

# The interplay of glycogen metabolism and differentiation provides an insight into the developmental biology of *Streptomyces coelicolor*

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Mycelial colonies of the developmentally complex actinomycete *Streptomyces coelicolor* growing on solid medium contain glycogen in two distinct locations. Phase I deposits are found in a substrate mycelium region bordering the developing aerial mycelium. Their production involves GlgBI, one of two glycogen branching enzyme isoforms. Phase II deposits occur in the upper regions of aerial hyphae, in long tip cells that are dividing, or have just divided, into unigenomic prespore compartments. Their formation involves a second branching enzyme isoform, GlgBII. To find out if the gene for the second isoform, *glgBII*, is regulated by any of the well-studied *whiA*, *B*, *G*, *H* or *I* genes needed for sporulation septation, *glgBI* or *glgBII* was disrupted in a set of *whi* mutants, and the glycogen phenotypes examined by transmission electron microscopy. In the *whiG* mutants, deposits were found throughout the aerial mycelium and the adjacent region of the substrate mycelium, but the morphology of all the deposits, i.e. whether they were in the form of granules of branched glycogen or large blobs of unbranched glycan, depended solely on GlgBI. In contrast, the *whiA*, *B*, *H* and *I* mutations had no obvious effect on the pattern of glycogen deposition, or on the spatial specificity of the branching enzyme isoforms (though phase II glycogen deposits were reduced in size and abundance in the *whiA* and *B* mutants, and increased in the *whiH* mutant). These results indicate that *glgBII* is regulated (directly or indirectly) by *whiG*, and not by any of the other *whi* genes tested, and that the aerial hyphae of a *whiG* mutant are atypical in being physiologically similar to the substrate hyphae from which they emerge. A new role for aerial hyphae is proposed.

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## INTRODUCTION

Glycogen is a highly branched, starch-like glucose polymer with approximately 95%  $\alpha$ -1,4 linkages and 5%  $\alpha$ -1,6 branching linkages. In bacteria, three enzymes typically catalyse glycogen biosynthesis from glucose 1-phosphate: ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA) and glycogen branching enzyme (GlgB) (Preiss, 1984).

Glycogen metabolism has a complex interplay with the colony development of streptomycetes. In these actinomycetes, spore germination is followed by the growth of a dense network of branched hyphae (the substrate mycelium), which after about 2 days gives rise to a fuzzy white growth of aerial hyphae (which are often coiled). The initially multigenomic apical compartments of aerial hyphae then

undergo multiple septation, ultimately giving rise to chains of unigenomic spores (Chater & Losick, 1997).

Transmission electron microscopy (TEM) has revealed two localized phases of net glycogen deposition during colony development (Braña *et al.*, 1986; Plaskitt & Chater, 1995; Bruton *et al.*, 1995). Phase I deposits are found in a region of the substrate mycelium bordering the aerial mycelium, and phase II deposits are present in the apical compartments of the aerial mycelium as they are undergoing sporulation septation. Glycogen is virtually absent from three other cell-types: the young vegetative hyphae; the regions of aerial hyphae ('stalks') immediately below the sporulating apical compartment; and mature spores. It has been suggested that some of the carbohydrate that makes up glycogen is transferred from one phase to the next in the form of trehalose ( $\alpha$ -1,1-linked diglucose), which is diffusible, and metabolically inert compared with sugars that retain a reducing end (Schneider *et al.*, 2000).

In *Escherichia coli*, distinct unbranched aggregations of

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Abbreviation: TEM, transmission electron microscopy.

polysaccharide have been observed by TEM of stained thin sections in *glgB* (branching enzyme) mutants (Lares *et al.*, 1974). Bruton *et al.* (1995) observed similar structures when they disrupted either of two branching enzyme genes (*glgBI* and *glgBII*) in the model species *Streptomyces coelicolor* A3(2). Each *S. coelicolor* mutant was affected in just one phase of glycogen deposition. In the *glgBI* mutant, abnormal phase I deposits (small numbers of large irregular blobs instead of the large numbers of dispersed small granules typical of wild-type deposits) occupied much of the volume of the hyphae at the interface of the substrate and aerial mycelium, while phase II deposits in aerial hyphae appeared normal. On the other hand, the *glgBII* mutant displayed normal phase I deposition, but the phase II deposits were abnormal, occurring as small numbers of large blobs in the immature spore compartments of the aerial hyphae. The abnormal phase II deposits were less frequent, and much more regularly rounded in appearance, than the unbranched phase I deposits of the *glgBI* mutant (Bruton *et al.*, 1995).

In this study, we have investigated further the interplay of glycogen and development in *S. coelicolor*, using a representative set of mutants in the 'early' *whi* developmental genes. These mutants develop abundant aerial mycelium, but lack the regular multiple sporulation septa typical of the wild-type, and produce no spore-like bodies (Hopwood *et al.*, 1970; Chater, 1972; McVittie, 1974; Schwedock *et al.*, 1997; Flårdh *et al.*, 1999). The early *whi* genes are involved, directly or indirectly, in the regulation of later sporulation genes including those for the grey spore pigment (reviewed by Chater, 2001). The mutants therefore have white, instead of grey, aerial mycelium.

The most studied early *whi* gene, *whiG*, encodes a member of a specialized subgroup of sigma ( $\sigma$ ) factors, whose best-known members are involved in motility in various bacteria (Chater *et al.*, 1989). The level of  $\sigma^{\text{WhiG}}$  is critical in initiating sporulation (Chater *et al.*, 1989), and *whiG* mutants have long, straight, undifferentiated aerial hyphae. Mutants in the remaining well-studied early *whi* genes display at least some coiling of aerial hyphal tips (Chater *et al.*, 1989; Flårdh *et al.*, 1999). Among these genes, *whiA* encodes a protein of unknown function with orthologues in several other Gram-positive bacteria (Aínsa *et al.*, 2000); *whiB* belongs to a gene group whose products code for actinomycete-specific small cysteine-rich regulatory proteins of unknown structure (Davis & Chater, 1992; Soliveri *et al.*, 2000); *whiH* encodes a member of the GntR family of transcription factors (Ryding *et al.*, 1998); and *whiI* encodes an atypical member of the kind of response regulator usually associated with histidine protein kinases in bacterial two-component systems (Aínsa *et al.*, 1999). Both *whiH* and *whiI* are direct targets for RNA polymerase holoenzyme containing  $\sigma^{\text{WhiG}}$  (Ryding *et al.*, 1998; Aínsa *et al.*, 1999).

We set out to investigate the dependence of the two *glgB* genes on *whiA*, *B*, *G*, *H* and *I*, using three isogenic sets of

*whi* mutants. These were wild-type for the *glgB* genes, mutant in *glgBI*, or mutant in *glgBII*. Their patterns of glycogen deposition were analysed by TEM. The mutant glycogen branching phenotypes presented cytological markers that could be used to ascertain whether and where the phase I and/or phase II glycogen branching enzymes were active in a particular *whi* mutant. The results showed that phase II deposition is closely dependent on *whiG*, and that glycogen present throughout the aerial hyphae of a *whiG* mutant is branched by the action of *GlgBI*. This observation has suggested that there may be an additional, sporulation-independent, and ecologically meaningful role for aerial growth.

## METHODS

**Strains and media.** The *S. coelicolor* A3(2) derivative J1508 (*hisA1 uraA1 strA1 Pgl<sup>-</sup> SCP1<sup>NF</sup> SCP2<sup>-</sup>*: Ikeda *et al.*, 1984) was the origin of all strains utilized in this study. Media and conditions for *Streptomyces* culture, mating and protoplast transformation were as described in Kieser *et al.* (2000).

**Construction of *whi* mutants.** To introduce *hyg*-disrupted versions of *whi* genes into J1508, DNA from previously constructed disruptants of *S. coelicolor* M145 (Ryding *et al.*, 1998; Flårdh *et al.*, 1999; Aínsa *et al.*, 2000) was denatured and used to transform J1508 protoplasts as described by Oh & Chater (1997). Details of the constructed strains are given in Table 1.

**Construction of *whi glgB* mutants.** The *whi* mutant derivatives of J1508 were mated with J1861 and J1859. These are both also J1508 derivatives, but with the *glgBI* and *glgBII* genes, respectively, disrupted by prophages KC887 and KC883 carrying *tsr* (encoding thiostrepton resistance) (Table 1). Recombinants resistant to both

**Table 1.** *S. coelicolor* strains used in this study

Strain	Relevant genotype	Reference
J1508	<i>hisA1 uraA1 strA1 Pgl<sup>-</sup> SCP1<sup>NF</sup> SCP2<sup>-</sup></i>	Ikeda <i>et al.</i> (1984)
J1859	J1508::KC883 ( <i>glgBII</i> disrupted)	Bruton <i>et al.</i> (1995)
J1861	J1508::KC887 ( <i>glgBI</i> disrupted)	Bruton <i>et al.</i> (1995)
J1827	J1508 <i>whiA</i> :: <i>hyg</i>	This study
J1828	J1508 <i>whiB</i> :: <i>hyg</i>	This study
J1829	J1508 <i>whiG</i> :: <i>hyg</i>	This study
J1830	J1508 <i>whiH</i> :: <i>hyg</i>	This study
J1831	J1508 <i>whiI</i> :: <i>hyg</i>	This study
J1833	J1508 <i>whiA</i> :: <i>hyg glgBII</i> ::KC883	This study
J1834	J1508 <i>whiB</i> :: <i>hyg glgBII</i> ::KC883	This study
J1835	J1508 <i>whiG</i> :: <i>hyg glgBII</i> ::KC883	This study
J1836	J1508 <i>whiH</i> :: <i>hyg glgBII</i> ::KC883	This study
J1837	J1508 <i>whiI</i> :: <i>hyg glgBII</i> ::KC883	This study
J1839	J1508 <i>whiA</i> :: <i>hyg glgBI</i> ::KC887	This study
J1840	J1508 <i>whiB</i> :: <i>hyg glgBI</i> ::KC887	This study
J1841	J1508 <i>whiG</i> :: <i>hyg glgBI</i> ::KC887	This study
J1842	J1508 <i>whiH</i> :: <i>hyg glgBI</i> ::KC887	This study
J1843	J1508 <i>whiI</i> :: <i>hyg glgBI</i> ::KC887	This study

hygromycin and thiostrepton were readily obtained, and Southern blotting was used to confirm the disruption of the relevant *whi* and *glgB* genes.

**Electron microscopy.** Preparation of samples and transmission electron microscopy of thin sections were as previously described by Thiéry (1967) and Plaskitt & Chater (1995). Colonies were grown for 4 days on minimal medium (MM) containing mannitol (0.5%) as carbon source. In order to recognize aerial hyphae of *whi* mutants in thin sections (in the absence of stages of sporulation) we used three kinds of information (as in Plaskitt & Chater, 1995): position in the colony; the absence of a surrounding matrix of agar, which showed as a weakly stained and somewhat granular background; and the occurrence, where appropriate and available, of curved hyphal compartments. The examples shown in the figures are chosen to illustrate the morphology of the deposits, and are not necessarily representative of the full range of glycogen abundance levels.

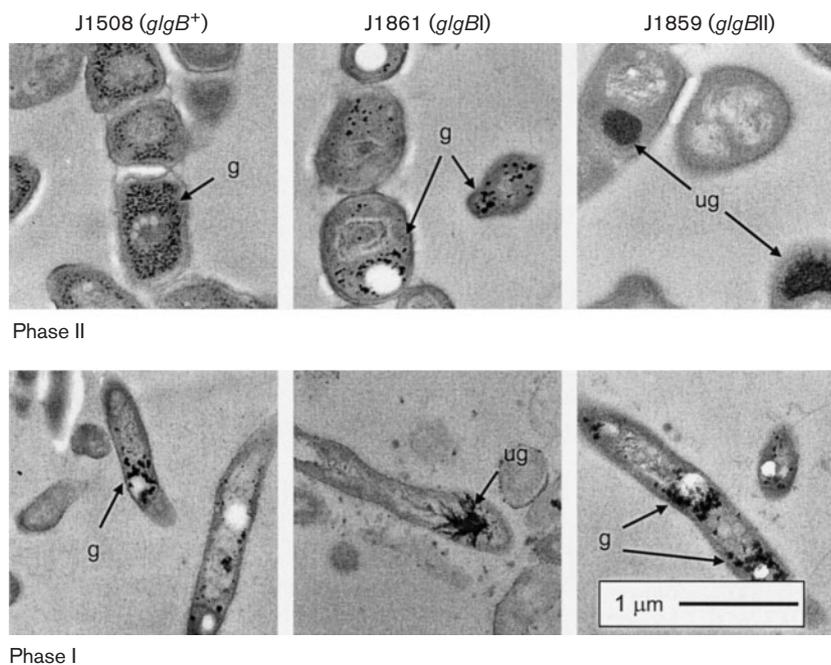
## RESULTS

### Glycogen deposition in *whi* mutants

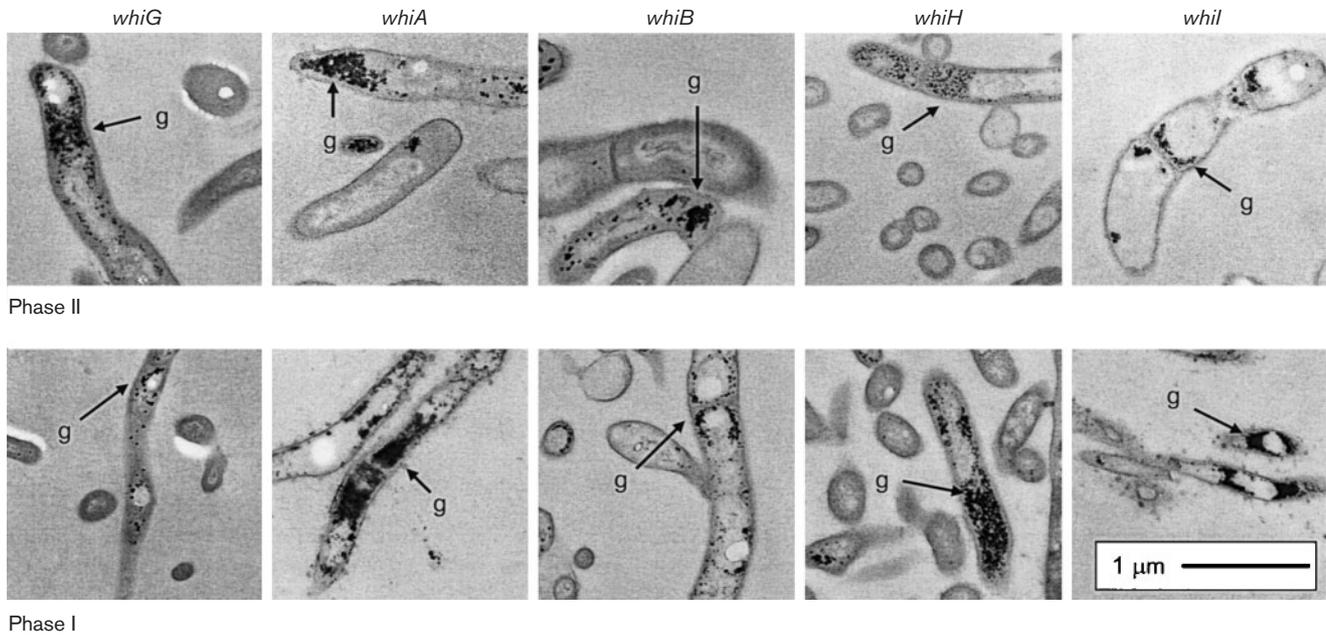
Plaskitt & Chater (1995) found that glycogen was present in the aerial hyphae of a series of *whi* gene point mutants obtained by mutagenesis of the wild-type *S. coelicolor* A3(2). In the *whiA*, *whiB*, *whiH* and *whiI* mutants these deposits were spatially separated from phase I (substrate mycelium associated) glycogen, suggesting that the developmentally distinct phases were undisturbed in these mutants. In contrast, in the *whiG* mutant glycogen was present in nearly all cell compartments of the colony, other than young vegetative mycelium, making it impossible to recognize the two discrete phases of glycogen deposition. Here we first set out to verify these observations using a series of constructed null *whi* mutants, all containing the hygromycin-resistance cassette (*hyg*), in an isogenic genetic background

(J1508 was chosen because it accumulated comparatively large amounts of glycogen: Schneider *et al.*, 2000). Thin sections of colonies that had been grown on solid medium were stained with silver proteinate to reveal polysaccharide deposits, and examined by TEM. After this treatment, glycogen granules are seen as many small dark spots dispersed in the cytoplasm (sometimes densely packed), and unbranched polyglucose (in branching enzyme mutants) is seen as large blobs, typically one per hyphal compartment.

The previously observed glycogen deposition phenotypes of the parental strain J1508 and its *glgBI*- and *glgBII*-disrupted strains (J1861 and J1859 respectively) (Bruton *et al.*, 1995; Plaskitt & Chater, 1995) were confirmed. J1508 (Fig. 1) and its *whiA*, *B*, *H* and *I* derivatives (Fig. 2) all showed phase I glycogen granules in many hyphal compartments of the substrate mycelium just below the air-agar interface, with a glycogen-free zone in the aerial mycelium above it. The upper parts of the aerial mycelium of J1508 contained phase II glycogen granules associated particularly with the initiation of spore compartments. Thick-walled mature spores contained little or no glycogen. Likewise, the *whiA*, *B*, *H* and *I* mutants contained some glycogen in the upper parts of the aerial hyphae. Compared with the parental strain J1508 and with each other, the amounts were low in the *whiA* and *whiB* mutants, fairly low in the *whiH* mutant, and quite abundant in the aerial mycelium of *whiI*. In contrast to all the other strains, the *whiG* mutant displayed large amounts of glycogen dispersed throughout the undifferentiated aerial mycelium (Fig. 2). All these observations were similar to those of Plaskitt & Chater (1995), though in general we found relatively few curled aerial hyphae in the J1508-derived *whiA*, *B*, *H* and *I* mutants compared with previous observations on



**Fig. 1.** Glycogen deposition in the wild-type strain J1508 and the *glgBI*- and *glgBII*-disrupted strains J1861 and J1859. In silver-stained thin sections of J1508, glycogen (g) can be observed in the substrate mycelium (phase I deposition) and in immature spore chains (phase II deposition). Sections of the *glgBI* mutant (J1861) display abnormal aggregations of unbranched polysaccharide (ug) in their substrate mycelium, while phase II glycogen deposition is unaffected. The *glgBII* mutant (J1859) displays normal phase I glycogen deposition, but blobs of unbranched polysaccharide in phase II.



**Fig. 2.** Glycogen deposition in a *whi* mutant set. Sections of 4-day-old colonies of *whi* mutants: J1827 (*whiA* mutant), J1828 (*whiB* mutant), J1829 (*whiG* mutant), J1830 (*whiH* mutant) and J1831 (*whiI* mutant) were stained for polysaccharide deposition and analysed by TEM. g, glycogen granules.

equivalent A3(2)- and M145-derived mutants. This indicated that neither the newly constructed mutants nor the earlier mutants had unsuspected secondary mutations influencing glycogen deposition.

### Effect of *glgB* mutations on glycogen deposition in *whi* mutants

The J1508-derived *glgBI* (J1861) and *glgBII* (J1859) mutant strains were crossed with each J1508 *whi::hyg* mutant to produce equivalent sets of *whi glgBI* and *whi glgBII* double mutants in the J1508 genetic background.

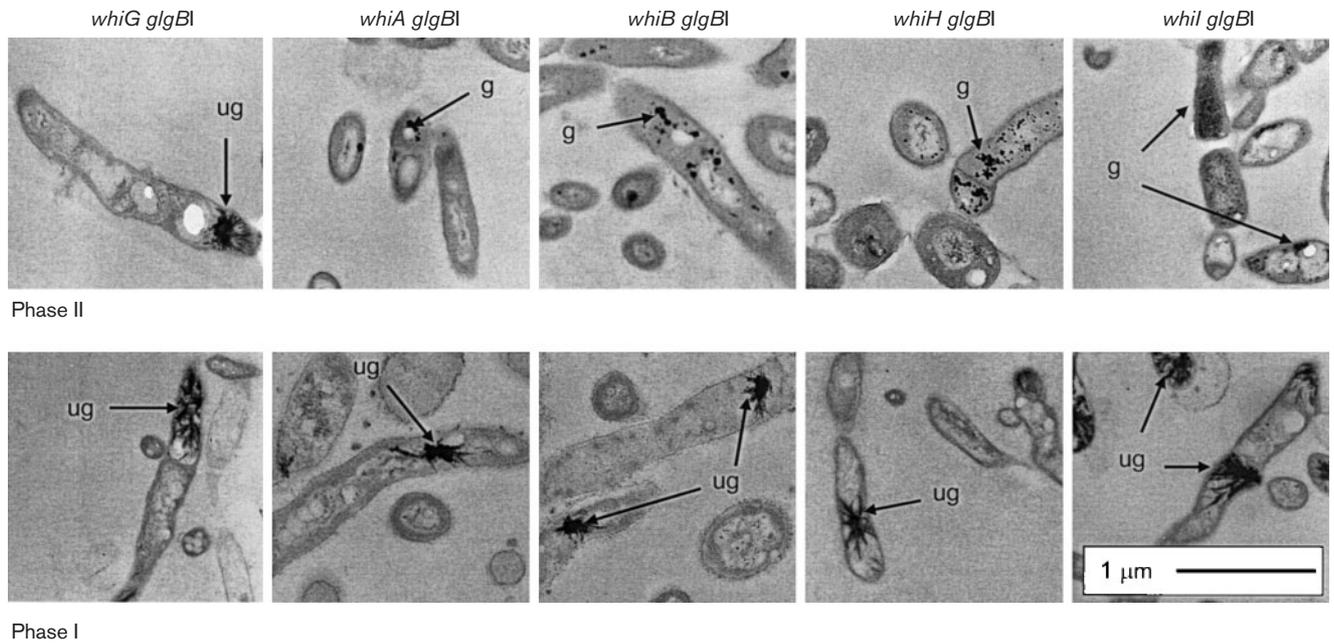
If, as Plaskitt & Chater (1995) suggested, phase II glycogen deposition is positively regulated, directly or indirectly, by the *whiG* gene product ( $\sigma^{WhiG}$ ), all glycogen present in a *whiG* mutant, even that in the uppermost parts of the developmentally defective aerial hyphae, would have been processed by the phase I branching enzyme GlgBI. In a *whiG glgBI* colony all granules of glycogen should therefore be replaced by large blobs of unbranched polysaccharide throughout the colony. This was indeed observed (Fig. 3), and no 'normal' glycogen was observed anywhere in the *whiG glgBI* colony. Thus, in the *whiG* mutant, all glycogen branching, even in the aerial mycelium, depends on the activity of GlgBI, showing not only that GlgBII is inactive in the *whiG* mutant, but also that the usual shut-off of net glycogen deposition in the growing aerial hyphae does not take place. On the other hand, since two spatially separate phases of glycogen deposition had been found in

the other *whi* mutants examined, it seemed likely that both branching enzymes were active in a spatially appropriate manner in those mutants. Thus, we predicted that the substrate mycelium of double mutants of *glgBI* with *whiA*, *whiB*, *whiH* and *whiI* should contain large irregular aggregations of polysaccharide, while normal dispersed granules of glycogen, reflecting activity of GlgBII, were expected in the aerial hyphae. This prediction was borne out (Fig. 3).

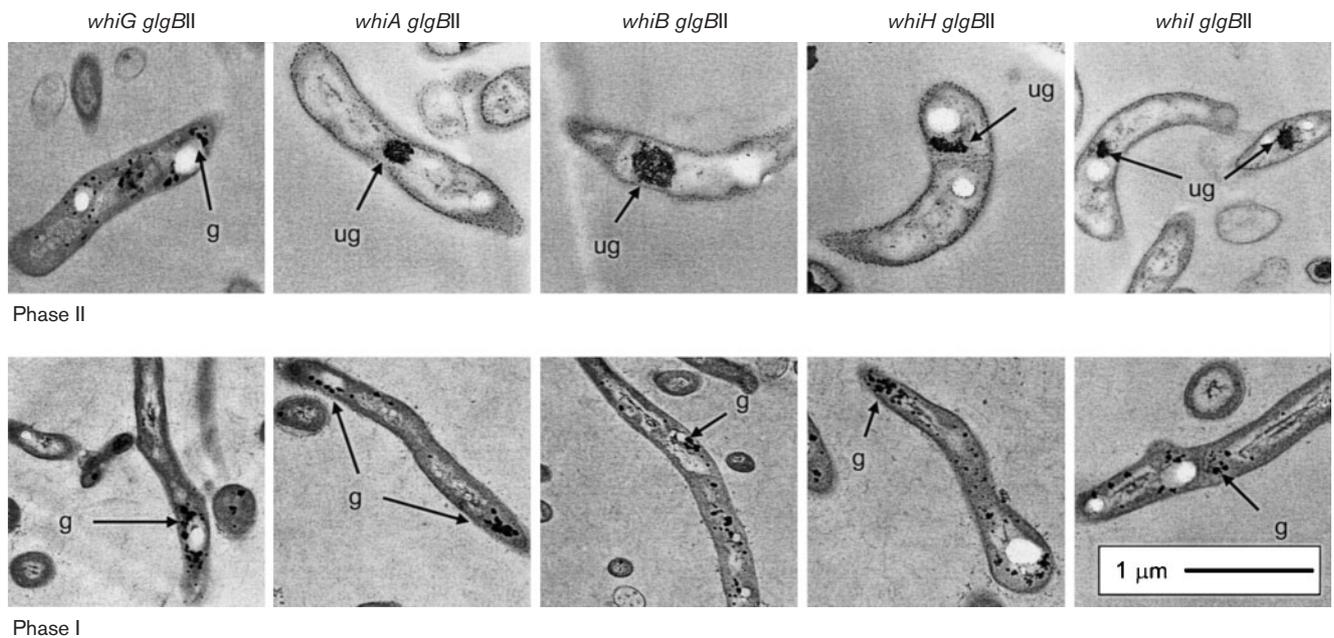
As a corollary of these results, we expected the *glgBII* double mutants with *whiA*, *whiB*, *whiH* and *whiI* mutations to have mutant blobs of unbranched polysaccharide in their aerial mycelium. The TEM studies confirmed these predictions (Fig. 4). On the other hand, a *whiG glgBII* mutant was expected to be indistinguishable from a *whiG glgB<sup>+</sup>* mutant, since *glgBI*, which had been implicated in branching of all the glycogen throughout the colony of a *whiG* mutant, was still present. Data such as those in Fig. 4 confirmed this prediction.

## DISCUSSION

The aim of this study was to clarify the pattern of dependence of aerial-mycelium-associated (phase II) glycogen branching on five well-characterized developmental regulatory genes involved in sporulation. We found that the spatial patterns of activity of *glgBI* and *glgBII* were unperturbed by mutations in four of the genes (*whiA*, *B*, *H* and *I*), although the amounts of phase II glycogen were affected in some of the mutants, being particularly low in the *whiA*



**Fig. 3.** Glycogen deposition in a *whi glgBI* mutant set. Sections of 4-day-old colonies of *whi glgBI* mutants: J1839 (*whiA glgBI* mutant), J1840 (*whiB glgBI* mutant), J1841 (*whiG glgBI* mutant), J1842 (*whiH glgBI* mutant) and J1843 (*whiI glgBI* mutant) were stained for polysaccharide deposition and analysed by TEM. g, glycogen granules; ug, deposits of unbranched polysaccharides.



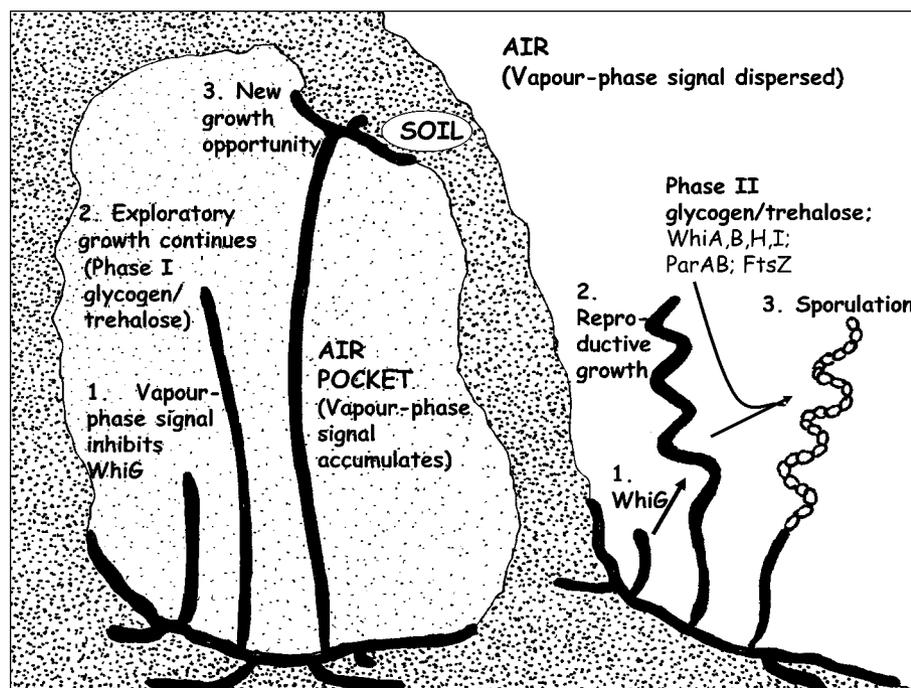
**Fig. 4.** Glycogen deposition in a *whi glgBII* mutant set. Sections of 4-day-old colonies of *whi glgBII* mutants J1833 (*whiA glgBII* mutant), J1834 (*whiB glgBII* mutant), J1835 (*whiG glgBII* mutant), J1836 (*whiH glgBII* mutant) and J1837 (*whiI glgBII* mutant) were stained for polysaccharide deposition and analysed by TEM. g, glycogen granules; ug, deposits of unbranched polysaccharides.

and *whiB* mutants and rather high in the *whiH* mutant. The low levels may indicate either that phase II glycogen polymerization takes place at a reduced rate in the *whiA* and *whiB* mutants, or that it is short-lived, as appears to be the case in wild-type sporulating hyphae. The comparatively abundant glycogen seen in the *whiH* mutant may indicate that it is long-lived, perhaps indicating a role for WhiH either in shutting off the synthesis of phase II glycogen or in activating spore-maturation-associated glycogen degradation.

Different results were obtained with *whiG* mutants, in which we found no evidence of any GlgBII activity; instead the aerial hyphae contained abundant glycogen whose branching depended specifically on GlgBI. The expression of *glgBII* therefore depends on *whiG*, but not on *whiH* or *I* (both of which are themselves directly and completely *whiG*-dependent; Ryding *et al.*, 1998; Aínsa *et al.*, 2000) or *whiA* or *B* (which are *whiG*-independent; Aínsa *et al.*, 1999). The abundance of aerial-mycelium-associated glycogen in *whiG* mutants may reflect the absence of WhiH from such mutants, if WhiH does indeed influence the quantity of glycogen in aerial hyphae (see above).

A search of the DNA sequence upstream of the *glgBII* operon (as far as the diverging coding sequence SCO7337, and including SCO7336, an uncharacterized small gene that may also be part of the operon: Schneider *et al.*, 2000) revealed some regions similar to the consensus '-10' sequence for  $\sigma^{\text{WhiG}}$ -dependent promoters, but we were not successful in attempts to identify the transcript of the *glgBII* operon, so we have yet to establish a role for these sequences. None of the potential promoter sequences was conserved in the genome of *Streptomyces avermitilis* (Ikeda *et al.*, 2003). This indicates that the  $\sigma^{\text{WhiG}}$ -dependence of *glgBII* is likely to be indirect, perhaps via a *whiG*-dependent regulator that is not essential for sporulation, and which has therefore not been revealed by isolating sporulation-defective mutants.

It seems that in a *whiG* mutant, glycogen is deposited in the aerial hyphae as if they were physiologically equivalent to the part of the substrate mycelium from which aerial hyphae emerge. A plausible interpretation of this observation is that, in the wild-type, emerging aerial hyphae are initially like those of the *whiG* mutant but, as they extend, the  $\sigma^{\text{WhiG}}$  form of RNA polymerase may become active and



**Fig. 5.** Tentative model for the involvement of WhiG and phase I and phase II glycogen in two alternative developmental fates for aerial hyphae. The right-hand part of the figure depicts typical reproductive development, in which phase II glycogen-related metabolism coincides with the activity of genes such as *ftsZ* (cell division) and *parAB* (chromosome partitioning) at the time of formation of pre-spore compartments (Chater, 2001). The left-hand part of the figure illustrates the hypothesis that, in tiny enclosed spaces in soil, aerial hyphae may make a distinct developmental decision, and grow in a non-reproductive 'exploratory' manner until they encounter another, and potentially colonizable, surface. It is proposed that the accumulation of signalling molecules in the vapour phase of an enclosed space might determine the choice of this developmental pathway. Note that in the exploratory aerial hyphae the phase I-associated glycogen branching/trehalose system operates, in contrast to the operation of the phase II-associated system in sporulating reproductive aerial hyphae.

then cause commitment to a sporulation-specific cell fate. In the absence of  $\sigma^{\text{WhiG}}$ , this developmental decision cannot be made, and an alternative non-reproductive kind of aerial growth continues.

Interestingly, scanning electron micrographs of soil-growing streptomycetes (Wellington *et al.*, 1990) clearly indicate that hyphae can extend across air spaces to form bridges between soil surfaces. This observation makes us wonder if the extended non-reproductive aerial growth seen in *whiG* mutants might ever occur and have a role in the life of the wild-type *S. coelicolor*. We suggest that, in some soil microenvironments, some aerial hyphae may fail to activate  $\sigma^{\text{WhiG}}$ , giving a *whiG* mutant phenocopy, and take on an alternative exploratory role, which could lead to the colonization of nearby soil surfaces (Fig. 5). The repeated deposition of glycogen in newly formed compartments during the extension of such hyphae may help to fuel further growth into the air. The occurrence of these deposits implies some form of transport of glycogen precursors from the substrate mycelium. Because such exploratory growth would not be useful unless other colonizable surfaces were nearby, a sensing mechanism based on the increased accumulation of volatile signals in partially enclosed small spaces could be imagined, which might account for the tufts of long white hyphae that are often seen to emerge from sporulating agar surface cultures of streptomycetes on prolonged incubation on Petri dishes or slants.

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