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ORIGINAL PAPER

Constitutive expression of *ftsZ* overrides the *whi* developmental genes to initiate sporulation of *Streptomyces coelicolor*

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Abstract The filamentous soil bacteria *Streptomyces* undergo a highly complex developmental programme. Before streptomycetes commit themselves to sporulation, distinct morphological checkpoints are passed in the aerial hyphae that are subject to multi-level control by the *whi* sporulation genes. Here we show that *whi*-independent expression of FtsZ restores sporulation to the early sporulation mutants *whiA, whiB, whiG, whiH, whiI* and *whiJ*. Viability, stress resistance and high-resolution electron microscopy underlined that viable spores were formed. However, spores from sporulation-restored *whiA* and *whiG* mutants showed defects in DNA segregation/condensation, while spores from the complemented *whiB* mutant had increased stress sensitivity, perhaps as a result of changes in the spore sheath. In contrast to the *whi* mutants, normal sporulation of *ssgB* null mutants—which fail to properly localise FtsZ—could not be restored by enhancing FtsZ protein levels, forming spore-like bodies that lack spore walls. Our data strongly suggest that the *whi* genes control a decisive event towards sporulation of streptomycetes, namely the correct timing of developmental *ftsZ* transcription. The biological significance may be to ensure that sporulation-specific cell division will only start once sufficient aerial mycelium biomass has been generated. Our data shed new light on the longstanding question as to how *whi* genes control sporulation, which has intrigued scientists for four decades.

Keywords Checkpoint · Transcription · Cell division · Feedback control · Actinomycete

Introduction

Bacterial cells do not commit themselves lightly to dramatic changes in their lifestyle. Radical morphological changes, such as sporulation, stalk formation or swarming are typically made out of need for survival and controlled by major transcriptional checkpoints (Chater 2001; Grossman 1995; Wang and Levin 2009). At important junctures in the onset of cellular development, transcriptional control is coupled to the completion of landmark morphological events. It has been proposed that checkpoints also govern the activity of the *whi* genes that control the onset of sporulation in *Streptomyces* (Chater 2001). These soil-dwelling Gram-positive bacteria undergo

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complex morphological development and in connection to the developmental programme antibiotics and other natural products are produced (Hopwood 2007; van Wezel and McDowall 2011). Initially, streptomycetes form a network of branched hyphae, the vegetative or substrate mycelium, consisting of syncytial cells separated by occasional cross-walls (Wildermuth and Hopwood 1970). Once the checkpoint for the initiation of differentiation has been passed, the formation of an aerial mycelium is initiated, using the vegetative mycelium as a substrate (Chater 2001). The aerial hyphae then undergo several morphological stages before committing themselves to sporulation (Flärdh and Buttner 2009). In sporulation-committed aerial hyphae, FtsZ organises into spiral-shaped intermediates along the length of the aerial hyphal cell, which then assemble into multiple foci at the septum sites, eventually forming ladders of up to a hundred Z-rings (Grantcharova et al. 2005; Schwedock et al. 1997; Willemse and van Wezel 2009). The *ftsZ* gene is regulated from three promoters, one constitutive (p3), one primarily transcribed during vegetative growth (p1) and one primarily transcribed during sporulation (p2). Interestingly, deletion of the p2 promoter prevents sporulation-specific cell division, but vegetative division appears unaffected (Flärdh et al. 2000).

Genes essential for sporulation are called *whi* (white) genes, characterised by the white appearance of mutants due to the lack of the WhiE spore pigment (Chater 1972). Six *whi* loci, designated *whiA*, *whiB*, *whiG*, *whiH*, *whiI* (Chater 1972) and *whiJ* (Ryding et al. 1999) were identified that presumably form checkpoints in the development of aerial hyphae and spores (Chater 2001). The respective mutants fail to make the transitions through particular stages of aerial development. The *whiG* gene encodes a sigma factor that is required for early aerial mycelium development, with the mutant producing erect aerial hyphae that fail to coil (Chater 1989); *whiB* encodes a small, cysteine-rich transcription factor with many homologues in streptomycetes and mycobacteria (Soliveri et al. 2000), and mutants of *whiB* (as well as of the less well characterised *whiA*) form abundant and coiling aerial hyphae (presporulation stage). Deletion of the *gntR*-family regulatory gene *whiH* results in sporulation mutants that produce occasional sporulation septa (Flärdh et al. 1999; Ryding et al. 1999), and a direct role for WhiH in enhancing developmental *ftsZ* transcription has been proposed (Flärdh et al. 2000). The *whiI* gene encodes a response regulator-like protein controlling both early and late sporulation events (Tian et al. 2007), while *whiJ* encodes a likely DNA binding protein that may act as a repressor of development (Ainsa et al. 2010). Several paralogues of *whiJ* exist in streptomycetes that are typically flanked by *abaA*-like genes associated with control of antibiotic production (Gehring et al. 2000).

Still relatively little is known of how sporulation-specific cell division is controlled. In most bacteria the positioning and timing of septum formation involves the action of negative control systems such as Min, which prevents Z-ring assembly at the cell poles (Raskin and de Boer 1997; Marston et al. 1998), and nucleoid occlusion that prevents formation of the Z-ring over non-segregated chromosomes (Wu and Errington 2004). We recently showed that the formation of Z-ladders depends on the SsgB protein, which is a member of the SsgA-like proteins (SALPs), an emerging family of sporulation proteins found exclusively in morphologically complex actinomycetes (Noens et al. 2005; Traag and van Wezel 2008). Of the SALPs, SsgA (van Wezel et al. 2000a; Jiang and Kendrick 2000) and SsgB (Keijser et al. 2003) are essential for sporulation. Interestingly, the transcription of *ssgA* does not depend on the classical *whi* genes *whiABGHIJ* (Traag et al. 2004), which may be explained by the fact that SsgA is also involved in processes involving remodelling of the peptidoglycan during normal growth, such as germination, branching and tip growth (Noens et al. 2007) as well as submerged sporulation (Yamazaki et al. 2003).

In this work we show that sporulation is restored to the *whi* mutants by expression of *ftsZ* from a constitutive promoter, strongly suggesting that the sporulation proteins WhiA, WhiB, WhiG, WhiH, WhiI and WhiJ form a checkpoint system to correctly time the expression of FtsZ, a complicated regulatory system that may serve to ensure the production of sufficient aerial biomass prior to undergoing sporulation.

Results

FtsZ accumulation in *whi* mutants

In an attempt to identify possible global causes for the sporulation block in the *whi* mutants we traced back the steps leading to sporulation-specific cell division.

This cell division process is hampered in all *whi* mutants, perhaps because transcription from the developmental *ftsZ*p2 promoter is not yet activated (Flärdh et al. 2000; Ryding et al. 1999). Transcript analysis revealed that transcriptional activity of *ftsZ*p2 was absent in *whiA* and *whiB* mutants and very low in *whiG* mutants, while in *whiH, whiI* and *whiJ* mutants there was transcriptional activity, but without the strong upregulation during development that is typical of wild-type cells. To establish how the reduced expression of the p2 promoter affects FtsZ protein accumulation, protein extracts were prepared from solid-grown cultures when robust aerial mycelium was formed and analysed by Western analysis using polyclonal antibodies against FtsZ. This revealed that FtsZ was absent in mycelia of *whiA* and *whiB* mutants as well as in the control *ftsZ* mutant, while it was strongly reduced in *whiG* mutants. However, FtsZ was detected in protein samples of the *whiH, whiI* and *whiJ* mutants, which are all stalled at a later stage of aerial development (Fig. 1).

Restoration of sporulation by nondevelopmental expression of *ftsZ*

In order to find out if the reduced expression of FtsZ in the *whi* mutants may be a determining factor in their failure to initiate sporulation-specific cell division, we forced FtsZ expression in aerial hyphae of the *whi* mutants using integrative vector pSCF7 that expresses *ftsZ* from the constitutive *ermE* promoter, resulting in FtsZ protein levels that are around twice as high as in wild-type cells [(van Wezel et al. 2000b); see also Fig. 1]. While overexpression of FtsZ from a multi-copy vector strongly inhibits development, the enhanced levels resulting from integrative vector pSCF7 lead to a slight delay of colony development in original transformants but otherwise sporulation is normal (van Wezel et al. 2000b). As controls we used pSCF1 and pSCF5 (Table S1), which are low-copy shuttle vectors harbouring *ftsZ* and *ftsQ* or *ftsZ* alone, respectively, with in both cases the *ftsZ* gene preceded by its natural promoter region, or the empty vectors (pSET152 as control for pSCF7 and pHJL401 as control for pSCF1). Both plasmid systems are suitable for genetic complementation (van Wezel et al. 2000c). The functionality of pSCF7 was confirmed by its ability to restore sporulation to the *ftsZ* null mutant (Fig. S1). Western analysis of the pSCF7 transformants showed that the construct enhanced FtsZ protein levels in all *whi* mutants (Fig. 1). FtsZ protein levels were restored to around wild-type levels for *whiA, whiB, whiG* and *whiI* mutants. Surprisingly, FtsZ levels were reproducibly enhanced in *whiH* and *whiJ* mutants as compared to the parental strain M145 harbouring the same plasmid. A second lower band was also observed in these mutants, perhaps reflecting degradation of FtsZ. In contrast, a similar experiment with samples taken from mycelia grown in TSBS media and harvested at transition phase, which corresponds to the onset of development in surface-grown cultures, showed restoration of FtsZ levels by

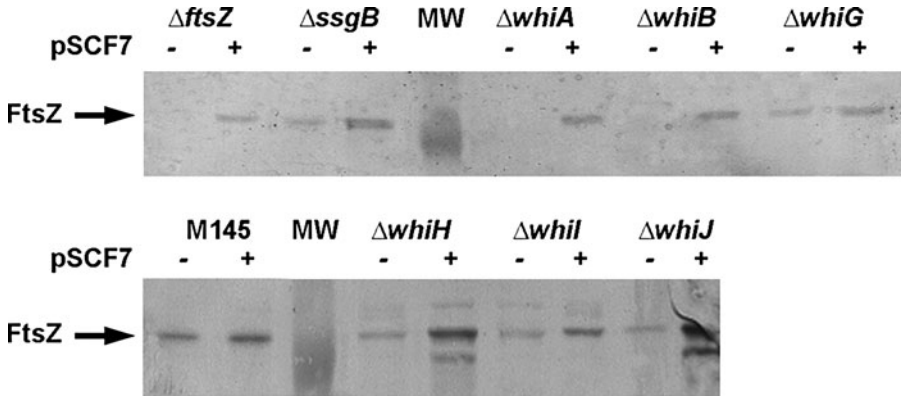


Fig. 1 FtsZ levels in the *whi* mutants and restoration by pSCF7. Western analysis using polyclonal antibodies against FtsZ demonstrating FtsZ protein levels in protein extracts of *S. coelicolor* M145 (wild-type strain) as well as its sporulation mutant derivatives carrying complete deletions of the *whiA, whiB, whiG, whiH, whiI, whiJ* or *ssgB* genes. Note that introduction of pSCF7 restores FtsZ to levels similar to those found in the wild-type strain, except for *whiH* and *whiJ* mutants, which show enhanced FtsZ protein levels

pSCF7 to wild-type levels in all six *whi* mutants (Fig. S2).

Excitingly, while the *whi* mutants harbouring control plasmid all showed the white phenotype typical of sporulation mutants, the constitutive expression of FtsZ resulted in the production of grey spore pigment, suggesting that sporulation had been restored to all of the *whi* mutants (Fig. 2). In contrast, transformation with pSCF1 expressing *ftsZ* from the natural promoter region did not restore sporulation to any of the *whi* mutants. Since pSCF1 also contains the *ftsQ* gene, we further tested pSCF5 (which contains a 1.6 kb DNA fragment harbouring only *ftsZ* and its promoter region; Table S1), and introduction of this plasmid also failed to restore sporulation to the *whi* mutants (not shown). A mutant lacking the *ssgB* gene, which encodes a structural protein required for the proper localization of FtsZ, was also analysed. *ssgB* mutants harbouring pSCF7B (a variant of pSCF7 based on pHM10a, which integrates at the minicircle attachment site) showed light grey pigmentation indicating that development had progressed enough to produce the WhiE spore pigment, while no pigmentation was observed for pSCF1 or the empty vectors. However, no intact spores were identified as was apparent from transmission electron microscopy and stress sensitivity tests (see below).

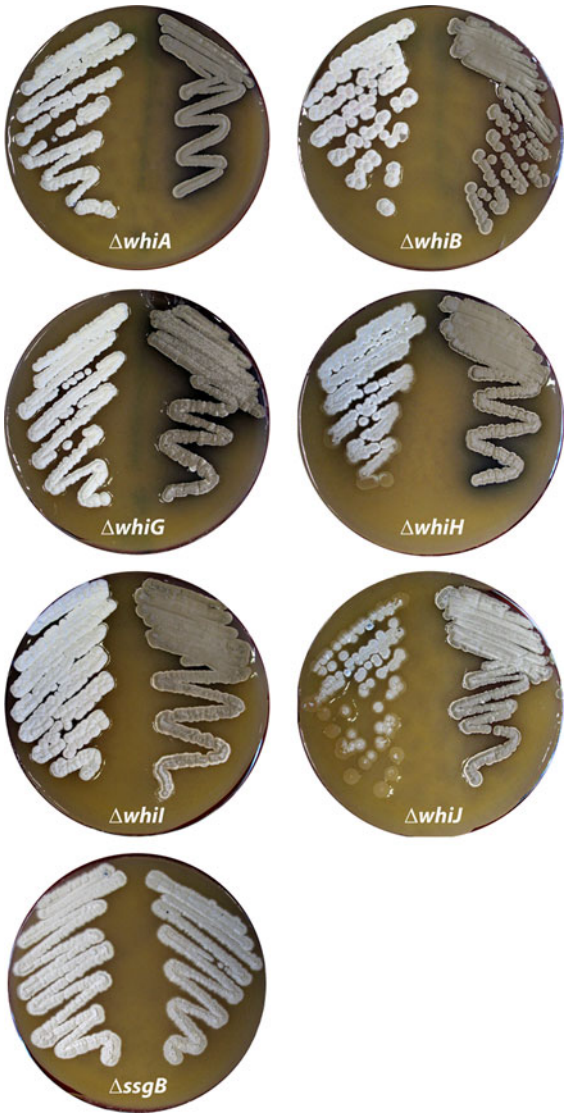


Fig. 2 Expression of FtsZ from a constitutive promoter rescues the sporulation block of *whi* mutants. The *image* shows the respective *whi* mutants (*whiA, whiB, whiG, whiH, whiI,* or *whiJ*) with control plasmid (*left*), and the mutants carrying pSCF7 (*right*), which expresses *ftsZ* from the constitutive *ermE* promoter. The *ssgB* null mutant is presented as a control. Note that *grey* pigmentation is restored to the *whi* mutants but only partially to the *ssgB* mutant (see Fig. 3 and Fig. S3 for cryo-SEM images). Single colonies of the *whiJ* mutant reproducibly showed delayed aerial development. Strains were grown for 4 days on SFM agar plates at 30°C

After initial scrutiny by phase-contrast microscopy, all original strains and their transformants were analysed in detail by high-resolution imaging. Cryo-scanning electron microscopy (cryo-SEM) indeed revealed that introduction of pSCF7 restored sporulation to the *whiA*, *whiB*, *whiH*, *whiI* and *whiJ* mutants, forming the abundant coiling spore chains similar to the parental strain M145 (Fig. 3 and Fig. S3), with similar lengths of the spore chains (those of sporulation-restored *whiJ* mutants were in fact longer, see below). Spore sizes (0.95–1.12 ± 0.19 μm; averages of >100 spores measured by SEM) were very similar to wild type spores (1.03 ± 0.19 μm). Partial restoration of sporulation was observed for the *whiG* mutant carrying pSCF7, which revealed wild-type spore chains, but with strongly reduced amount of spores, namely around 10% of wild-type levels (M145). Conversely, the *whiJ* mutant sporulated so abundantly that we could not observe non-sporulating sections of the aerial hyphae (Fig. S3). As could already be gleaned from the Whi phenotype of the transformants, cryo-SEM showed that neither pSCF1 (expressing *ftsZ* from the natural promoters) nor the empty plasmid could restore sporulation to any of the mutants (Fig. 3). Some spore-like bodies were also observed in the *ssgB* mutant expressing FtsZ from the *ermE* promoter (Fig. 3; Fig. S3), with a significantly larger

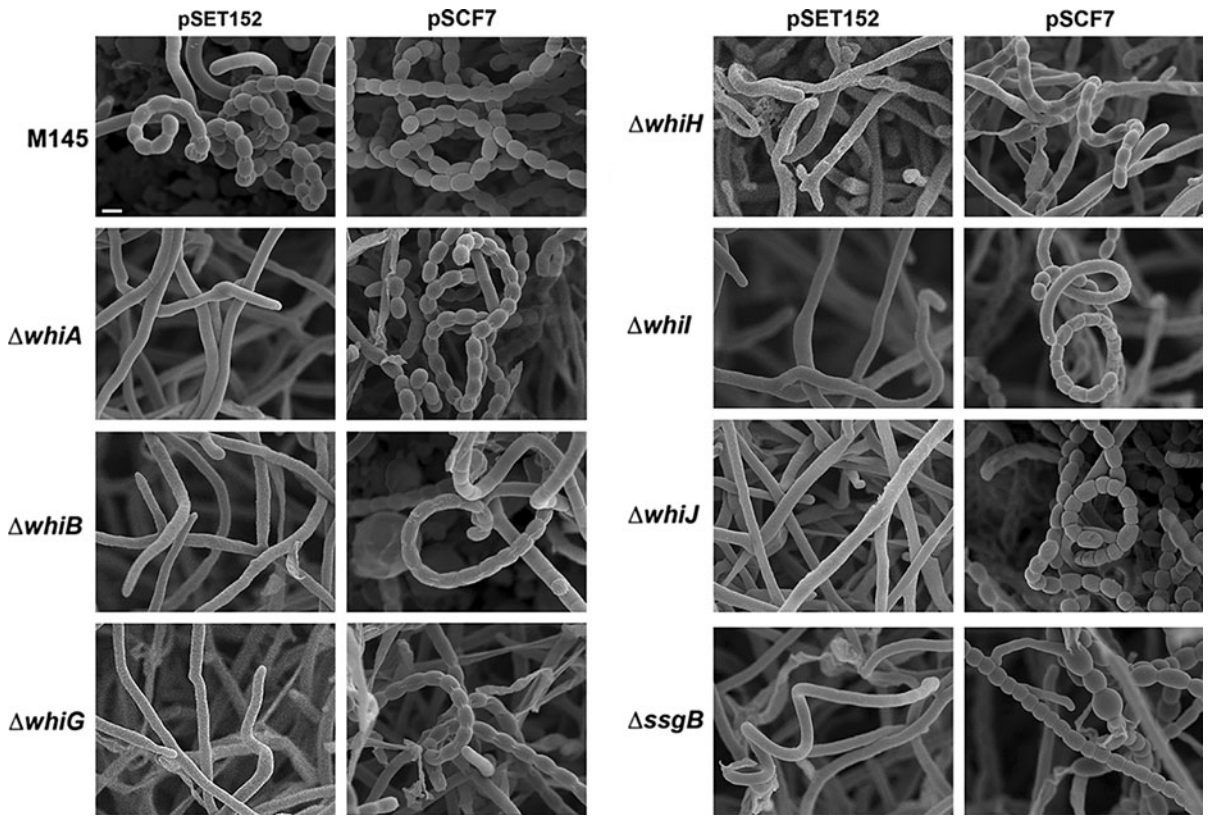
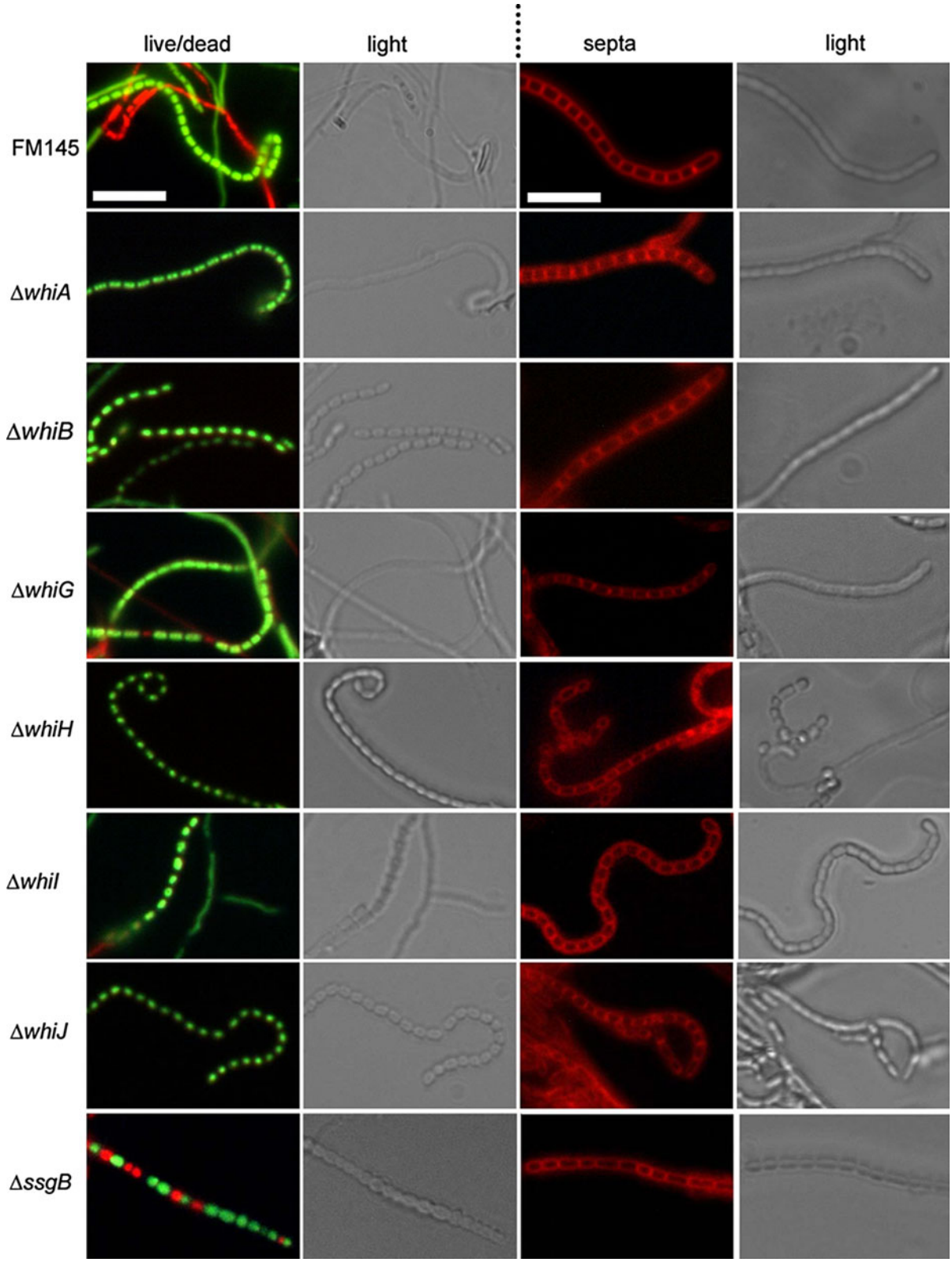


Fig. 3 Cryo-scanning electron micrographs of mature spore chains of the *whi* mutants expressing FtsZ. *Column 1*, *S. coelicolor* M145 (parental strain) and its *whiA, whiB* and *whiG* mutants harbouring control plasmid; *column 2*, same strains as shown in *column 1* but now containing plasmid pSCF7, which expresses *ftsZ* from the constitutive *ermE* promoter; *column 3*, *whiH, whiI, whiJ* and *ssgB* mutants containing control plasmid; *column 4*, same strains as shown in *column 3*, but now containing plasmid pSCF7 (or pSCF7B for the *ssgB* mutant). Note that the constitutive expression of *ftsZ* restores sporulation to all *whi* mutants, while *ssgB* mutants produce spore-like bodies with highly variable sizes (see Fig. 5), most likely due to incorrect localization of FtsZ. For high-resolution TEM images of the spore chains see Fig. 5. All strains were grown for 5 days on SFM agar plates at 30°C. See Fig. S3 for lower magnification. All images presented at the same scale. *Bar* (*top left*), 1 μm

variation in size (0.95 ± 0.30 μm; *F*-test $P < 0.001$) and only 44% in the size range of 0.9–1.3 μm that is typical of wild-type spores (Fig. S4).

The sporulation-restored *whi* mutants produce viable spores

To establish to what extent sporulation had really been restored to the *whi* mutants, spores were analysed in more detail. Firstly, fluorescence microscopy was used to visualise septum formation (using the membrane dye FM 5-95) and for live/dead staining (combination of the green-fluorescent nucleic acid stain SYTO 82 for viable cells and the red-fluorescent nucleic acid stain propidium iodide (PI) for dead cells). The *whi* mutants themselves occasionally produced septa—with similar spacing as vegetative cross walls—but lack the ladders of septa typical of normal sporulation; such occasional septum formation is typical of aerial hyphae that fail to initiate sporulation-specific cell division (Grantcharova et al. 2005; Willemse et al. 2011). However, all *whi* mutants transformed with pSCF7 produced the septal ladders typical of sporulation-specific cell division (Fig. 4). These results are in line with the wild-type appearance of the spore chains (Fig. 3; Fig. S3). In terms of live/dead staining, no aberrant spores were detected in the pSCF7 transformants of *whiA*, *whiB, whiH, whiI* or *whiJ* mutants, and close to 100% of the spores gave rise to a colony (Fig. 4; Table 1), suggesting that the restoration of sporulation to these *whi* mutants results in spores with similar viability as the wild-type strain.


live/dead
light
septa
light
FM145
ΔwhiA
ΔwhiB
ΔwhiG
ΔwhiH
ΔwhiI
ΔwhiJ
ΔssgB

◀**Fig. 4** Viability and formation of septal ladders during sporulation of sporulation-restored *whi* mutants. Live/dead staining (*left*) and septum staining (*right*) is shown for the *whi* and *ssgB* mutants harbouring pSCF7. FM145 was used as the control. Live cells were identified with syto-82, dead cells with propidium iodide. Septa were highlighted by the membrane stain FM5-95. Note that several spores of the *whiG* and *ssgB* mutants were not viable (*red*; *dark* in b/w), fitting with the viability count and the incomplete restoration of sporulation. Note the multi-lobed DNA produced by *whiA* and *whiG* mutants (see Fig. S5 for deconvolution images). Transformants were grown against microscopy cover slips for 3 days on SFM agar plates. *Bar*, 5 μm

Table 1 Viability of spores obtained from pSCF7 transformants of the *whi* mutants, the *ssgB* mutant and the parental strain (*S. coelicolor* M145)

Strain	Viability (%)[a]	*N*	*P* value[b]
M145	100	248	Reference
Δ*whiA*	99	269	$P > 0.99$
Δ*whiB*	100	307	ND
Δ*whiG*	87	215	$P < 0.02$
Δ*whiH*	100	250	ND
Δ*whiI*	100	200	ND
Δ*whiJ*	100	364	ND
Δ*ssgB*	41	266	$P < 0.001$

N, number of spores counted

[a] Based on live/dead staining experiments

[b] probability that the spores of the respective complemented mutants have similar viability as those of the parental strain M145 (*ND* not determined)

However, over 10% of the spores obtained from the *whiG* mutant with pSCF7 were either dead (stained with propidium iodide) or empty, and in line with these imaging results, spore preparations of *whiG*-pSCF7 had about 10% reduced viability. Of the spore-like bodies produced by the *ssgB* transformants only 41% were viable, with a particularly low viability for the spores that were significantly smaller than wild-type spores. For distribution of spore sizes and viability of the spores of the *ssgB* transformants in comparison to wild-type spores see Fig. S4.

The *whiA* and *whiG* mutants with pSCF7 showed two or three well-separated DNA foci or 'chromosomal lobes' rather than uniform staining, although the total amount of DNA is similar to that in wild-type cells (Fig. 4; see Fig. S5 for deconvolution images). This aberrant DNA segregation/condensation was confirmed by transmission electron microscopy (see below). Such lobed DNA is sometimes found in wild-type spores and may reflect a spore-specific nucleoid state in prespores (Dagmara Jakimowicz, personnel communication); apparently, this effect is strongly enhanced in *whiA* and *whiG* mutants. The incomplete indentation of the hyphae in light images (Fig. 4) suggests that most sporulating hyphae were still in a pre-sporulation stage (~80%). Similar multi-lobed DNA and incomplete indentation was observed for the *whiG* transformants (Fig. 4 and S4). This suggests that although sporulation in *whiA* and *whiG* mutants is restored, the maturation process is slowed down.

Subsequently, thin sections of the spores from the various pSCF7 transformants were prepared and analysed by high resolution transmission electron microscopy (TEM). This revealed apparently normal spores for the *whiB, whiH, whiI* and *whiJ* transformants, although the spore sheaths of the whiB transformant was notably fragmented (Fig. 5). Interestingly, while also *whiA* and *whiG* transformants had apparently normal spore walls and sizes, both showed the multi-lobed DNA also observed with fluorescence microscopy (see white arrows in Fig. 5). This strongly suggests that WhiA and WhiG are required for proper DNA condensation, even in a situation where the sporulation block has been circumvented and (viable) spores were eventually produced. In contrast to the sporulation-restored *whi* mutants, the 'spore chains' that were observed for the *ssgB* mutant harbouring pSCF7B were highly aberrant, with the generally misshapen bodies having an entirely different appearance; the DNA had a spiky appearance and was surrounded by a white electron-lucent mass of unknown nature (Fig. 5), while the cell wall was as thin as that of aerial hyphae (see insert in Fig. 5 for a magnified example).

Finally, the spores were tested for resistance to heat and lysozyme treatment. Therefore, spores were diluted and incubated for 0–20 min either at 60°C or in a solution containing 2 μg/ml lysozyme (Table 2). As expected, the spore-like bodies obtained from the *ssgB* transformants were very sensitive to exposure to both heat and lysozyme, with hardly any colonies formed even after 5 min of either treatment. Spores from the *whiA, whiG, whiH* and *whiI* pSCF7 transformants showed similar resistance to heat treatment as the parental strain M145. However, spores of the *whiB* transformants were significantly more sensitive to heat

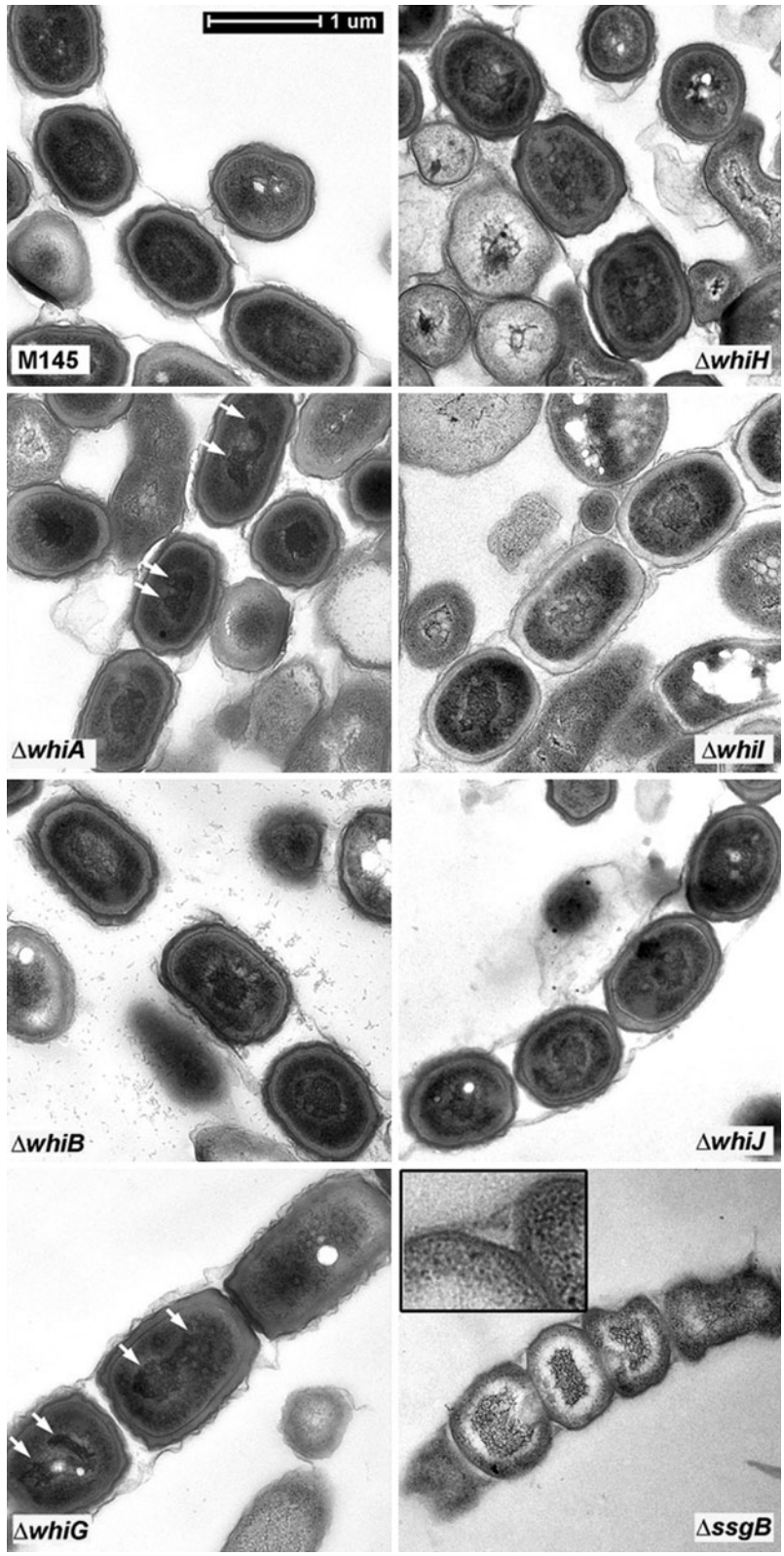


Fig. 5 Transmission electron micrographs of sporulation-restored *whi* mutants. Thin sections of spore chains of pSCF7 transformants of *S. coelicolor* M145 and its *whi* and *ssgB* mutants were analysed at high resolution by transmission electron microscopy. For each of the transformants a representative spore chain is presented. *Arrows* indicate multi-lobed chromosomes in *whiA* and *whiG* transformants. *Insert* in the image for the *ssgB* transformant shows magnification of the thin cell wall of the spore-like bodies produced by the transformants. *Bar* = 1 μm

Table 2 Heat and lysozyme resistance of spores

Strain[a]	Heat treatment (min)[b]				Lysozyme treatment (min)[b]			
	0	5	10	20	0	5	10	20
M145	100	67	54	44	100	63	60	65
Δ*whiA*	100	100	63	63	100	79	50	56
Δ*whiB*	100	54	23	8	100	18	21	20
Δ*whiG*	100	54	50	30	100	82	89	89
Δ*whiH*	100	100	66	43	100	100	100	100
Δ*whiI*	100	45	40	34	100	41	51	48
Δ*whiJ*	100	45	33	30	100	29	31	33
Δ*ssgB*	100	1	0	0	100	4	0	0

Survival (in percentage) of spores after heat treatment (60°C for 0–20 min) or lysozyme treatment (2 mg/ml, 0–20 min) of the spores

[a] All strains harboured plasmid pSGF7

[b] Colony forming units at $t = 0$ set to 100%. Spores of all transformants were freshly prepared from SFM agar plates prior to treatment. Data are averages from three independent spore preparations

treatment, with only around 10% of the spores surviving after 20 min of incubation at 60°C, while transformants of the other *whi* mutants and of the parent M145 showed at least 30% viability after 20 min of incubation at 60°C (Table 2). The same was observed for lysozyme treatment, with only around 20% survival for spores from *whiB* transformants after only 5 min incubation with lysozyme (Table 2). This may be due to the integrity of the sheath around the spores, which was found shattered in almost all spore chains, with small fragments surrounding the spores (see Fig. 5), while it was mostly intact (although occasionally broken at a single position) in the other transformants. Surprisingly, spores from the *whiG* and *whiH* transformants were more resistant to lysozyme treatment than the other transformants, with 90 and 100% survival after 20 min, respectively. The enhanced resistance of spores obtained from *whiG* and *whiH* transformants was reproducible, but scrutiny of many TEM images did not reveal statistically relevant differences in, for example, cell wall integrity or thickness as compared to wild-type spores. Transformants of Δ*whiA,* Δ*whiI*, Δ*whiJ* and the parent M145 showed a significant decline already after 5 min of lysozyme treatment, followed by stabilization during longer incubation.

Discussion

The main finding presented in this work is that the sporulation deficiency of the *whiA, whiB, whiG, whiH, whiI,* and *whiJ* sporulation mutants can be overcome by transcription of *ftsZ* from a nondevelopmental (constitutive) promoter. This provides compelling evidence that the correct timing of developmental *ftsZ* transcription is (one of) the major function(s) of the *whi* regulatory genes and the primary reason for the developmental arrest of the *whi* mutants. This finding contrasts the canonical view that the main function of the *whi* genes is to activate genes and processes relating to the various stages of aerial development, from early aerial growth to the onset of sporulation, and is supported by the earlier observation that the developmental transcription of *ftsZ* is essential for sporulation (Flärdh et al. 2000). Apparently, most if not all other processes required for aerial development and for the onset of sporulation-specific cell division can take place in the absence of at least one of the *whi* genes. Therefore we propose that the Whi transcription factors form a regulatory network, with their transcription tied closely to morphological checkpoints, which is directed at ensuring the correct timing of developmental FtsZ production.

Live/dead staining, viability tests (cfu counting), analysis of heat and lysozyme resistance and TEM revealed that the spores of the sporulation-restored *whi* transformants were viable and stress resistant. Indeed, their spores had apparently normal spore walls, except for the spore-like bodies produced by *ssgB* pSCF7B transformants, which lacked a spore wall, rendering them hypersensitive to heat shock and lysozyme treatment (discussed below). Surprisingly, spores of

the *whiG* and *whiH* transformants were significantly more resistant to lysozyme treatment than wild-type spores, perhaps as a result of structural differences in the spore wall, although these could not be detected by electron or fluorescence microscopy. Conversely, spores from *whiB* transformants were more sensitive to both heat and lysozyme treatment than the parental strain M145. TEM images revealed a shattered sheath around the spore chains of the sporulation-restored *whiB* mutants, suggesting that the higher stress sensitivity may be caused by structural changes in the protective sheath.

The failure to completely restore sporulation to the *whiG* mutant indicates that WhiG plays an important role in the control of other events during aerial development. It is not clear what the exact WhiG regulon is, but it includes *whiH* and *whiI* (Flärdh et al. 1999; Ainsa et al. 1999; Ryding et al. 1998), which can both be fully restored to normal sporulation by increasing FtsZ levels. Interestingly, both sporulation-restored *whiA* and *whiG* mutants showed multilobed DNA in the spores (as shown by both TEM and fluorescence microscopy), suggesting a defect in DNA condensation in these transformants. It was shown recently that WhiA, WhiG and WhiI are all required for the transcription of *hupS*, encoding one of the two Hu-like nucleoid-associated proteins in streptomycetes (Salerno et al. 2009). Such defective transcription of *hupS* may well explain the defects in DNA segregation in sporulation-restored *whiA* and *whiG* mutants, although it was not apparent in sporulation-restored *whiI* transformants. Deletion of *smc* (for structural maintenance of chromosomes) or *scpAB* (for segregation and condensation proteins) also strongly affects DNA condensation and segregation of the chromosomes during sporulation (Dedrick et al. 2009; Kois et al. 2009), but it is unknown if their transcription is controlled by the Whi proteins. While it seems likely that chromosome condensation and segregation are linked to the morphological checkpoints discussed above, strains in which sporulation septation was blocked by mutation of a sporulation-specific *ftsZ*p2 promoter (Flärdh et al. 2000) showed normal activation of *parAB* transcription and proper localization of ParB in the aerial hyphae, which seems to argue against strong coupling between cell division and DNA partitioning (Jakimowicz et al. 2006). The coordination of cell division and chromosome partitioning during sporulation requires further investigation.

In contrast to the Whi proteins, which all carry DNA binding domains and are therefore likely regulatory proteins, SsgB has a structural role and acts in a way similar as ZipA, with both proteins connecting the Z-ring to the cell wall and stimulating the polymerization of FtsZ in vitro (Hale and de Boer 1997; Willemse et al. 2011). In line with the idea that nondevelopmental expression of FtsZ overrides specifically the Whi-mediated control system, and not just any sporulation mutant, introduction of an FtsZ-expressing plasmid in *ssgB* null mutants did not restore sporulation. Some deformed spore-like bodies were produced, similar to *ssgB* mutants complemented with *ssgB* orthologues from other actinomycetes (Xu et al. 2009), but considering among others the extremely high sensitivity to lysozyme and heat treatment and the obvious lack of a typical spore wall (see insert in Fig. 5) these could not be described as proper spores. The lack of a spore wall is the opposite phenotype of *crp* null mutants, which produce spores with a wall that is more than twice as thick as that of wild-type spores (Piette et al. 2005). Comparing the *crp* and *ssgB* mutants could provide interesting leads towards the study of proteins involved in (the control of) spore-wall synthesis.

It was suggested previously that the failure of *whiA* and *whiB* mutants to sporulate was due to their inability to stop aerial growth prior to sporulation (Flärdh et al. 1999). However, the restoration of sporulation by the expression of FtsZ in these mutants argues against this idea. It appears that the *growth cessation checkpoint* in aerial hyphae is most likely linked to the localization of SsgB at the future septum sites, as shown by the large-colony phenotype of *ssgB* null mutants, which form extremely large ('immortal') colonies (Keijser et al. 2003). Such a large-colony phenotype is not shown by any of the other *whi* mutants (data not shown).

Surprisingly, enhanced expression of FtsZ in *whiJ* mutants resulted in what is best described as *hyper*-sporulation, producing extremely long spore chains, whereby the entire aerial hyphae are converted into spores (Fig. S3). Earlier work suggested that WhiJ may repress developmental genes. Mutant J2452 contains a *hyg* cassette inserted downstream of the part of *whiJ* that encodes the DNA binding domain, and expression of this domain most likely causes the Whi phenotype (Ainsa et al. 2010). In contrast, complete deletion of *whiJ* does not visibly affect

development (Ainsa et al. 2010), and the formation of extremely long spore chains which apparently include the entire aerial hyphae by the sporulation-restored *whiJ* mutants (presented in this work), suggests that such repression includes controlling the length of the sporogenic part of the aerial hyphae.

Concluding remarks and future perspective

In terms of the control of aerial development several important questions remain to be answered. For example, what other processes are controlled by the *whi* genes besides the correct timing of sporulation-specific FtsZ expression? Many attempts have been made to identify each of the individual Whi regulons and their primary (direct) target genes, such as for WhiB and WhiH, but have been hampered by the poor functionality of proteins heterologously produced in *E. coli*, perhaps as a result of incorrect posttranslational processing. However, modern technologies such as ChIP-on-chip now allow determining the primary response regulons in vivo. Another well-studied developmental regulatory network is that governed by the *bld* genes. While originally primarily considered for their role in controlling the events that take place during the switch from vegetative to aerial growth (Nodwell et al. 1999), the pleiotropic control of late developmental genes by BldD (den Hengst et al. 2010) and the sharp increase of *bldN* transcription during sporulation (Bibb et al. 2000) indicate that at least some of the Bld proteins also control gene expression during aerial growth and sporulation. Detailed insight into the regulatory networks controlled by the Bld and Whi proteins will further our understanding of precisely how the highly complex sporulation process in streptomycetes is controlled.

Materials and methods

Bacterial strains and plasmids

For strains and plasmids see Table S1. pSCF7 is an integrative vector based on pSET152 (Bierman et al. 1992), with *ftsZ* expressed from the constitutive *ermE* promoter (van Wezel et al. 2000b). All plasmids were introduced by protoplast transformation. Derivative pSCF7B was created by cloning the insert of pSCF7 into the hygromycin resistant integrative vector pHM10a, which allows integration at the minicircle attachment site (Motamedi et al. 1995). All mutants described in this work were derived from the wild-type strain *S. coelicolor* M145. The *whi* mutants J2401 (M145 *whiA*::*hyg*), J2402 (M145 *whiB*::*hyg*), J2400 (M145 *whiG*::*hyg*), J2403 (M145 *whiH*::*hyg*), J2450 (M145 *whiI*::*hyg*) and J2452 (M145 *whiJ*::*hyg*) were obtained from the John Innes Centre strain collection. The *ftsZ* null mutant HU133 (*ftsZ::aph*; (McCormick et al. 1994)) was a kind gift from Joe McCormick. GSB1 has the *ssgB* gene replaced by the apramycin resistance cassette *aacC4* (Keijser et al. 2003).

Protein extracts and western analysis

For preparation of protein extracts from solid-grown cultures, mycelia were grown on SFM agar plates (Kieser et al. 2000) overlaid with cellophane discs, and when robust aerial mycelium was formed (around 48 h after inoculation) mycelia were scraped from the surface, washed and resuspended in 10 mM Tris–HCl (pH 7); for liquid-grown cultures we used TSBS media (tryptic soy broth with 10% sucrose) and cultures were grown until transition phase. Mycelia were then sonicated, followed by 15 min centrifugation at $30{,}000 \times g$ to remove the cell debris. The protein concentrations were determined using a Bradford protein assay reagent (Bio-Rad). 20 µg of protein was analysed by SDS–PAGE and blotted to nitrocellulose filters. Subsequently, blots were incubated with 1:1000000 dilution of FtsZ antibodies. Following washing and incubation with GARAP as secondary antibody, alkaline phosphatase detection was used to visualise the bands. Two independent replicates were performed for all samples.

Microscopy

Electron microscopy

Morphological studies on surface grown aerial hyphae and/or spores by cryo-scanning electron microscopy were performed using a JEOL JSM6700F scanning electron microscope as described previously (Colson et al. 2008). Transmission electron microscopy (TEM) for the analysis of cross-sections of hyphae and spores was performed with a Philips EM410 transmission

electron microscope as described previously (van Wezel et al. 2000a).

Fluorescence microscopy

Cell membranes were stained with FM® 5-95 and the ratio of live/dead cells was determined by staining nucleic acids with green-fluorescent SYTO® 82 (540/560 nm) and the red-fluorescent PI (propidium iodide; 535/617 nm). Staining and imaging was done as described previously (Willemse et al. 2011). All dyes were obtained from Molecular Probes, Inc (Eugene). All images were background corrected setting the signal outside the hyphae to zero.

Viability tests

Freshly harvested spores were diluted in 20% glycerol to a concentration of 4×10^6 colony forming units (cfu) per ml before treatment. Subsequently, 2 ml of spores were treated with lysozyme (2 mg/ml) for 5, 10 and 20 min, or incubated at 60°C (heat treatment) for 5, 10 and 20 min. At each time point a 10 µl aliquot was taken and plated following a tenfold dilution series onto SFM agar plates, Plates were then cultivated for 2 days at 30°C followed by cfu counting. Percentage of survival was calculated as the number of colonies grown on the plates after treatment at 60°C divided by the number of colonies grown on plates without treatment at 60°C in percentage.

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