Association of early sporulation genes with suggested developmental decision points in *Streptomyces coelicolor* A3(2)

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Cytological analysis of a series of *Streptomyces coelicolor* A3(2) mutants with disruptions of early sporulation (*whi*, for white aerial mycelium) genes in an isogenic background has provided new information about the role of *whiH*, and confirmed and extended previous knowledge about *whiG*, *whiA* and *whiB*. The characteristic straight aerial hyphae of *whiG* mutants contained normally spaced vegetative-like septa, while mutants in *whiA* or *whiB* had abnormally long and coiled aerial hyphae almost devoid of septation. *whiG*, *whiA* and *whiB* were all absolutely required for sporulation septation, and for all visible signs of nucleoid condensation and partitioning and other changes associated with later stages of sporulation. On the other hand, *whiH* appeared to enhance low basal levels of these processes. Thus, *whiH* mutant aerial hyphae were divided into loosely coiled fragments of variable sizes by what appeared to be a few sporulation septa. These fragments showed some spore-like characteristics and contained condensed and aberrantly partitioned nucleoids. *whiG*, *whiA* and *whiB* were epistatic to *whiH* on the criterion that they prevented such fragments from forming in double mutants. These spore-like features and the synthesis of clearly detectable levels of the *whiE*-directed grey spore pigment were not due to any residual activity of previously studied *whiH* alleles since they were retained by a constructed *whiH* null mutant. A model is presented that explains the mutant phenotypes by proposing two early developmental decision points involved in commitment to sporulation septation, one requiring *whiG* and the other requiring *whiA* and *whiB*.

Keywords: cell division, *whi* genes, aerial mycelium, checkpoints

INTRODUCTION

During growth of rod-shaped unicellular bacteria such as *Escherichia coli* and *Bacillus subtilis*, cell wall extension takes place on lateral walls, and every DNA replication cycle is followed by partitioning of the sibling chromosomes, coordinated with the ingrowth of a centrally located septum at the site of incipient cell separation (Donachie, 1993; Park, 1996). None of these observations holds for the vegetative growth of streptomycetes to generate a substrate mycelium: most cell wall growth is at the hyphal tips; sibling DNA molecules do not show clearcut partitioning; septation occurs far

(typically about 20 µm) from growing tips to give rise to adjacent multigenomic compartments; and cell separation does not take place. Quasi-exponential growth is possible only because of the emergence of branches (Prosser & Tough, 1991).

The situation for streptomycetes is further complicated by their reproductive strategy, with which this paper is concerned. From the older parts of the substrate mycelium, new branches grow into the air to form an aerial mycelium, which eventually generates long chains of unigenomic spores. In the extensively studied organism *Streptomyces coelicolor* A3(2), some aspects of aerial hyphal wall structure change after an initial growth period, because a typical aerial hypha has a straight stalk surmounted by a somewhat coiled apical region (Wildermuth, 1970; Wildermuth & Hopwood, 1970). The onset of sporulation in such aerial hyphae

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Abbreviations: 7-AAD, 7-aminoactinomycin D; DAPI, 4',6-diamidino-2-phenylindole; Fluo-WGA, fluorescein-conjugated wheat germ agglutinin.

requires a switch to a different mode of septation and chromosome partitioning from that in vegetative hyphae. The key cell division protein FtsZ assembles into a large number of rings (sometimes as many as 50), forming a regular ladder (Schwedock *et al.*, 1997). The rings are the precursors of sporulation septa, which delimit prespore compartments into which single nucleoids are partitioned. Sporulation septa differ morphologically from vegetative septa, having a structure that leads to full separation of adjacent cells and not simply to hyphal crosswalls (Wildermuth & Hopwood, 1970). Maturation of the spores involves induction of late sporulation genes, rounding up and thickening of the cell wall and synthesis of a grey polyketide spore pigment (for reviews, see Chater, 1989, 1993, 1998; Chater & Losick, 1997).

Several so-called *whi* (white) mutants have been isolated which form sporulation-defective aerial hyphae lacking the dark grey spore pigment (Hopwood *et al.*, 1970; Chater, 1972). Previous work indicated that mutations in *whiG*, *whiA*, *whiB* or *whiH* block development at early stages and prevent sporulation septation (Chater, 1972; Hopwood *et al.*, 1970; McVittie, 1974), and more recently Schwedock *et al.* (1997) used immunofluorescence microscopy to show that *whiG*, *whiB* and *whiH* mutants fail to assemble ladders of FtsZ rings in aerial hyphae. Of these 'early' *whi* genes, *whiG* encodes an alternative sigma factor (Chater *et al.*, 1989); *whiA* encodes a protein of unknown function with orthologues in several other Gram-positive bacteria (N. J. Ryding, J. A. Ainsa, N. Hartley, C. J. Bruton & K. F. Chater, unpublished); *whiB* belongs to a group of genes unique to actinomycetes, which code for small highly charged and cysteine-rich proteins of unknown function (Davis & Chater, 1992; J. Soliveri, J. Gomez, W. R. Bishai & K. F. Chater, unpublished); and *whiH* encodes a member of the GntR family of transcription factors (Ryding *et al.*, 1998). It would be of considerable interest to understand how these genes, directly or indirectly, interact with the cell division machinery to control sporulation septation.

Despite an early suggestion to the contrary (Chater, 1972), it has become increasingly clear that the phenotypes of early *whi* mutants do not directly correspond to intermediate stages of normal development of wild-type spore chains. Rather, it seems that certain growth and/or morphological processes continue in the mutants after the point at which normal development is blocked, leading to mutation-specific terminal phenotypes. With the exception of the work by Schwedock *et al.* (1997), previous descriptions of these phenotypes have utilized only phase-contrast microscopy or transmission electron microscopy and have mostly dealt with uncharacterized presumptive point mutations which may retain residual activity to various degrees (Chater, 1972, 1975; Hopwood *et al.*, 1970; McVittie, 1974; Plaskitt & Chater, 1995; Ryding *et al.*, 1998). The availability of defined gene disruptions and the recent development of a technique for transformation with denatured chromosomal DNA (Oh & Chater, 1997) have made it

straightforward to construct a series of defined null *whiG*, *whiA*, *whiB* and *whiH* mutants in an isogenic background. We have analysed hyphal morphology, septation patterns and distribution of nuclear material in fully developed aerial mycelium of these mutants using scanning electron and fluorescence microscopy. Based on these analyses, the *whiG*, *whiA* and *whiB* genes can be associated with blocks at specific early stages of morphological development, which may represent developmental decision points in the sporulation process. Furthermore, it is shown that *whiH* affects a different stage of sporulation than previously thought, and is not absolutely required for some of the later sporulation processes.

METHODS

Bacterial strains and growth conditions. The strains of *Streptomyces coelicolor* A3(2) used in the main part of this study are listed in Table 1. The following white, non-sporulating derivatives of the wild-type were also used: C47, C63, C66, C78, C90, C96, C109, C119, C219, C220, C240, C250 and C251 (Chater, 1972; Hopwood *et al.*, 1970). The mutations in C109, C119, C219 and C250 were genetically mapped to the *whiH* locus (Chater, 1972), and the mutations in these and the remaining strains were all complemented by cloned *whiH* DNA on the low-copy-number pIJ698 (Ryding *et al.*, 1998; N. J. Ryding, personal communication). The *whiH*63, *whiH*109, *whiH*119 and *whiH*209 mutant alleles have been sequenced (Ryding *et al.*, 1998). *E. coli* strain DH5 α (Hanahan, 1983) was used for plasmid construction, and ET12567 (MacNeil *et al.*, 1992) was used for obtaining non-methylated plasmid DNA (*S. coelicolor* cannot be transformed by methylated DNA; Kieser & Hopwood, 1991) for transformation of *Streptomyces*. Media and general techniques for growth and transformation of bacterial strains and manipulation of DNA were as described for *Streptomyces* by Hopwood *et al.* (1985) and for *E. coli* by Sambrook *et al.* (1989). Surface cultures for studies of sporulation-related phenotypes were grown on minimal medium (MM; Hopwood *et al.*, 1985) agar with 0.7% mannitol as carbon source.

Construction of $\Delta whiH::ermE$ mutation. Plasmid pIJ599 carries a *SacI* fragment containing *whiH* and surrounding sequences in a derivative of the *tsr* (thiostrepton resistance)-containing pDH5 vector (Hilleman *et al.*, 1991; Ryding *et al.*, 1998). The *whiH* promoter and the first half of the *whiH* coding region were deleted from this plasmid by digesting with *MunI* and *BamHI* and replacing the 497 bp *whiH* fragment with an *EcoRI*-*BglII* fragment containing the *ermE* gene from plasmid pIJ4026 (Bibb *et al.*, 1985), which confers resistance to erythromycin and lincomycin (Fig. 1). The resulting plasmid, pIJ6304, was passed through *E. coli* strain ET12567, denatured in alkali (Oh & Chater, 1997) and used to transform *S. coelicolor* strain J1501 to lincomycin resistance (200 $\mu\text{g ml}^{-1}$). Transformants were screened for thiostrepton sensitivity to indicate loss of the vector replicon, and the disruption of the *whiH* locus in those strains was confirmed by Southern blotting. All strains in which *whiH* had been replaced with $\Delta whiH::ermE$ showed a characteristic *whiH* phenotype (see this paper). The $\Delta whiH::ermE$ mutation from one such strain, J2409, was transferred to strain M145 by chromosomal DNA transformation to create strain J2408, as described below.

Transfer of gene disruption alleles. Total DNA was prepared from previously constructed *whi* disruption mutants (Table 1)

Table 1. *Streptomyces coelicolor* A3(2) strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
Strains		
C119	<i>whiH119</i> SCP1 ⁺ SCP2 ⁺ Pgl ⁺	Chater (1972)
J1501	<i>hisA1 uraA1 strA1</i> SCP1 [−] SCP2 [−] Pgl [−]	Chater <i>et al.</i> (1982)
J1862	J1501 <i>whiG::hyg</i>	C. Whatling
J2221	J1501 <i>whiB::hyg</i>	Oh & Chater (1997)
J2031	J1501 <i>whiA::hyg</i>	N. J. Ryding
J1979	J1508 <i>sigF::tsr</i>	Porůčková <i>et al.</i> (1995)
J2210	M145 <i>whiH::hyg</i>	Ryding <i>et al.</i> (1998)
J2400	M145 <i>whiG::hyg</i>	This paper
J2401	M145 <i>whiA::hyg</i>	This paper
J2402	M145 <i>whiB::hyg</i>	This paper
J2404	C119 <i>whiG::hyg</i>	This paper
J2405	C119 <i>whiA::hyg</i>	This paper
J2406	C119 <i>whiB::hyg</i>	This paper
J2407	C119 <i>sigF::tsr</i>	This paper
J2408	M145 Δ <i>whiH::ermE</i>	This paper
J2409	J1501 Δ <i>whiH::ermE</i>	This paper
J2412	M145 Δ <i>whiH::ermE</i> Δ <i>whiE::hyg</i>	This paper
J2413	M145 <i>whiA::hyg</i> Δ <i>whiH::hyg</i>	This paper
J2414	M145 <i>whiB::hyg</i> Δ <i>whiH::hyg</i>	This paper
M145	Prototrophic, SCP1 [−] SCP2 [−] Pgl ⁺	Hopwood <i>et al.</i> (1985)
YU106	<i>proA1 argA1 redE60</i> Δ <i>whiE::hyg</i> SCP1 [−] SCP2 [−]	Yu & Hopwood (1995)
Plasmids		
pIJ698	Hyg ^R Thio ^R	Kieser & Melton (1988)
pIJ2157	pIJ698 with <i>whiB</i> ⁺ in 5.7 kb insert	Davis & Chater (1992)
pIJ6201	pIJ698 with <i>whiH</i> ⁺ in 4 kb insert	Ryding <i>et al.</i> (1998)
pIJ6204	pIJ698 with <i>whiA</i> ⁺ in 7 kb insert	Ryding (1995)
pIJ6301	pIJ698 with <i>whiG</i> ⁺ in 2 kb insert	N. J. Ryding

using the Kirby-mix procedure of Hopwood *et al.* (1985). The structures of the *whi* gene disruption alleles are summarized in Fig. 1, with the exception of the Δ *whiE::hyg* allele in which ORFs I–VII of the *whiE* gene cluster are replaced by the *hyg* marker (Yu & Hopwood, 1995). Protoplasts of recipient strains were transformed with around 2 µg alkali-denatured chromosomal DNA from donor strains as described previously (Oh & Chater, 1997). After protoplast regeneration, the antibiotic resistance markers used to disrupt the genes were selected. The presence of disrupted alleles and absence of the corresponding wild-type alleles were verified by PCR or Southern blotting, and the absence of other unsought morphological mutations was confirmed in complementation tests with the corresponding gene on the low-copy-number SCP2-derived pIJ698 vector (Table 1).

Microscopical methods. For scanning electron microscopy, colonies were mounted on the surface of an aluminium stub with O. C. T. compound (Agar Scientific) as the mounting medium. The stub was then plunged into liquid nitrogen slush at approximately −210 °C to cryopreserve the material. The sample was then transferred onto the cryostage of a CT1500HF cryo-transfer system (Oxford Instruments) attached to a Philips XL30 FEG scanning electron microscope (Philips Electron Optics). Sublimation of surface frost was performed at −95 °C for 3 min before sputter-coating the sample with platinum for 2 min at 10 mA, at colder than −110 °C. After sputter-coating, the sample was moved onto

the cryo-stage in the main chamber of the microscope, held at approximately −140 °C. The sample was viewed at 3 kV and photographs were taken using Ilford FP4 120 roll film.

Cultures for light and fluorescence microscopy were set up by inserting a sterile coverslip at a 45 ° angle into MM agar and inoculating in the acute angle along the glass surface (Chater, 1972). Coverslips were removed after 2–4 d incubation at 30 °C and cells on the coverslip surface were either stained with 4',6-diamidino-2-phenylindole (DAPI) for nucleoids (Kwak & Kendrick, 1996), or stained for the cell wall, with fluorescein-conjugated wheat germ agglutinin (Fluo-WGA) after fixation, treatment with lysozyme and blocking with BSA (Schwedock *et al.*, 1997). To stain DNA in the Fluo-WGA-treated material, 7-aminoactinomycin D (7-AAD) was included at a final concentration of 10 µg ml^{−1} (both fluorescent reagents were obtained from Molecular Probes). The samples were then washed five times with PBS (Sambrook *et al.*, 1989), and mounted for microscopy in PBS containing 50 % glycerol. Samples were studied and photographed using a Zeiss Axiophot microscope equipped for phase-contrast and epifluorescence microscopy. No filters were used for observation of DAPI staining, Zeiss filter set 09 with an added filter cutting off at 560 nm was used for Fluo-WGA, and filter set 15 was used for 7-AAD. Photographs were taken with Kodak Technical pan film; exposure was at 25 ASA with normal development for phase-contrast illumination, and at 100 ASA with development in HC-110, dilution D, 6 min, for fluor-

Fig. 1. *whi* gene disruptions used in this study. The *whiG*::*hyg* allele has a 13 codon *Bam*HI segment of *whiG* replaced by the *hyg* cassette (Chater *et al.*, 1989). The *whiA*::*hyg* allele has the *hyg* cassette inserted in a *Pst*I site after the first 21 codons of *whiA* (N. J. Ryding, N. Hartley, C. J. Bruton, J. A. Aínsa & K. F. Chater, unpublished). In the *whiB*::*hyg* allele, *hyg* replaces a *Bgl*II-*Eco*47III fragment containing all of *whiB* (Oh & Chater, 1997). The *whiH*::*hyg* allele has *hyg* inserted in a *Bam*HI site in the middle of the *whiH* coding region (Ryding *et al.*, 1998). The Δ *whiH*::*ermE* allele is described in this paper. For references, see Table 1.

escence illumination. Negative images were scanned, processed using Adobe Photoshop software and assigned monochromatic red, green or blue colours to resemble the samples as observed in the microscope.

RESULTS

Aerial hyphal morphology and septation patterns in *whiG*, *whiA*, *whiB* and *whiH* disruption mutants: *whiH* mutants attain some morphological features associated with later stages of sporulation

A series of strains with defined disruptions of the early sporulation regulatory genes *whiG*, *whiA*, *whiB* and *whiH* were constructed in an isogenic background by transforming the prototrophic, plasmid-free *S. coelicolor* A3(2) strain M145 with chromosomal DNA from existing disruption mutants. To compare the effects of these mutations on morphological differentiation, colonies that had developed on MM for 5 d were analysed by scanning electron microscopy (Fig. 2). As is typical for *S. coelicolor* wild-type strains, the parent M145 colonies were covered with abundant spore chains, which were straight or loosely coiled and often contained several tens of spores (Fig. 2a). None of the

mutants formed spore chains. The *whiG*::*hyg* mutant J2400 formed long straight aerial hyphae (Fig. 2b), very similar to those observed in previously characterized *whiG* point mutants (Chater, 1972). Slight constrictions indicating septa were frequently visible. Branches were rare. The *whiA*::*hyg* and *whiB*::*hyg* strains J2401 and J2402 closely resembled each other in possessing tightly coiled aerial hyphae (Fig. 2c–d). The coiled regions appeared to lack septal constrictions and branches, and were often very long (see below). The excessive length was further reflected in the scarcity of hyphal tips in fields of J2401 and J2402 compared to those of the other mutants. Similar coils were observed by phase-contrast microscopy of most of the originally isolated *whiA* and *whiB* mutants, although one *whiA* mutant showed considerable fragmentation (Chater, 1972). Finally, and in agreement with previous results from optical microscopy (Chater, 1972; Ryding *et al.*, 1998), the *whiH*::*hyg* strain J2210 produced loosely coiled aerial hyphae with occasional deep constrictions (Fig. 1e–f).

The patterns of septation in aerial hyphae that had been allowed to develop the mutation-specific characteristic terminal phenotypes were analysed by staining lysozyme-treated cell walls with Fluo-WGA, which binds to oligomers of peptidoglycan. DNA was stained with 7-AAD. Fig. 3 shows a typical developing spore chain of the wild-type strain M145. As described previously for another morphologically wild-type strain, J1508 (Schwedock *et al.*, 1997), ladders of sporulation septa formed synchronously, reaching right up to the tip of the hypha, and single nucleoids were partitioned into the spore compartments. In the phase-contrast microscope, the spore compartments appeared darker than other hyphae and contrasted with the stalk leading up to the spore chains. This difference became more accentuated as sporulation progressed. As exemplified by the piece of a more mature spore chain in the top righthand corner of Fig. 3(a–c), the walls of maturing spores lose susceptibility to staining with Fluo-WGA (Schwedock *et al.*, 1997).

The straight aerial hyphae of J2400 (*whiG*) contained crosswalls with a mean spacing of 15 μ m (Fig. 4a–b). These septa did not lead to cell separation and appeared more similar to vegetative crosswalls than sporulation septa. The presence of such vegetative-like septa correlates well with the shallow constrictions of the aerial hyphal walls seen in the scanning electron microscope (Fig. 2b). The tightly coiled regions of aerial hyphae in J2401 (*whiA*) were virtually devoid of septa, as shown by the absence of Fluo-WGA-staining crosswalls, and by the continuity of the 7-AAD-staining DNA (Fig. 5a–c). A very similar pattern was seen in J2402 (*whiB*). Although septa were usually absent from the coiled regions of the *whiA* and *whiB* mutants, rare single septa could be found (Fig. 5b–c). Many coils had 10–20 or even more turns without septa. Given the mean circumference of the coil of 6.2 ± 0.5 μ m (measured for both mutants in scanning electron micrographs like those in Fig. 2c–d), these coiled aseptate regions were far longer than the hyphal compartments delimited by septa

Fig. 2. Scanning electron micrographs of the surface of colonies of the wild-type strain M145 (a) and its *whi* mutant derivatives J2400 (*whiG*) (b), J2401 (*whiA*) (c), J2402 (*whiB*) (d) and J2210 (*whiH*) (e–f). Colonies had developed for 5 d on mannitol MM plates before being prepared for electron microscopy. Indentations indicating septation are indicated by arrows. Bars, 10 μ m.

Fig. 3. Characteristic aerial hyphae of the wild-type strain M145. (a) Phase-contrast micrograph; (b, c) fluorescence micrographs of hyphae stained with Fluo-WGA (b) and 7-AAD (c). The figure shows representative aerial hyphae from colonies that had developed for 3 d on mannitol MM plates before being prepared for microscopy. Bar, 10 μ m.

in the *whiG* mutant J2400. Such lengths would correspond to chains of around 100 or more spores, which are rarely seen in the M145 strain, even when looking at young Fluo-WGA-stained aerial hyphae where septa can be counted before the spore chains start to break up (data not shown).

Unlike developing spore chains of the wild-type, aerial hyphae of J2400 (*whiG*), J2401 (*whiA*), or J2402 (*whiB*) did not become dark in phase-contrast illumination, nor did they fail to stain with Fluo-WGA. In contrast, the loosely coiled aerial hyphae of *whiH* mutants were often fragmented into segments of different lengths, the fragments appeared dark in phase-contrast and their cell walls stained very poorly or not at all with Fluo-WGA. This was first observed with strain J2210 (*whiH::hyg*; data not shown), and subsequently with the

Fig. 4. Characteristic aerial hyphae of *whiG* and *whiH* disruption mutants. Phase-contrast (a, c) and fluorescence (Fluo-WGA) (b, d) micrographs of J2400 (*whiG*) (a, b) and J2408 (*whiH*) (c, d) show representative aerial hyphae from colonies that had developed for 3 d on mannitol MM plates before being prepared for microscopy. The small arrows in (b) indicate the widely spaced crosswalls found in *whiG* mutant aerial hyphae (other, more faintly staining positions of fluorescence did not correspond to crosswalls when viewed in different focal planes). The arrow in (c) indicates a hyphal fragment of the kind referred to in the text as phase-dark. Bar, 10 μ m.

Fig. 5. Characteristic aerial hyphae of strain J2401 (*whiA::hyg*). Phase-contrast micrograph (a), and fluorescence micrographs of hyphae stained with Fluo-WGA (b) and 7-AAD (c). The panels show a representative coiled aerial hypha from a colony that had developed for 3 d on mannitol MM plates before being prepared for microscopy. Arrows point to a probable septum. The results with J2402 (*whiB::hyg*) were indistinguishable from those shown here for J2401 (*whiA::hyg*). Bar, 10 μ m.

Δ *whiH::ermE* null mutant J2408 (Fig. 4c–d), the construction of which is described below. The fragmentation of aerial hyphae of *whiH* mutants, and the deep constrictions observed in electron micrographs of such hyphae, suggested that a few sporulation septa had formed, which was in stark contrast to the other *whi* mutants analysed here.

A *whiH* null mutant produces perceptible amounts of the *whiE*-encoded grey spore pigment

The production of aerial hyphal fragments with spore-like characteristics in the *whiH::hyg* strain J2210 indicated that *whiH* may not be required for all late sporulation processes. This was further supported by the previous observations that *whiH* mutants develop a slightly grey aerial mycelium while *whiG*, *whiA* and

whiB colonies remain pure white (Ryding *et al.*, 1998) and by low, but detectable, transcription of the major operon of *whiE* spore pigment genes and of *sigF*, encoding a late sporulation σ factor, in a *whiH* point mutant (Kelemen *et al.*, 1996, 1998). However, the possibility could not be excluded that previously studied *whiH* mutants retained residual *whiH* activity because they contained either missense mutations, a frameshift in the C-terminal half, or uncharacterized mutations (Chater, 1972; Ryding *et al.*, 1998). Also, the *whiH::hyg* insertion mutation, which gives rise to a very low level of grey pigmentation, allows expression of the N-terminal half of *WhiH*, including a putative DNA-binding motif (Ryding *et al.*, 1998). A knockout mutation of *whiH* was therefore constructed by deleting the promoter and the first half of the gene and replacing it with the *ermE* gene. When introduced into M145 to

Fig. 6. Pigmentation of aerial mycelium on the surface of strains M145, J2210, J2408 and J2412 (mannitol MM, 4 d). J2412 (a *whiE whiH::ermE* double mutant) is creamy white whereas the *whiH::ermE* single mutant (J2408) is almost as grey as the wild-type M145. The *whiH::hyg* mutant (J2210), which retains the *whiH* promoter and much of the *whiH* gene, is pale grey.

produce strain J2408, this $\Delta whiH::ermE$ allele gave aerial hyphae which were morphologically and cytologically very similar to those of other *whiH* mutants (see Fig. 4c–d and below). The aerial mycelium of J2408 colonies accumulated readily perceptible grey pigment, to a level intermediate between J2210 (*whiH::hyg*) and M145 (Fig. 6). The extent of grey pigmentation was medium-dependent (e.g. J2408 appears white on soya flour mannitol agar), which may explain why *whiH* mutants had appeared no less white than any other white colony mutants in the original screen (Hopwood *et al.*, 1970; see also Ryding *et al.*, 1998).

The grey pigment of the wild-type is of polyketide origin, and its synthesis is directed by the *whiE* gene cluster (Davis & Chater, 1990; Yu & Hopwood, 1995). To find out whether the grey pigment of *whiH* mutants was the same as that of the wild-type, the *whiE::hyg* mutation of Yu & Hopwood (1995) was introduced into J2408. The resulting strain, J2412, lacked the grey pigment and had, like other *whiE* mutants, a very pale cream-coloured aerial mycelium (Fig. 6), but retained all other morphological characteristics of *whiH* mutants (data not shown). We concluded that the greyness of *whiH* mutants is indeed due to the *whiE*-encoded spore pigment, and therefore that *whiE* expression is not completely dependent on *whiH*. This result was consistent with the previous finding of a low level of *whiE* mRNA in a *whiH* point mutant (Kelemen *et al.*, 1998).

Condensation and aberrant partitioning of nucleoids in aerial hyphae of *whiH* mutants

Spore formation involves accurate segregation of one nucleoid to each prespore compartment (although oc-

casional abnormal events occur, as is evident from Fig. 3a–c). We used DAPI staining of methanol-fixed aerial hyphae to examine how nucleoid partitioning was affected by the *whi* mutations. As reported for several *Streptomyces* species (Kwak & Kendrick, 1996; Miguélez *et al.*, 1998; Schwedock *et al.*, 1997), separation of nucleoids is not seen in the wild-type strains until septa have started forming. Thereafter, nucleoids stain more intensely and become smaller (Fig. 7a–b). No such condensation was seen in J2400 (*whiG*), J2401 (*whiA*) and J2402 (*whiB*), nuclear material remaining more or less continuous throughout the straight or coiled aerial hyphae (Fig. 7c–e). However, away from apical parts, in particular in J2400, and in older parts of hyphae, the staining was more patchy and unevenly distributed (data not shown). Results obtained with 7-AAD staining of paraformaldehyde/glutaraldehyde-fixed mutant aerial hyphae agreed with the results from DAPI staining (data not shown). In contrast, the *whiH* mutant J2210 displayed a distinctly different pattern of nucleoid staining. In the phase-dark and often loosely coiled fragments that formed in many aerial branches, nucleoids were partitioned into bodies of variable sizes and distributed unevenly along the length of the fragments, leaving large DNA-free spaces between them (Fig. 7f). There were no nucleoids close to the poles of the phase-dark fragments, and the total length of DNA-containing sections was about equal to the total length of DNA-free sections. Otherwise, no obvious pattern of distribution could be discerned. The nucleoids fluoresced intensely and appeared to be condensed, contrasting sharply with the non-condensed DNA in the hyphal stems leading up to the phase-dark fragments. Often, the stems appeared to be lysed in ageing material, while the phase-dark fragments persisted. Further analysis revealed this pattern of DNA staining in all of 13 independent *whiH* mutants obtained after UV or NTG (nitrosoguanidine) mutagenesis (listed in Methods section; representative examples shown in Fig. 7 g–j).

A previous report indicated that *sigF*, encoding a sigma factor required for some late stages of sporulation, may be needed for condensation of DNA in mature spores (Potůčková *et al.*, 1995). To determine whether *sigF* influenced the *whiH* phenotype, a *sigF::tsr whiH119* double mutant, J2407, was constructed. It lost the grey pigmentation of the parent strain C119 (as expected, since a major *whiE* promoter is unexpressed in *sigF* mutants; Kelemen *et al.*, 1998), but the aerial hyphal morphology and DNA condensation and segregation pattern were indistinguishable from C119 (data not shown). We have no explanation for the disparity between our data showing *sigF*-independent DNA condensation in the *whiH* mutant, and the earlier results of Potůčková *et al.* (1995) obtained with a *whiH*⁺ strain.

whiG, *whiA* and *whiB* are epistatic to *whiH* in morphological studies of double mutants

Since *whiH* was not absolutely required for parts of the wild-type sporulation processes, while *whiG*, *whiA* and *whiB* mutants were completely blocked at early stages of

Fig. 7. DAPI staining of characteristic aerial hyphae of the wild-type strain M145 (b), J2400 (*whiG::hyg*) (c), J2401 (*whiA::hyg*) (d), J2402 (*whiB::hyg*) (e), J2210 (*whiH::hyg*) (f), C63 (*whiH63*) (h), C119 (*whiH119*) (i) and C219 (*whiH219*) (j). Phase-contrast micrographs of strains M145 (a) and C63 (g) are also shown. Bar, 10 μ m.

sporulation, *whiG*, *whiA* and *whiB* mutations would be expected to be epistatic to *whiH* mutations. However, early phase-contrast microscopic studies of double mutants had indicated that the epistasis pattern was $G > H > A, B$ (Chater, 1975). Those results were based on highly non-isogenic strains arising from genetic crosses. Exploiting our ability to construct strains in an isogenic background, and using the easily scorable DAPI staining pattern, the epistasis was re-examined. The

whiG::hyg, *whiA::hyg* and *whiB::hyg* mutations were transferred to strain C119, which contains a point mutation (*whiH119*) causing an amino acid substitution near the supposed WhiH DNA-binding domain, and has a phenotype like that of the *whiH* null mutant (Ryding *et al.*, 1998). Derivatives of the double mutants containing functional alleles of the relevant *whi* genes on low-copy-number plasmids were also studied as controls expected to show the relevant single mutant phenotypes.

Fig. 8. DAPI staining of aerial hyphae of the double *whi* mutants J2404 (*whiG::hyg whiH119*) (a), J2405 (*whiA::hyg whiH119*) (c), and J2406 (*whiB::hyg whiH119*) (e), and of the same strains where the *whiG::hyg* had been complemented by *whiG* on plasmid pIJ6301 (b), *whiA::hyg* had been complemented by *whiA* on pIJ6204 (d) and *whiB::hyg* had been complemented by *whiB* on pIJ2157 (f), respectively. Bar, 10 μ m.

Table 2. Phenotypes of constructed *whiA whiH* and *whiB whiH* double mutants in the presence and absence of plasmid-borne functional alleles

Strain	Plasmid	Colour of aerial mycelium*	Morphology of aerial hyphae†
M145	—	Dark grey	Sporulation
J2408 (<i>whiH</i>)	—	Pale grey	Short, phase-dark fragments
J2413 (<i>whiH whiA</i>)	—	White	Long, loose coils
	pIJ698 (vector)	White	Long, straight hyphae or very loose coils
	pIJ6201 (<i>whiH</i> ⁺)	White	Long, tight coils
	pIJ6204 (<i>whiA</i> ⁺)	Very pale grey	Short, phase-dark fragments
J2414 (<i>whiH whiB</i>)	—	White	Long, loose coils
	pIJ698 (vector)	White	Long, straight hyphae or very loose coils
	pIJ6201 (<i>whiH</i> ⁺)	White	Long, tight coils
	pIJ2157 (<i>whiB</i> ⁺)	Very pale grey	Short, phase-dark fragments

* Evaluated after 5 d growth on mannitol MM medium.

† Examined by phase-contrast microscopy after 4 d growth on mannitol MM medium.

In agreement with previous epistasis data, *whiG* was epistatic to *whiH*: the aerial hyphae of strain J2404 (*whiG whiH*) were indistinguishable in morphology and DNA staining pattern from those of J2400 (*whiG*) (Fig. 8a). The phenotype of the double mutant was not affected by introduction of *whiH*⁺ on pIJ6201 (not shown), but the complementation of *whiG* by introducing pIJ6301 gave a characteristic *whiH* phenotype (Fig. 8b). J2405 (*whiA whiH*) was similar to the *whiA* single mutant, although the coils were slightly less tight (Fig. 8c). The *whiB whiH* strain J2406 had a clearly reduced tightness of the coils in comparison to the *whiB* single mutants (Fig. 8e). However, the coils of the *whiA whiH* and *whiB whiH* mutants were very long, appeared non-septated and had continuously dispersed nuclear material, in this respect closely resembling the *whiA* and *whiB* single mutants. Complementation of *whiA*::*hyg* in J2405 by pIJ6204, and of *whiB*::*hyg* in J2406 by pIJ2157, restored the *whiH* phenotypes completely (Fig. 8d, f), while introduction into either strain of the *whiH*⁺ plasmid pIJ6201 only increased the tightness of the coils (data not shown). In summary, *whiG*, *whiA* and *whiB* mutations were epistatic to *whiH119* on the criteria that they completely abolished the formation of phase-dark hyphal fragments, the occasional sporulation-like septation, and the separation and condensation of nucleoids that occurred in the *whiH119* single mutant. In addition, they abolished the pale grey pigmentation of C119 and gave pure white aerial mycelium (data not shown).

The only detectable effect of *whiH119* when it was combined with *whiA* or *whiB* disruptions was the decreased degree of coiling of aerial hyphae. To further investigate this and to exclude the possibility that it was related to any residual activity of the *whiH119* allele, double mutants were constructed also in J2408 ($\Delta whiH::ermE$). Both *whiA*::*hyg* and *whiB*::*hyg* abolished the grey pigmentation of the J2408 parent and prevented the formation of phase-dark aerial hyphal

fragments with condensed and separated nucleoids, thus exactly reproducing the results obtained with the C119 strain (Table 2). Aerial hyphae of both double mutants were long coils which appeared looser than the *whiA*::*hyg* (J2401) or *whiB*::*hyg* (J2402) single mutants. Plasmid-borne functional alleles of *whi* genes were introduced into the double mutants. The vector itself (pIJ698) reduced the tightness of the coiling of aerial hyphae further, so that the double mutants containing pIJ698 had mostly straight or slightly wavy aerial hyphae (Table 2). As expected, pIJ6204 (*whiA*⁺) restored the *whiH* phenotype to J2413, and pIJ2157 (*whiB*⁺) restored the *whiH* phenotype to J2414. The *whiH*⁺-containing plasmid pIJ6201 gave rise to tight coiling of aerial hyphae in both J2413 and J2414, reminiscent of the phenotype of *whiA* or *whiB* single mutants (Table 2). These results showed that a *whiH* null allele also caused reduced coiling in *whiA* or *whiB* mutants.

DISCUSSION

By examining the morphology and cytology of a series of *whi* disruption mutants in an isogenic background, we found that inactivation of *whiG*, *whiA* and *whiB* completely prevented all traces of sporulation septation and later stages of spore chain development, while the *whiH* phenotype was more complex. In the following, the significance of these results and earlier observations on the mutant phenotypes are discussed. A model for commitment to sporulation is suggested, involving two crucial developmental decision points defined by *whiG* and *whiA/whiB*, respectively (Fig. 9). Additional stages or decision points may possibly be recognized when further *whi* genes are analysed in detail. The model also suggests how the characteristic *whiG*, *whiA*, *whiB* and *whiH* phenotypes relate to the stage at which normal development is blocked in the respective mutant.

Fig. 9. A model for commitment of aerial hyphae to sporulation, involving postulated developmental decision points. The first decision (1), which depends on WhiG protein, is between continued vegetative-type growth and adoption of a type of aerial growth specialized for sporulation. The second decision (2), which depends on WhiA and WhiB proteins, is between continued extension growth and orderly growth cessation, and is a prerequisite for WhiH protein to properly activate sporulation septation and genome partitioning (3). The terminal phenotypes of *whi* mutants blocked at specific stages of development are depicted in the lower part of the figure.

***whiG* and the primary commitment of aerial hyphae to sporulation**

The aerial hyphae of *whiG* mutants are straight and septa are laid down with a spacing similar to that reported for vegetative growth of the closely related organism *Streptomyces granaticolor* (Kretschmer, 1982), in accord with the observation of widely spaced FtsZ rings in *whiG* aerial hyphae (Schwedock *et al.*, 1997). The structure of the septa in *whiG* aerial hyphae, as seen by transmission electron microscopy of thin sections, is typical of vegetative septa (Plaskitt & Chater, 1995). *whiG* is epistatic to *whiA*, *whiB* and *whiH* (Chater, 1975; this paper), as well as to another *whi* gene, *whiI* (Chater, 1975), which was not studied here because it was cloned only recently (J. A. Aínsa, H. D. Parry & K. F. Chater, unpublished). Furthermore, additional copies or increased expression of *whiG* cause premature and ectopic sporulation, implying that it is a crucial determinant of the decision to sporulate (Chater *et al.*, 1989; Méndez & Chater, 1987). Altogether, these observations indicate that there is a *whiG*-dependent developmental decision point that commits aerial hyphae to sporulation. This decision leads, among other things, to coiling of the apical parts of hyphae, indicative of a switch in the structure of the cell wall (Fig. 9). This could involve a change in the synthesis of new cell wall as it is laid down in the apical region after the *whiG*-dependent switch. Alternatively, it could be accounted

for by modification of existing peptidoglycan, an alternative that would raise the further question of how to explain the abrupt discontinuity between straight stem and coiled apex. A continual failure to carry out the *whiG*-dependent developmental decision would give rise to continued growth of straight and in many respects undifferentiated aerial hyphae (Fig. 9).

The *whiG* gene product is an RNA polymerase σ factor, σ^{WhiG} (Chater *et al.*, 1989; Tan *et al.*, 1998), of a subclass whose homologues in other bacteria (e.g. σ^{FlhA} in *Salmonella typhimurium*) are subject to regulation by anti- σ factors responsive to morphological checkpoints (Hughes *et al.*, 1993). It has previously been suggested that the morphologically coupled release of σ^{WhiG} from an anti- σ factor may be critical in initiating sporulation (Losick & Shapiro, 1993; Kelemen *et al.*, 1996). As one result, σ^{WhiG} RNA polymerase would transcribe a gene or genes influencing cell wall structure. We suggest that the transition from straight to curled growth of a hypha occurs very soon after σ^{WhiG} RNA polymerase has become active, and provides a visible record of the time at which the checkpoint was successfully negotiated. Another *whiG*-dependent gene encodes a possible glycine-betaine-binding protein, perhaps contributing to turgor for continued growth of the apical compartment after it has been cut off from direct contact with the substrate mycelium (Tan *et al.*, 1998). Indeed, this septation event might itself provide the checkpoint,

as suggested by Losick & Shapiro (1993); a consequent transient limitation of nutrients or of turgor might be more direct sources of the σ^{WhiG} -activating signal. Further σ^{WhiG} targets include the sporulation regulatory gene *whiH* (Ryding *et al.*, 1998).

A putative dependence of sporulation septation on growth cessation may provide the basis of a *whiA*- and *whiB*-mediated developmental checkpoint

The *whiG*-dependent switch in cell wall structure apparently works in *whiA* and *whiB* mutants since these develop tightly coiled aerial hyphae. The epistasis of *whiG* to *whiA* and *whiB* is consistent with this conclusion (Chater, 1975). Mutants in *whiA* or *whiB* lack sporulation septation or nucleoid segregation and condensation, and develop abnormally long and coiled aerial hyphae. This identifies a putative second developmental decision point required for formation of sporulation septa and for appropriate limitation of aerial growth (Fig. 9). On the other hand, aerial hyphae of *whiH* mutants are not abnormally long, so they appear to retain proper control of the decision to stop aerial growth.

The fact that *whiH* mutants make reduced numbers of sporulation septa, yet cease growth properly, indicates that a full commitment to sporulation septation is not a prerequisite for orderly growth cessation. This leads us to suggest tentatively that, instead, growth cessation is a prerequisite for sporulation septation, and that *whiA* and *whiB* affect elongation more directly than septation. Nevertheless, it remains possible that a *whiA/whiB*-dependent initiation of sporulation septation (even if just a few septa are formed as in *whiH* mutants) could lead to cessation of hyphal elongation.

Properly controlled aerial hyphal cell wall structure, as reflected in the extent of coiling of aerial hyphae, as well as other sporulation events such as sporulation septation, nucleoid condensation, spore pigment synthesis and transcription of *sigF* and *whiE*, all require *whiA* and *whiB* (Kelemen *et al.*, 1996, 1998). Although these dependences may conceivably be fairly directly exerted at the level of transcription of the relevant genes, it is quite possible that some or all of the events are more directly affected by the morphological checkpoint in which *whiA* and *whiB* are implicated. The sequences of the *whiA* and *whiB* genes are known but have not yet provided any unambiguous clues about their molecular function (Davis & Chater, 1992; N. J. Ryding, J. A. Aínsa, N. Hartley, C. J. Bruton & K. F. Chater, unpublished).

Some later sporulation processes are initiated in *whiH* mutants

Inactivation of *whiH* does not fully block all aspects of spore chain development. First, all *whiH* alleles examined to date, including the $\Delta\textit{whiH}::\textit{ermE}$ null allele, allow synthesis of detectable amounts of a grey spore pigment. We show here that this pigmentation is directed by the *whiE* gene cluster which is responsible

for the grey polyketide pigment of wild-type spores. Consistent with this, very low levels of transcription of the late sporulation loci *sigF* and *whiE* were detectable in a *whiH* point mutant (Kelemen *et al.*, 1996, 1998), whereas no transcription of these loci was detected in *whiG*, *whiA* and *whiB* strains. Second, a few irregularly spaced apparent sporulation septa divide *whiH* mutant aerial hyphae into shorter fragments, and only a few widely spaced FtsZ rings were reported in *whiH::hyg* aerial hyphae by Schwedock *et al.* (1997). Some of these rings may have corresponded to the occasional putative sporulation septa formed in *whiH* strains. Third, the fragments formed from *whiH* aerial hyphae become phase-dark and their cell wall becomes resistant to staining with Fluo-WGA in a manner reminiscent of developing spore chains. Fourth, nucleoids in these fragments condense and are partitioned (albeit aberrantly).

We interpret these observations and the epistasis of *whiG*, *whiA* and *whiB* to all these aspects of *whiH* to mean that the first two developmental decision points do not require *whiH* (Fig. 9). The *whiH* mutants initiate later sporulation processes, but execute them incorrectly or only to a limited extent. In the simplest model, *whiH* would act after *whiG*, *whiA* and *whiB* and be dependent on these genes. Indeed, a direct dependence of *whiH* transcription on σ^{WhiG} has been demonstrated (Ryding *et al.*, 1998). The situation appears, however, to be more complex than this, as *whiH* mutations clearly reduce the tight coiling that develops in *whiA* and *whiB* aerial mycelium, indicating that the wild-type *whiH* gene promotes this hypercoiling, and is therefore likely to be active during aerial hyphal growth, at least in *whiA* and *whiB* mutants. However, this situation in the mutants may not reflect a normal activity: *whiH* single mutants show a degree of coiling similar to that of developing wild-type aerial hyphae.

WhiH resembles a family of DNA-binding regulatory proteins responsive to carboxylate-containing intermediates of carbon metabolism (Ryding *et al.*, 1998). It has therefore been suggested that WhiH may sense some such metabolic intermediate, whose concentration changes during aerial growth (particularly at growth cessation), giving rise to two forms of WhiH: the form whose proportion increases later can be designated WhiH*. It is possible that later events are regulated by or indirectly require WhiH*. These would include increased expression of genes required for sporulation septation and for orchestration of the partitioning of DNA after the final one or two rounds of replication. Possibly, the level of WhiH* may be important in transmitting information about *whiA/whiB*-dependent cessation of tip growth to genes whose up-regulation mediates subsequent events. On this view, *whiH* would provide a point of information exchange to coordinate the *whiG*-dependent regulatory 'cascade' (of which *whiH* is a part) with the apparently *whiG*-independent 'cascade' represented by *whiB* (Soliveri *et al.*, 1992) and, perhaps, *whiA* (J. A. Aínsa & K. F. Chater, unpublished).

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