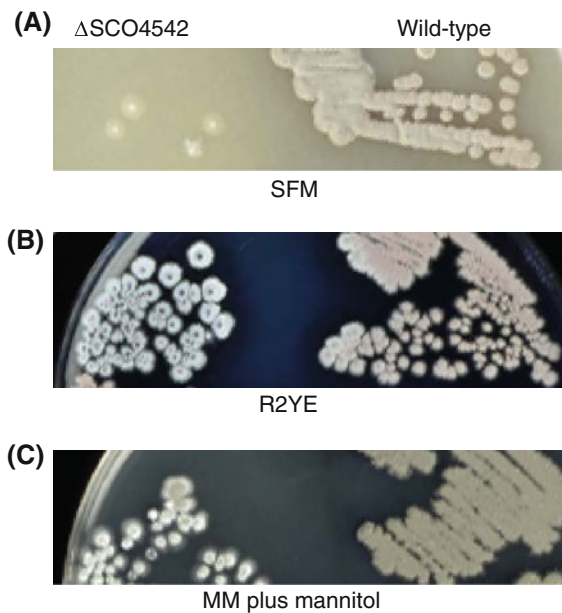
(A) ΔSCO4542   Wild-type

SFM

(B)

R2YE

(C)

MM plus mannitol

Fig. 4 The phenotype of a ΔSCO4542 mutant is medium-dependent (5 day cultures). The ΔSCO4542 colonies are bald on SFM, but on R2YE they have abundant white aerial mycelium. On MM plus mannitol aerial mycelium is sparse and white (the *grey* areas on some colonies lack obvious aerial growth)

the wild-type) (Fig. 4). It thus appeared that on SFM, SCO4542 is normally needed for aerial growth and actinorhodin production; while on R2YE that requirement is bypassed, and aerial hyphae can grow, but not differentiate into spores. Thus, the action of SCO4542 shows some sensitivity to environmental cues.

Because of the possibility that SCO4542 protein might interact with WhiJ, we went onto test the phenotype of a SCO4542/SCO4543 double deletion mutant constructed by PCR-targeting. The mutant was morphologically indistinguishable from the wild-type M145 parent on any of the media tested (result not shown). Since the morphological defects resulting from SCO4542 deletion could be bypassed by eliminating the repressor encoded by *whiJ* (SCO4543), we propose that SCO4542 acts via WhiJ.

Discussion

A cascade of negatively acting steps regulating development and secondary metabolism

The transposon mutagenesis study of Gehring et al. (2000) and our results reported here have identified a

further sporulation regulatory gene, *whiJ*. Unexpectedly, and unlike any of the previously studied *whi* genes, *whiJ* is not required for sporulation—a complete deletion of *whiJ* had no phenotypic effect. Instead, the morphological deficiencies of the *whiJ* mutants result from alleles that retain partial function.

These results indicate that WhiJ is a repressor of development, whose function in the wild-type is more or less irrelevant to conditions encountered in the laboratory, but which presumably has some adaptive benefit under natural conditions. Genomic analysis suggested that the adjacent gene, SCO4542, might be involved in some interplay with *whiJ*, and indeed a SCO4542 mutant also had a white colony phenotype on R2YE, though the SCO4542 mutant was devoid of aerial mycelium on SFM medium. Remarkably, the SCO4542 mutant phenotype was entirely suppressed by simultaneous deletion of *whiJ*.

It appears that sporulation in *Streptomyces* is subject to many checkpoint controls, particularly involving exposure to stress (e.g. Cho et al. 2001; Kelemen et al. 2001; Sevcikova et al. 2001; Chater 2001; Viollier et al. 2003; Chater and Horinouchi 2003; Lee et al. 2005). The observations on SCO4542 and *whiJ* are explicable by a model in which SCO4542 protein is involved in transmitting a checkpoint signal to WhiJ (Fig. 5). In the absence of this signal transmission, WhiJ binds to one or more targets in the genome and thereby prevents the expression of genes involved in development. The various phenotypic phenomena that we have described can then be explained as follows. In the laboratory, the checkpoint perceived via SCO4542 protein is constitutively passed, so that SCO4542 protein prevents WhiJ from binding to target DNA. In this situation, WhiJ would play no part in regulating development, hence the absence of phenotypic effects of deleting the *whiJ* gene. On the other hand, the deletion specifically of SCO4542 would result in the constitutive binding of WhiJ to its target(s), and therefore the repression of development indicated by a bald colony phenotype.

To explain the conditional white phenotype of the SCO4542 mutant, we suggest that WhiJ may undergo a SCO4542-independent change during growth on certain media, such as R2YE, with the second form (WhiJ*) being ineffective in repressing early development, but effective in repressing later stages. On this model, *whiJ* mutant alleles that give a white
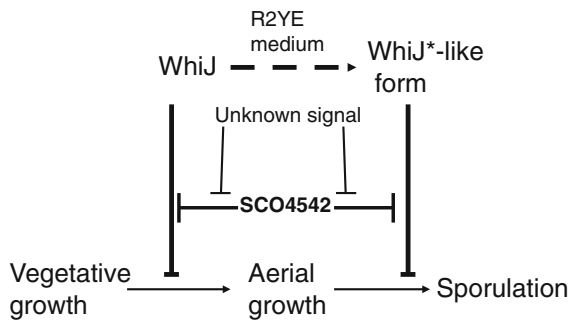
Fig. 5 Model for the negatively acting cascade encoded by *whiJ* and SCO4542. WhiJ is a repressor of unidentified genes needed for aerial growth and/or antibiotic production. WhiJ is sensitive to SCO4542 protein, which interferes with repression. In turn, SCO4542 protein is sensitive to some unknown signal, which results in release of WhiJ from SCO4542 protein, thereby repressing development. This signal is presumed to be absent under laboratory conditions, so development is independent of WhiJ. Under certain conditions (such as growth on R2YE medium), WhiJ is modified to a form (WhiJ*) that cannot repress aerial growth, but does repress sporulation of aerial hyphae. WhiJ* is also sensitive to SCO4542 protein. The forms of WhiJ in mutants such as C53 and C77 are presumed to be insensitive to SCO4542 protein

colony phenotype would mimic the WhiJ* form. Since the constructed disruptant J2542 and the transposon-induced mutant of Gehring et al. (2000) would presumably express just the N-terminal helix-turn-helix-containing domain, it is tempting to speculate that WhiJ* is a form in which an interaction between the N-terminal domain and the rest of the protein is prevented. The change to WhiJ* might either be a direct response of WhiJ to a signal or be mediated by some other, converging, signal transduction system that can interact with WhiJ.

It seems likely that each of the many pairs of genes paralogous with SCO4543 and SCO4542 may be functioning in a similar manner, with the SCO4542 homologue being involved in transmitting a signal to the SCO4543 homologue and thereby changing its regulatory action.

Implications of the frequent association of *whiJ* paralogues with SCO4544 paralogues

In this paper we do not report on mutations in SCO4544, which is also a member of a paralogous family frequently represented in *whiJ*-like clusters, and which will need to be accommodated as the models for *whiJ* action become increasingly refined.

Here we make two observations: a strain in which the three genes SCO4542-4544 are all deleted has a medium dependent phenotype of being bald and overproducing antibiotics (N Bird and KF Chater, unpublished); and the SCO4544 product is a member of a protein family at least one of whose members is able to interact with a sigma factor and with two anti-anti-sigma factors, and is highly likely to act as an anti-sigma factor (Kim et al. 2008). Furthermore, two of these genes (SCO0673 and SCO0868) are next to genes annotated as encoding anti-anti-sigma factors, while a third, SCO7277, is next to a gene encoding a sigma factor of the cognate family. This family of sigmas includes at least two members, σ^F and σ^N, whose function is primarily developmental (Potuckova et al. 1995; Dalton et al. 2007), and several that function primarily in stress responses, but which also influence development (Cho et al. 2001; Kelemen et al. 2001; Sevcikova et al. 2001; Viollier et al. 2003; Lee et al. 2005). It has been suggested that there may be highly complex cross-talk in the regulation of activity of sigma factors of this family (Kim et al. 2008). The evidence here that this regulation is also likely to be sensitive to signal input from *whiJ*-like gene sets appears to complicate matters even further.

The distribution of *whiJ*-like gene sets

Clusters similar to the *whiJ* cluster are widespread and numerous in sequenced genomes of other streptomycetes, but they are generally absent from any other bacterial genomes. The only exceptions are a few examples in other mycelial actinomycetes (Chater and Chandra 2006). Strikingly, though, the *whiJ* cluster is flanked on both sides by genes that are absent from other sequenced *Streptomyces* genomes, and does not itself appear to have orthologues in those genomes as judged by reciprocal BLAST analysis.

Not all such gene sets are conserved among different *Streptomyces* genomes, perhaps indicating that different species perceive an incompletely congruent set of signals as part of their adaptation to a particular range of ecological niches. Thus, the nature of the suite of such sets in any one organism may play a part in speciation. A mechanism for the acquisition of *whiJ*-family clusters by lateral gene transfer is suggested by the presence of such a cluster in the

terminal inverted repeats of SCP1, a highly transmissible linear plasmid of *S. coelicolor* that has been shown to integrate stably into the chromosome (Bentley et al. 2004).

There is little information to indicate the nature of the signals, or the target genes regulated by WhiJ-like proteins, or the areas of physiology whose activity is influenced by these regulatory complexes. However, one *whiJ*-like cluster, *abaA*, has been found to influence secondary metabolism (Fernández-Moreno et al. 2002), while mutations in an isolated SCO4542-like gene, *bldB*, cause severe impairment of development (Merrick 1976; Eccleston et al. 2002). We have also found that mutations in the complex *whiJ*-like cluster SCO2859-2869 give rise to changes in both morphological differentiation and antibiotic production (M Korberska, K Fowler and KF Chater, unpublished). It therefore seems possible that a significant fraction of such genes may have the potential to repress morphological and physiological development. Thus, the systematic elimination of all the *whiJ*-like gene sets from a genome might be expected to lead to increased readiness to differentiate. In this connection, it will be of interest to enumerate *whiJ*-locus-like gene clusters in *Streptomyces venezuelae*, a remarkably rapidly growing streptomycete that sporulates very quickly even in liquid medium (Flärdh and Buttner 2009).

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