Functional and evolutionary implications of a survey of various actinomycetes for homologues of two *Streptomyces coelicolor* sporulation genes

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In *Streptomyces coelicolor* A3(2) the *whiB* and *whiG* genes are essential for sporulation, their deduced products being a possible transcriptional activator and an RNA polymerase sigma factor, respectively. In a survey of DNA from diverse actinomycetes by Southern blotting, all samples tested hybridized with *whiB*, but only those representing genera capable of producing sporulating aerial mycelium hybridized with *whiG*. It is postulated that *whiB* may play a more intimate role in hyphal fragmentation processes (including sporulation) than *whiG*. The *whiB* and *whiG* homologues (*whiB-Stv* and *whiG-Stv*) of *Streptoverticillium griseocarneum* were cloned and sequenced, and subjected to functional tests in *S. coelicolor whiB* and *whiG* mutants. The genes were closely similar, but not identical, to their *S. coelicolor* counterparts at the DNA and deduced protein levels, and both *Stv. griseocarneum* gene products could function well in *S. coelicolor*. However, studies with hybrid transcription units suggested that the promoter region of *whiB-Stv* is somewhat inefficient in *S. coelicolor*.

Introduction

Actinomycetes are Gram-positive prokaryotes with DNA rich in G+C. Many of them exhibit some degree of filamentous growth, with only distantly spaced crosswalls, alternating with fragmentation into cells containing single genomes (Goodfellow & Cross, 1983). In some genera the filamentous habit persists throughout vegetative growth, and quasi-exponential growth is achieved by branching, giving a mycelium. Such mycelial

organisms often produce morphologically specialized structures, such as sporangia in *Actinoplanes* or aerial hyphae in *Streptomyces*, where large numbers of uni-nucleoidal spores can be formed which allow dispersal to take place. This developmental complexity has been most studied in *Streptomyces coelicolor* A3(2) (see Chater, 1989, for a recent general review). One focus of attention has been on genes which are needed for the formation of the specialized sporulation septa that divide aerial hyphal tips into spore compartments (Wildermuth & Hopwood, 1970), but are dispensable for normal growth. These early sporulation genes were identified by morphological studies (Chater, 1972; McVittie, 1974) of a collection of mutants unable to form the normal grey spore pigment (Hopwood *et al.*, 1970). Because their aerial mycelium remained white on prolonged incubation, these mutants were designated *whi* mutants. Genetic analysis (Chater, 1972) revealed at least six loci (*whiA*, *B*, *C*, *G*, *H* and *I*) at which mutations caused deficiencies in sporulation septation. Among these, *whiA*, *B*, *G* and *H* mutations seemed to cause a complete absence of sporulation septa (Chater, 1972; McVittie, 1974) (Davis & Chater, 1992; Mendez & Chater, 1987; Chater *et al.*, 1989). Two of the corresponding genes, *whiB* and *whiG*, have been cloned and sequenced. The *whiB* gene product, WhiB, is deduced to contain only 87

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amino acids, including four cysteine and two tryptophan residues as well as an unusually high content of charged residues (acidic at the amino terminus and mostly basic at the carboxy terminus) (Davis & Chater, 1992). No obvious similarities of *WhiB* to other known or deduced proteins were discovered, though a number of transcription-factor-like aspects of *WhiB* were pointed out (Davis & Chater, 1992). The deduced *whiG* gene product, however, closely resembles the RNA polymerase sigma factors σ^D of *Bacillus subtilis* and σ^F of enteric bacteria (Chater *et al.*, 1989; Helmann, 1991; Starnbach & Lory, 1992), and is here termed σ^{WhiG} , though there is no direct evidence of its mode of action. It is required (presumably directly) for transcription of promoters with sequence features resembling the consensus features of σ^D - and σ^F -dependent promoters (Tan & Chater, 1993). Additional copies of *whiG* cause excessive and ectopic sporulation, implying that σ^{WhiG} crucially controls a developmental switch (Chater *et al.*, 1989).

Both *whiB*- and *whiG*-related sequences were detectable by Southern blotting in more than 20 *Streptomyces* spp. tested (Chater *et al.*, 1989; Blanco *et al.*, 1993), bearing out the reasonable expectation that sporulation in all *Streptomyces* spp. would involve some common elements. It is less easy to predict whether homologous genes are required for sporulation or fragmentation processes in other actinomycete genera: information that may be relevant to understanding the functions of the genes and the evolution of differentiation in this morphologically diverse order. To help in addressing these questions, we here describe the use of *whiB* and *whiG* sequences as probes in Southern blots against various actinomycetes and other bacteria, leading to the cloning, sequencing and functional testing of homologues from a *Streptovorticillium* species.

Methods

Bacterial strains, plasmids and transformation. The cultivation and manipulative procedures were as in Hopwood *et al.* (1985) and Sambrook *et al.* (1989). *Streptomyces coelicolor* A3(2) derivatives were: C70 (*whiB70*) and C71 (*whiG71*), both derived from the wild-type after mutagenesis (Chater, 1972); J171 (*whiB218 cysD18 strA1 NF*; Chater, 1972) and J1501 [*hisA1 uraA1 strA1 Pgl*⁻ ($\equiv \phi C31^S$) SCP1⁻ SCP2⁻; Chater *et al.*, 1982]. *Streptomyces lividans* 66 (John Innes Institute strain 1326) was the host for some transformation experiments. The source of DNA for cloning of *whiB* and *whiG* homologues was *Streptovorticillium griseocarneum* NCIMB 40447. Actinomycetes were maintained on R2YE agar (Hopwood *et al.*, 1985). Morphological studies were done on suitably supplemented MM containing 0.5% mannitol as carbon source (Hopwood *et al.*, 1985). pIJ558 (provided by Dr N. K. Davis), containing an 819 bp *BclI*-*BstEII* fragment including *whiB* inserted into pUC19 (Yanisch-Perron *et al.*, 1985), was the source of *whiB* probe DNA. pIJ486 (Ward *et al.*, 1986) was used as a general vector for *Streptomyces*. *Escherichia coli* JM101 was the host for M13 mp18, M13 mp19 and pUC19 derivatives (Norlander *et al.*, 1983). Transformation conditions for *Streptomyces* were as in

Hopwood *et al.* (1985), and for *E. coli* they were as in Sambrook *et al.* (1989). Eight plasmids containing permutations of *whiBP1*, *whiBP2* and the *whiB* ORF from *S. coelicolor* and the equivalent regions of *whiB-Stv* were constructed from the following modules (coordinates from Fig. 7 and Davis & Chater, 1992): *S. coelicolor* P1, 181 bp *BclI*-*BglII* fragment; *Stv.* 'P1', ca. 500 bp fragment extending from the upstream end of the sequence to the *BglII* site; *S. coelicolor* P2, 116 bp *BglII*-*NdeI* fragment from a mutagenized *whiB* sequence in which the CACATG hexamer including the *whiB* start codon had been modified to CATATG (*NdeI* site) by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987); *Stv.* 'P2', 116 bp *BglII*-*NdeI* fragment from a similarly mutagenized *whiB-Stv* gene; *S. coelicolor whiB* ORF, 531 bp *NdeI*-*BstEII* fragment from the modified (*NdeI* site-containing) *whiB* clone; *whiB-Stv* ORF, 293 bp *NdeI*-*XmaIII* fragment from the similarly modified *whiB-Stv* clone. The constructions were assembled in pIJ2181, a pUC19 derivative from which the *NdeI* site had been removed by filling-in the ends of pUC19 linearized by *NdeI*. The completed constructions were inserted into pIJ486, using the *HindIII* and *EcoRI* sites, and they were all oriented anticlockwise in pIJ486 so that they were preceded by a transcription terminator present in the vector (Ward *et al.*, 1986).

Sources of total DNA. DNA was also isolated from the following *Streptovorticillium* species: *Stv. griseocarneum* NCIMB 40447; *Stv. albireticuli* ATTC 19721; *Stv. baldacci* ATCC 23654; *Stv. distallicum* DSM 40846; *Stv. hachijoense* ATCC 19769; *Stv. ladakanum* ATCC 27441; *Stv. orinoci* ATCC 23202; and *Stv. rectiverticillium* ATCC 25491, as well as from *Streptosporangium viridogriseum* subsp. *viridogriseum* ATCC 25242 and *Actinoplanes brasiliensis* (Lepetit). The following strains were donated by H. M. Kieser: *Amycolatopsis mediterranea* ATCC 13685; *Arthrobacter simplex* NCIB 9770; *Bacillus subtilis* 168; *Corynebacterium fascians* (= *Rhodococcus fascians*) JII 2767; *Micromonospora chalybeata* var. *florida* (J. E. Davies); *Nocardia corallina*; and *Saccharopolyspora erythraea* NRRL 2338. R. Melton provided DNA from *Mycobacterium tuberculosis* var. *bovis* BCG. D. A. Hodgson provided DNA from *Myxococcus xanthus*. J. A. Downie provided DNA of *Rhizobium leguminosarum* 8400 and its derivative lacking the Sym plasmid.

Techniques for DNA manipulation in vitro. Conventional procedures were used for nuclease digestion, ligation, Southern blots, plaque lifts, DNA preparation, random priming (Amersham, oligolabelling kit) and polymerase chain reaction (Hopwood *et al.*, 1985; Sambrook *et al.*, 1989). Hybond-N membranes (Amersham) were used for Southern blotting, and cellulose nitrate membranes (Sartorius) for plaque hybridization. Hybridization conditions were as in Hopwood *et al.* (1985), with the most stringent wash being done at 65 °C in 0.1% SDS, 3 × SSC.

DNA sequencing. The dideoxy chain-terminating method (Sanger *et al.*, 1977) was used on single-stranded DNA templates of exonuclease-III-generated clones (Henikoff, 1986) in M13 mp18 or mp19, using the Sequenase kit (US Biochemical Corp.) with universal primer or oligonucleotide primers based on earlier rounds of sequencing. Open reading frames were identified by the program FRAME (Bibb *et al.*, 1984). Further processing and analysis of the sequences were by the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

Microscopy. For phase-contrast microscopy, samples were prepared by the cover-slip method (Chater, 1972). Electron microscopy of uranyl-acetate-stained thin sections was as in Chater *et al.* (1989). Scanning electron microscopy (model SX-25, I.S.I.) was done on gold- and palladium-coated cultures (Cross *et al.*, 1973) grown on Bennett agar against cover slips inserted into the agar at an angle (Locci *et al.*, 1969).

Results

DNA hybridizing with the whiB gene of S. coelicolor is present in representatives of many actinomycete genera

DNA samples from representatives of nine actinomycete genera other than *Streptomyces* (Fig. 1) were digested with *Pvu*II and subjected to Southern blotting. The filters were probed, using hybridization conditions of intermediate stringency, with a 32 P-labelled *whiB* probe consisting of a 467 bp *Apa*I fragment that contained the 264 bp *whiB* ORF and some flanking non-coding DNA (extending 122 bp upstream and 81 bp downstream of *whiB*). With one exception, a single perceptible signal was obtained with every DNA sample (Fig. 2a), in the following order of decreasing signal strength: *Streptoverticillium* and *Arthrobacter*, *Amycolatopsis*, *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Actinoplanes*, *Micromonospora* and *Saccharopolyspora*. We have not tested whether the two bands obtained with *Arthrobacter* DNA reflected two *whiB* homologues, or the presence of an internal *Pvu*II site, or incomplete digestion. In another experiment (not shown) seven other *Streptoverticillium* spp. gave signals of similar intensity to that shown in Fig. 1 for *Stv. griseocarneum* DNA, though to *Pvu*II

fragments mostly of different sizes; and a weaker signal was also obtained with a *Streptosporangium* sp. Overall, these results suggested the universal occurrence of *whiB* homologues among actinomycetes, raising the question of whether they might also occur in other, phylogenetically distant, bacterial genera. In a limited survey, the *whiB* probe was used to analyse DNA of *Bacillus subtilis*, which is Gram-positive and has about 45% G+C in its DNA, and three Gram-negative species: *Escherichia coli* (ca. 50% G+C), *Myxococcus xanthus* (ca. 70% G+C) and *Rhizobium leguminosarum* (ca. 60% G+C, containing the SYM plasmid pRL1 which encodes many – but not all – of the functions needed for the *Rhizobium*–legume symbiosis: Downie & Surin, 1990). A clear signal was obtained with *R. leguminosarum* DNA, but not with the other samples (Fig. 3). The signal was unchanged in DNA from strain 8400 lacking pRL1. A more detailed study than is currently possible would be necessary to evaluate further whether relatively diverged *whiB* homologues are present in *B. subtilis*, *E. coli* or *M. xanthus*.

Close homologues of the whiG gene of S. coelicolor are absent from some actinomycetes

The same set of actinomycete DNA samples was analysed for the presence of DNA hybridizing with *whiG*, using a probe obtained by PCR amplification of the part of the *whiG* sequence encoding much of region 2 of σ^{WhiG} (nt 288–529, coordinates of Chater *et al.*, 1989) – a region particularly similar at the amino acid level to σ^D of *B. subtilis* (Helmann *et al.*, 1988) and σ^F of *Salmonella typhimurium* (Ohnishi *et al.*, 1990), and therefore likely to be particularly highly conserved even at the DNA level among diverse actinomycetes, with their high G+C content and phylogenetic relatedness. Surprisingly, and in contrast to the results for *whiB*, homologous sequences were detected only in the *Saccharopolyspora*, *Streptoverticillium* (including seven spp. that are not shown) and *Amycolatopsis* spp., and were absent from the *Arthrobacter*, *Corynebacterium*, *Nocardia*, *Mycobacterium*, *Actinoplanes* and *Micromonospora* spp. (Fig. 2b). The use of a presumptively conserved region of *whiG* as a probe, rather than the whole *whiG* gene, and of relatively non-stringent hybridization conditions, probably explains why we were readily able to detect a signal in *Amycolatopsis mediterranea*, which had previously appeared to lack a *whiG* homologue (Chater *et al.*, 1989).

Using the same probe, a putative *whiG* homologue was detected in DNA of *E. coli* JM101, presumably corresponding to the as yet unsequenced *E. coli* gene specifying σ^F , but none was detected in DNA of *B. subtilis*, despite the presumed presence of the *sigD* gene

Fig. 1. Actinomycete genera analysed by Southern blotting: their phylogenetic positions and morphology. The phylogenetic tree is based on partial sequencing of 16S rRNA and is redrawn from Goodfellow (1989). The vignettes of morphology are redrawn from Goodfellow & Cross (1983). To simplify the diagram, only those genera mentioned in the text are named. The molecular phylogenetic position of *Saccharopolyspora erythraea* and *Amycolatopsis mediterranea* have not been established. Both were formerly designated as streptomycetes, but more recent conventional taxonomy (Lechevalier, 1989) has grouped both with nocardioforms. *A. mediterranea* resembles *Nocardia* spp. in its morphology.

Fig. 2

Fig. 3

Fig. 2. Southern blot analysis of DNA from various actinomycetes, using (a) *whiB*- and (b) *whiG*-specific probes. DNA samples were digested with *PvuII*. The most stringent wash after hybridization was with $3 \times \text{SSC}$ at 65°C . See Results for details of probes, and Methods for details of blotting and hybridization. *Streptoverticillium* sp. 43/16 is synonymous with *Str. griseocarneum*.

Fig. 3. Southern blot analysis of DNA from unicellular bacteria, using (a) *whiB*- and (b) *whiG*-specific probes. The probes and conditions were as in Fig. 2. DNA samples were digested with *PvuII*.

encoding σ^D . Examination of the *B. subtilis sigD* DNA sequence (Helmann *et al.*, 1988) reveals that even the most conserved parts of the region corresponding to the probe do not have more than 50% sequence identity to *whiG* over extended segments, whereas the *Salmonella typhimurium fliA* gene, encoding σ^F (Ohnishi *et al.*, 1990) and presumably very similar to its *E. coli* homologue, has segments of several tens of nucleotides with 60–70%

identity to the *whiG* probe. It is not known whether *Rhizobium leguminosarum* or *Myxococcus xanthus*, which did not give signals, contain a comparable gene. Nevertheless, the apparent success in detecting a signal in an organism as remote from actinomycetes in phylogeny and DNA base composition as *E. coli* gives us confidence that the absence of a signal from many actinomycetes indicates the absence of a *whiG*-like gene.

Fig. 4. Scanning electron micrograph of *Stv. griseocarneum*. Bar marker, 5 μ m. Note the occurrence of multiple whorls of branches from the principal aerial hypha. Each branch itself forms a multiple branch point from which spore-forming hyphae develop.

Fig. 5. Restriction maps of cloned *Stv. griseocarneum* DNA that hybridized with *whiB*- and *whiG*-specific probes. The regions sequenced are shaded, and the positions and orientations of the *whiB-Stv* and *whiG-Stv* ORFs are shown by arrows.

Cloning from *Streptoverticillium griseocarneum* of DNA hybridizing with *S. coelicolor whiB* and *whiG* probes

To assist in understanding the significant features of *whiB* and *whiG*, the homologue of each was cloned from *Streptoverticillium griseocarneum*. The genus *Streptoverticillium* is closely related to *Streptomyces* (Goodfellow, 1989), but *Streptoverticillium* shows a distinctive morphology during aerial mycelium development (Fig. 4): it forms whorls of branches before sporulation, in contrast to the simple lateral branching (if any) of *Streptomyces* aerial hyphae (Locci, 1989). This difference in aerial mycelium branching pattern might conceivably be reflected – or caused – by differences in sporulation regulatory genes such as *whiG* or *whiB*. DNA of *Stv. griseocarneum* was subjected to Southern blotting analysis, to identify suitable fragments for cloning. As a result, *XmaI* fragments of ca. 1.9 kb (*whiB*-homologous) and ca. 2.4 kb (*whiG*-homologous) were isolated from agarose gels and inserted into the *XmaI* site of M13 mp18. The

desired clones, presumptively carrying *whiB-Stv* (pIJ2174) and *whiG-Stv* (pIJ2177), were identified by plaque hybridization. The restriction maps of representative examples are shown in Fig. 5.

The cloned *Streptoverticillium* DNA fragments partially restore sporulation to the appropriate *S. coelicolor* mutants

The two cloned segments of *Streptoverticillium* DNA were subcloned, as *HindIII*–*EcoRI* fragments, between the cognate sites of pIJ486, a multicopy vector for *Streptomyces* spp. The resulting plasmids (pIJ2176, containing *whiB-Stv*, and pIJ2179, containing *whiG-Stv*) were introduced into *S. coelicolor* strains J1501 (morphologically wild-type), C70 (a *whiB70* mutant) and C71 (a *whiG71* mutant). Microscopic analysis showed that C70 was induced to sporulate by pIJ2176, and C71 by pIJ2179. In neither case was complementation complete, as shown by the relatively low numbers of spores and the failure of white aerial mycelium of the colonies to develop the grey colour typical of abundantly sporulating wild-type colonies (though they were slightly greyer than the *whi* mutants carrying only the vector, pIJ486).

The presence of multiple copies of *whiB-Stv* (on pIJ2176) in the sporulation-proficient strain J1501 had no marked effect on colony morphology or sporulation. However, multiple copies of *whiG-Stv* (pIJ2179) caused J1501 to grow as small, black colonies with little obvious aerial mycelium, and electron microscopy (Fig. 6) revealed that the substrate mycelium of such colonies contained numerous spores – a feature previously shown to result from the presence of multiple copies of the *S. coelicolor whiG* gene (Chater *et al.*, 1989).

These experiments indicated that the two *Streptoverticillium* genes were probably structurally intact and functionally closely similar to their *S. coelicolor* homologues, but also suggested that neither gene was fully efficient in the heterologous host.

The sequence of *whiB-Stv* is highly similar to that of the *S. coelicolor whiB* gene

To localize *whiB-Stv* in the 1.9 kb fragment cloned, a nested set of exonuclease-III-generated unidirectional deletions of the M13 mp18 clone pIJ2174 was generated from the end of the insert closer to the universal sequencing primer site. Samples taken at different times during exonuclease III treatment were analysed by Southern blotting, using the *S. coelicolor whiB* probe. The hybridization signal was not detectable when more than 0.45 kb had been deleted, showing that the region hybridizing to the probe was close to the universal sequencing primer site. Computer analysis of the nucleotide sequence of this region (593 bp) revealed a *whiB*-like

Fig. 6. Electron micrographs of a thin section of a colony of *S. coelicolor* containing multiple copies of *whiG-Stv*. Strain J1501/pIJ2179 was grown on MM containing mannitol (0.5%) and thiostrepton ($5 \mu\text{g ml}^{-1}$). A colony was examined after 4 d. Large arrowheads show spore chains, and small arrowheads show the position of the agar surface.

ORF (*whiB-Stv*) preceded by a weak ribosome-binding site and 303 bp of upstream sequence (Fig. 7). An alternative translation start at a GTG codon at positions 238–240 is less likely because it is not preceded by a ribosome-binding site, it would invoke the use of several rarely used codons, and it would differ significantly from the *S. coelicolor* homologue (which has a TAA stop

codon corresponding to positions 253–255 in Fig. 7). Like *WhiB* protein from *S. coelicolor*, *WhiB-Stv* contains 87 amino acids. There are differences between the two proteins at only three positions, and each difference is conservative. At the nucleotide level, the two ORFs are 88% identical. In the non-coding 150 bp immediately upstream of the ORFs there is also extensive sequence identity: this region includes the developmentally regulated promoter *whiBP2* identified by Soliveri *et al.* (1992) (Fig. 7). Upstream of –150 the sequences diverge, although about 55 nt can be identified with punctuated similarity to the constitutive low level promoter *whiBP1* identified in *S. coelicolor* (Soliveri *et al.*, 1992; Fig. 7).

Experiments with hybrid whiB transcription units show that the product of whiB-Stv is fully functional in S. coelicolor

In view of the lack of knowledge about the molecular function of *WhiB* protein, we wished to establish whether the three amino acid differences between *WhiB-Stv* and *WhiB* of *S. coelicolor* were responsible for the apparently suboptimal complementation of the *whiB70* mutant by *whiB-Stv*. This was addressed by constructing a series of six hybrid transcription units, using modules that contained the *whiBP1*, *whiBP2* and *whiB*-coding regions of *S. coelicolor* and the equivalent segments of *Stv. griseocarneum*. These constructions exploited common *Bgl*II sites separating P1 from P2, and *Nde*I sites introduced at the start of each coding region by site-directed mutagenesis. The *Stv. griseocarneum* and *S. coelicolor whiB* regions were also reconstructed from the appropriate modules. The constructs were introduced into two different *S. coelicolor whiB* mutants, C70 (*whiB70* point mutant) and J171 (*whiB218* deletion). There were four main observations.

(i) All the constructs gave some degree of complementation, but this was much more obvious in the J171 deletion mutant, which was caused in every case to form sporulating grey aerial mycelium. In the C70 point mutant, complementation even by the reconstructed *S. coelicolor whiB* was manifested as a faint greyness in the aerial mycelium, and phase-contrast microscopy of impression preparations revealed mainly long, helical, non-sporulating aerial hyphae together with a few small areas containing spore chains (Fig. 8). These observations suggest that *whiB70* is partially dominant over the wild-type allele.

(ii) In J171, the reconstructed *S. coelicolor whiB* gene (i.e. with its own P1 + P2 region) gave rapid sporulation, whereas the *S. coelicolor whiB* ORF downstream of a promoter region that included either 'P1' or 'P2', or both, from *Stv. griseocarneum* gave full sporulation only after a further day. Thus, there is a difference in the

1 GACAGGGGCGCTCCGAGTGGTTCCTCTAAACATCTTCAGCCGGTGCAACGGGACGGGGCGG 60
 .GATCAGGTTCG...G..GC...T.T...A...AT..
 61 AGTGTCTCTCTCTTCGAAGAGGAACGGGATGACTGGACAGGGGGTGGAAAGTGTG 120
 .TC.C..GGGCG..C.TCCT..T.C[-----35nt-----]A.T.GTA...
 P1 ~~~~~
 121 CAATCTTGTGACATTTCGCCACTTTCGGCTTAGCCTTGGTTCGCGAGAGTGAATATGG 180
 G.GGT..A..G.GA...AA...A.T..A.G.G.....A..
 BglII
 181 GCCCAATAGCAGATCTCGGCTGACTGGCCCGGATCGGCACACTTGTAAATTCACCTG 240
C.....G.A.....
 P2 ~~~~~
 241 TCGTCTCCGACCCGATGGCGGCGAGCACCACGGGGACGCAATGACAGACGAGGGGG 300
A...G.AT..G.AA...TAG.T.....G.A.....
 V D D SacI
 301 M T E L F Q Q L L V E E A D E E L G W 19
 CACATGACCGAGCTGTTCGACCACTGCTGCTCGAGGAGCGGACGAGGCTCGGCTGG 360
 20 Q E R A L C A Q T D P E S F F P E K G G 39
 361 CAGGAGCGCGCGTGTGCGCCGACCGATCCTGAGTCTCTTCCCGAAAGGGCGGA 420
 40 S T R E A K K V C L A C E V R S E C L E 59
 421 TCCACGCGGGAGGCGAAGAAAGTCTGCTCGCTGTGAAGTCCGTTCCGAGTGCCTTGA 480
 60 Y A L A N D E R F G I W G G L S E R E R 79
 481 TAGGCCCTTGCGAAGCAGAACGCTTCGATTTGGGGCGGTTTGTCCGAGCGTGAGCGC 540
 80 R R L K K A A V stop 87
 541 CGCCGCTGAAGAAGCGCGGCTCTGAGGACCGGACCGAGCGGGCGCGG 593
 SmaI

Fig. 7. DNA sequence of *whiB-Stv*. A purine-rich potential ribosome-binding site is underlined. Dots under the sequence mark base positions at which the sequenced region upstream of the *S. coelicolor whiB* gene (Davis & Chater, 1992) is identical to that of *whiB-Stv*, base symbols indicating differences. Between nt positions 85 and 111, alignments were not obvious. The dots on wavy arrows show the positions of transcription start sites for the *S. coelicolor whiB* promoter region (Soliveri *et al.*, 1992). The three positions (F5V, E11D, E12D) at which the deduced polypeptide specified by *whiB-Stv* differs from those of *S. coelicolor* and *S. lividans* are indicated above the sequence. Selected restriction enzyme target sites are identified.

efficiency of the promoter regions from the two organisms in an *S. coelicolor* host.

(iii) In J171, the *whiB-Stv* coding region gave rapid and full complementation when expressed from the P1 plus P2 promoter region of *S. coelicolor*. This showed that the three conservative amino acid differences do not significantly affect the protein's function in *S. coelicolor*.

(iv) During microscopic observation of aerial mycelium from the various constructions in C70, in which the mutant phenotype was only partially corrected, we observed a single example of a multiple branch point (C70/pIJ2184, Fig. 8b; this triple branch was not a chance apposition of separate fragments, since it moved as one piece when nearby air bubbles coalesced during microscopic examination). We have never previously seen such branch points in *S. coelicolor* or its *whi* mutants, but they are typical of *Streptovercillium* spp. (Fig. 4).

The whiG genes of Stv. griseocarneum and S. coelicolor are highly similar

The part of the 2.3 kb *Streptovercillium* DNA fragment of pIJ2177 that hybridized to the *whiG* probe was located, by the exonuclease III method described in the

Fig. 8. Phase-contrast micrographs of aerial mycelium. (a) Typical partial complementation of C70 (*S. coelicolor whiB70*), in this case by a plasmid carrying the *S. coelicolor whiB* gene with an *NdeI* site introduced at the start of its coding sequence, and its natural promoter region. Arrows indicate a spore chain (the wild-type phenotype) and a coiled aerial hypha (the *whiB* phenotype). (b) A rare three-way branch in an aerial hypha of C70 (*whiB70*) bearing a plasmid containing the *S. coelicolor whiB* gene and P1 promoter, but with the P2 promoter region replaced by homologous DNA from *Stv. griseocarneum*. Bar markers, 10 µm.

1	TCGGGCTGAAGGGTTAAGCGATGACGGCGAGGCGAGCGATGCGCGAGCTCCGTACGG	60
61	CGCGCGACACCGGTGCGGCGCCAGGCGCGGCGCGCGCGCGAGCATATCCGCGCAT	120
	[← no alignment →]TC...CCTGTA.AGCCTTC.....A.G....T...T.	
121	CACCGAAGAGTTCCTCTTCCCTCAACCGCGAGCGCTCAGTCAAGCTACGCTCAGCGGAATC	180
	GGGT.CCCACCCCT.....A.....ATGC.....ATG.C.	
181	CGGCATTCTGACCCCTCGATCCACGTCAACAGGACGGCTCAGAGCGAGCAATGCCCC	240
	..CA.[←50nt→]GCA.....A...GG.....A.T..G.....	
4	HTSGSDRAAAPPAAARVGSV	23
241	AGCACACCTCGCGGTCCGACCGCGCGCTGCGCGCGCGCGCTCGCGCGCGCGCGCG	300
24	PAPSTLDELWRSYKASGDER	43
301	CTCCGCGCCCTCGAGCTCGAGCTGTGCGGTCTGTAAGGCTCGGCGCGAGAAC	360
44	LREQILIHYSPLVKYVAGR	63
361	GGCTGCGGAGCAGTGTCTCTGCTACTCGCGCTGTGTAATACGTGCGCGCGCGG	420
64	SVGLFPNVEQADFVSSGIF	83
421	TCAGCTCGGACTCGCGCCCAATGTGAGAGCGAGCTCTCTCTCGGAGCTCTCG	480
84	LIDAIKFKDPARAIKFKET	103
481	GGCTGATCGAGCCATCGAGAAATCGACCCCGCGAGGCACTCAAGTTCGAGACCTAC	540
	BamHI	
104	ITRIRGAMIDELRALDWMIPR	123
541	CCATACCCGGATCCGGGAGGAGTATCGAGCAACTGGCGCGCTGAGCTGGATCCCGC	600
124	SVRQKARAVRAYATLEAEL	143
601	GCTCGCTCGCGAGCGCGCGCGCTCGAGCGCGCTACGCCACGCTGAGCGCGGAGC	660
	* S * V D	
144	RRRTPTTEAEVAENGIALEE	163
661	TGCGCGCGACGCCACCGAGCGGAGGTGCGCGGAGATGGGCTCGCGCTGGAAGA	720
	A E	
164	HSVFSQLSLANVVALEDLH	183
721	TGCACAGCTTTTCAGCGAGTGTCTCGCGCAAGCTGTGCGCTCGAGAGAGCTGTGC	780
	G G	
184	AGDEGGERLSLMDTLEDTA	203
781	ACCGCGCGAGCGCGCGCGAGCGCTCAGCCTCATGACACGCTGAGGACACCGCG	840
	N * R	
204	DDPVEVAEDHELRLRLARAI	223
841	CGAGACCGCGTCTGAGTGGCGAGGACACGAGCTCGCGCGACTGTCTCGCGCTGCA	900
	* * * * *	
224	NLTPEREKTQVVTLYYYEGLT	243
901	TCAACACCTCCCGAGCGGAGAGACCTGCTACCTGCTACTACTACGAGGCGCTCA	960
	* * * * *	
244	LAEIGQVLGVTESRVSIHT	263
961	CGCTGCGAGATCGCGGCTCGGTCTCAGCGAGACCGCGGTCTGCTGAGTCCACA	1020
	PvuII	
264	KSVLQLRAKLADVGR stop	278
1021	CCAGTCTGCTCTCAGCTGCGGCGCAAGCTGCGGATGTGCGAGCTGAATCGTCTCTC	1080
1081	AGCGTCTCTCAGCGGTGGTTCCTAGAGTGAAGCGTCCCGAGGATTCGAGCGG	1140
1141	CTCGTGGCGGACACCGGAGTGCAGCGCGCGCGCTCTGGAACGCGCGCTCCCT	1200
1201	CGTCTCGAGCGGTACCGAGCGCTGACCTTCCCGCGCTCGCGAGCGCTCGGAGCT	1260
1261	GCGCGCTCTCTGCTATGAGTACTTCCGCTCGCGCGCGCGGTGCTGAGGACTGTCG	1320
1321	CGCGAC	1326

Fig. 9. DNA sequence of *whiG-Stv*. Symbols are as in Fig. 7, except that comparisons are with the *S. coelicolor whiG* sequence (Chater *et al.*, 1989). Two additional residues present in the *S. coelicolor whiG* gene product are shown in a triangle. A single base gap in the aligned *S. coelicolor* sequence is shown opposite position 120. In addition, amino acid residues that are marked with asterisks are identical in σ^D of *B. subtilis* (Helmann *et al.*, 1988), σ^F of *Salmonella typhimurium* (Ohnishi *et al.*, 1990) and σ^F of *Pseudomonas aeruginosa* (Sternbach & Lory, 1992). An imperfect inverted repeat appropriately positioned to act as a terminator is indicated by punctuated horizontal arrows.

preceding section, to a position about 1.3 to 1.6 kb from the universal-primer-proximal end of the insert (Fig. 5). The sequence of 1326 bp from this region was determined (Fig. 9). Computer analysis revealed a single ORF (termed *whiG-Stv*) preceded by a weak ribosome-binding site. The deduced protein product of the ORF is closely similar to the *S. coelicolor whiG* gene product (90% identity with one gap; at the DNA sequence level, there

is 85.4% identity). Apparent initiation and termination codons are found in the same positions in the two *whiG* genes. The comparison helps to resolve an uncertainty about the choice of probable translation start site in *S. coelicolor*, in which a second potential start site for *whiG* was suggested involving the GTG codon corresponding to valine 23 (Chater *et al.*, 1989): a GCG (alanine) codon occupies the corresponding position (21) in *whiG-Stv* (Fig. 9). It is therefore very likely that the ATG codon indicated in Fig. 9 corresponds to the correct start codon for the *S. coelicolor whiG* gene. None of the sequenced 233 bp upstream of the *whiG-Stv* ORF showed obvious coding characteristics. Alignment of this region with that upstream of the *S. coelicolor whiG* ORF showed regions of similarity separated by regions of divergence. Notably, the 35 bp immediately upstream of the ORFs differed at only six positions, and another comparably similar segment (five differences in 40 bp) was observed slightly further upstream (Fig. 9).

Discussion

Phylogenetic and evolutionary significance of the distribution of *whiB*- and *whiG*-related sequences

In all the actinomycete genera represented in this study, a form of fragmentation of rod-shaped or filamentous cells occurs that might be evolutionarily equivalent to sporulation in *S. coelicolor*, and which might therefore involve genes homologous with *whiB* and *whiG*. We found apparent *whiB* homologues in all the strains, whereas *whiG* homologues were of more limited occurrence. For one strain, *Stv. griseocarneum*, the genes were shown to function in *S. coelicolor*. We assume that the hybridizing sequences detected in the other strains are also *whiB* or *whiG* homologues and are functional, though it is not ruled out that some of them may be cryptic or their hybridization entirely fortuitous.

The *whiG* probe included the DNA specifying the region 2.4 of σ^{WhiG} , believed from studies of other σ factors to contact the -10 region of cognate promoters (reviewed by Lonetto *et al.*, 1992). The absence from several actinomycetes of DNA similar to the *whiG* probe therefore indicates a probable absence of σ^{WhiG} -like proteins and of the promoters that would be recognised by a σ^{WhiG} form of RNA polymerase holoenzyme: that is, the probable absence of a σ^{WhiG} regulon. Alternatively, there may be relatively high amino acid sequence divergence among σ^{WhiG} homologues: an interpretation that we do not favour, since σ^{WhiG} proteins from *S. coelicolor* or *Stv. griseocarneum* are rather similar to homologues from such distant organisms as *B. subtilis*, *Salm. typhimurium* and *P. aeruginosa* (Helmann, 1991); and since a hybridization signal was obtained with *E. coli* DNA. The organisms in which *whiG*-related sequences

were detected differ from those giving no signal, in that they represent genera which can form a sporulating aerial mycelium (Fig. 1). (*Nocardia corallina* is probably more correctly classified as a *Rhodococcus*, a genus that typically does not form aerial mycelium: Lechevalier, 1989.) It will be interesting in future to learn whether molecular phylogeny shows *A. mediterranea* and *Sac. erythraea* to be close to *Streptomyces* and *Streptovorticillium*, since the proposed role of *whiG* in the switch from indeterminate aerial growth to sporulation has potentially high adaptive, and therefore evolutionary, significance. In fact, both organisms were originally described as *Streptomyces* spp. (Margalith & Beretta, 1960; Labeda, 1987). On the parsimonious hypothesis that sporulation septation in *S. coelicolor* and fragmentation in other actinomycetes are indeed homologous processes, *whiG* would appear not to be centrally involved in either process, whereas the omnipresent *whiB* might play a more direct role. In those organisms from which *whiG* homologues are absent, transcription of the *whiB* homologues cannot depend, directly or indirectly, on a σ^{WhiG} form of RNA polymerase. This is consistent with results showing that *whiB* is transcribed from its normal promoters in a *whiG* mutant of *S. coelicolor* (Soliveri *et al.*, 1992).

The apparent absence of a *whiG* homologue from a motile-spored *Actinoplanes* (even using conditions of reduced stringency: results not shown) was perhaps surprising, because σ^{WhiG} homologues in other Gram-positive and Gram-negative bacteria are essential for the transcription of some of the structural genes for flagellum formation (Helmann, 1991). Some flagellar genes of other bacterial groups are transcribed by a form of RNA polymerase holoenzyme containing σ^{54} or its homologues (Helmann, 1991), so – among other, more unprecedented, possibilities – such a holoenzyme might conceivably account for the σ^{WhiG} -independent expression of flagellum genes in *Actinoplanes*.

Functional significance of sequence comparisons involving *whiB* and *whiG* homologues

The divergence between *S. coelicolor* and *Stv. griseo-carneum* is greater for *whiG* than for *whiB*. The *whiB-Stv* gene product differs at only three amino acid residues from that of the *S. coelicolor whiB*. At position 5, phenylalanine and valine are alternatives, suggesting a preference for a hydrophobic amino acid at this position; and the glutamate or aspartate alternatives at positions 11 and 12 suggest a requirement for acidic residues in these positions. Davis & Chater (1992) previously proposed that positions 11 and 12 contributed to the charged face of an amphipathic α -helix. The restoration of abundant sporulation to a *whiB* deletion mutant of *S.*

coelicolor by certain plasmids carrying the *whiB-Stv* coding region shows that these amino acid changes do not significantly impair function in the heterologous genetic background. The *whiB-Stv* promoter region appears to be somewhat inefficient in *S. coelicolor*, so it is possible that a relatively low level of *whiB-Stv* transcription may also be the natural situation in its proper host. On the basis of a single observation of a verticil-like structure in a poorly complemented *whiB* mutant of *S. coelicolor* (Fig. 8), it might even be proposed that the characteristic morphology of *Streptovorticillium* spp. results from a low level of *whiB* expression.

The comparison between the two WhiG proteins reveals more divergence than the WhiB comparison, with 28 differences in 279 positions (90% identity, compared with 97% identity for the WhiB comparison). When the two WhiG proteins are compared with σ^D (*B. subtilis*) and σ^F (*Salm. typhimurium* and *P. aeruginosa*), it emerges that, of the 68 positions identical between σ^D and the two σ^F sequences, no fewer than 57 (84%) are also identical in the *whiG* products (asterisks in Fig. 9), suggesting that these are functionally highly significant residues. At a further 90 positions, the two *whiG* products are identical to each other and to one of the other three σ factors. The differences of the *whiG-Stv* gene product from that of *S. coelicolor* do not seem to affect its ability to function in *S. coelicolor*, as judged by the ability of *whiG-Stv* DNA at high copy number to cause ectopic sporulation in *S. coelicolor*. We cannot readily explain why this phenotype is not seen when *whiG-Stv* is introduced into a *whiG* mutant of *S. coelicolor*.

Concluding remarks

Clearly, the comparative study of these developmental genes among actinomycetes can give information relevant to their function, regulation and evolution. Further analysis may require disruption of *whiB* and *whiG* homologues – a technique currently possible in only a few actinomycetes. These genetically manipulable actinomycetes include members of the genus *Mycobacterium*. The representative strain that we studied (*M. tuberculosis* var. *bovinus* BCG) contains a homologue of *whiB*, but not one of *whiG*. If the hypothesis that *whiB* could control fragmentation of these organisms is correct, interference with WhiB function might seriously inhibit their growth, and WhiB could be an interesting target for treatments aimed at mycobacterial infections (especially if *whiB* homologues are indeed absent from most non-actinomycete bacteria). Besides leprosy, tuberculosis, and probably Crohn's disease and Johne's disease, these infections include opportunistic infections of AIDS sufferers.

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