**SGM
SPECIAL
LECTURE****Taking a genetic scalpel to the *Streptomyces* colony**

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Background

Mature *Streptomyces* colonies grown on agar media are morphologically differentiated, since their lower parts consist of a branching vegetative mycelium and their upper surface is covered with aerial hyphae bearing chains of spores (Fig. 1). This morphological complexity was one of the initial attractions of streptomycetes as subjects for genetic analysis, along with their importance as producers of antibiotics. Indeed, in the early work of D. A. Hopwood – the father of *Streptomyces* genetics – there are as many papers on the anatomy of *Streptomyces coelicolor* A3(2) as on its genetics, and in his first major review (Hopwood, 1967) the example of how to assign genetic linkage was the S48 mutation (now known as *bldA1*; see below), which causes loss of aerial mycelium formation.

Another of the attractions of *S. coelicolor* A3(2) for a geneticist was its production of a diffusible blue pigment, which provided a potentially useful genetic marker. Later, it turned out that the blue pigment, and a red mycelium-bound pigment produced by the strain, were also antibiotics (actinorhodin and undecylprodigiosin, respectively), and could be used as models for the study of the general and specific mechanisms and regulation of antibiotic biosynthesis (e.g. Bibb, 1996) as well as providing a test-bed for understanding the genetic determination of antibiotic structure (e.g. Hopwood, 1997). If a surgical scalpel is used to section a suitably grown colony of *S. coelicolor* (Fig. 2), it is obvious that these pigments are produced in one region of the morphologically homogeneous substrate mycelium, revealing a further layer of developmental complexity ('physiological differentiation'; Chater, 1984). When colony sections are prepared for electron microscopy and subjected to suitable staining procedures, still more evidence of physiological differentiation becomes apparent: granules of the storage polysaccharide glycogen are visible at two distinct locations in the colony. One of these (phase I) approximately coincides with the region producing coloured secondary metabolites, and the

other (phase II) is specific for an early stage of spore chain development in aerial hyphae (Fig. 3) (Braña *et al.*, 1986; Plaskitt & Chater, 1995).

Anatomical studies provide an essential framework for analyses of the underpinning biochemical and molecular

Fig. 1. Spore chains in the aerial mycelium of *S. coelicolor* A3(2). Scanning electron micrograph kindly provided by Kim Findlay. Bar, 1 µm.

Fig. 2. Colony of *S. coelicolor* A3(2), sectioned vertically to reveal morphological and physiological differentiation. The vegetative mycelium of the colony (4 d old, grown on R2YE agar) is seen to grow into the agar for several millimetres. The surface is covered with a thin fluffy layer of spore-bearing aerial mycelium (am). In the centre of the colony, just below the aerial mycelium, is a red-pigmented layer (red) indicating the location of most active secondary metabolism. Bar, 0.5 mm. Photograph kindly supplied by Dr Jamie Ryding.

mechanisms of differentiation (Fig. 4). The primary sequences of the genes that determine these mechanisms provide a quite distinct reference point, and the search for these sequences, which has occupied much of my career, is now approaching completion. In a fanciful analogy, to which I shall not return, one might equate gross anatomy of a colony with a football stadium, and the genes with a football rule book and team sheet. Our problem then is to understand how these, respectively, very physical and almost abstract items translate into a vibrant, compelling football match. (Some might argue that the typically 4–5 d life cycle of a *Streptomyces* colony makes an analogy with cricket more suitable.)

Here I review how and what we have learned about *S. coelicolor* genes that play important roles in either the master control systems for differentiation, or the direct determination of biochemical pathways activated during differentiation. Although much work started from the isolation of mutants with altered morphology, reverse genetics, in which genes cloned by information from protein sequence (or from DNA sequencing) are used to generate mutants, is playing an ever-increasing part. Because this article is loosely based on the text of my Fred Griffith Review Lecture, it is biased towards work in which I have had some involvement, and should not be taken to be a comprehensive survey of the literature. For broader coverage, readers are referred to reviews by Hodgson (1992), Chater (1993), Champness & Chater (1994), Chater & Losick (1997) and Chater & Bibb (1997).

The *bld* genes: enigmatic pointers to extracellular signals and intracellular switches for secondary metabolism and aerial growth

Mutants of *S. coelicolor* that fail to develop a hairy surface layer of aerial mycelium are generally called *bld* (bald) mutants. Successive *bld* mutant hunts in different

laboratories (Merrick, 1976; Champness, 1988; Schauer *et al.*, 1991; Willey *et al.*, 1993; Nodwell *et al.*, 1996), followed by genetic analysis, have revealed more than a dozen *bld* genes (Hodgson, 1992; Champness & Chater, 1994). Other mutations that cause the bald phenotype have been generated by the use of cloned DNA to disrupt chromosomal genes: such genes have not usually been called *bld* genes, partly because they had already received other designations before the mutant phenotype became apparent (e.g. *ram*; Ma & Kendall, 1994), or because the biochemical function was known before the significance for aerial mycelium formation became apparent (thus *relA* mutants, which are defective in ppGpp synthetase, are bald on nitrogen-limited media; Chakraborty & Bibb, 1997). In other streptomycetes, different *bld* mutants have been discovered. Most notably, in some *Streptomyces griseus* strains, *bld* mutants of a particularly frequent kind are primarily defective in production of A-factor, an extracellular γ -butyrolactone signalling molecule; and the ability of certain genes to restore aerial mycelium to these mutants, when introduced at high copy number, has been very informative about the regulation of sporulation. From such information, Horinouchi (1996) proposed that the phosphorylation of two regulatory proteins by cognate membrane-bound protein kinases is important for the A-factor-dependent initiation of aerial growth in *S. griseus*. The important studies dealing with A-factor and similar molecules have been reviewed extensively by Horinouchi & Beppu (1994) and will be referred to only tangentially in this review.

For various studies of physiological characteristics that are likely to show some degree of developmental regulation, a subcollection of representative *S. coelicolor* *bld* mutants (*bldA*, *B*, *C*, *D*, *G* and *H*) has been used in several laboratories. The make-up of this collection is largely a historical accident – these mutants happened to have been isolated earlier. Willey *et al.* (1991) discovered that most of these mutants failed to produce a small extracellular protein, SapB, that is excreted into

Fig. 3. Glycogen deposits at two locations in a colony of *S. coelicolor* A3(2). The black dots are glycogen deposits stained by silver proteinate. Those occurring in hyphae at the interface of the vegetative and aerial mycelium are labelled phase I, and those occurring at an early stage of sporulation are labelled phase II. Note the absence of glycogen from the non-sporulating parts of the aerial hyphae and from more mature spores. Photograph kindly supplied by Kitty Plaskitt. Bar, 2 μ m.

the medium by Bld⁺ strains on some media and is also associated with the surface of aerial hyphae and spores. Nodwell *et al.* (1996) found that the same was true for a newly isolated mutant, *bldK*. Conversely, a mutant (*bld261*) isolated by screening for loss of SapB production had a Bld phenotype (Willey *et al.*, 1991). All

these mutants could be induced to develop aerial mycelium by adding purified SapB protein, so SapB can be considered to be a morphogen, a situation reminiscent of observations with the hydrophobins that coat the surface of fungal aerial mycelium (Chater, 1991); but there is a significant difference in that SapB, which contains only 17 amino acids, appears to be synthesized non-ribosomally, presumably by a peptide synthase mechanism (Willey *et al.*, 1993).

The SapB/*bld* mutant story proved to be even more interesting when it turned out that some *bld* mutants could be stimulated to develop aerial mycelium (and produce SapB) by growth near other *bld* mutants (Willey *et al.*, 1993). The matrix of interactions could be interpreted as a dependence sequence of at least five extracellular signals, with each participating *bld* mutant being deficient in one step of the cascade (Fig. 5). Each mutant appears to secrete signals up to the point of its deficiency, and responds to signals downstream of it. Thus the *bld261* mutant, which can be stimulated by all the other mutants that define the cascade, but cannot itself stimulate any of them, is presumably defective in the first signal, signal 1. Nodwell *et al.* (1996) proposed that signal 1 is an extracellular oligopeptide, since the complex *bldK* locus, whose involvement in the next step in the cascade is indicated by its stimulation only of the *bld261* mutant and by all the other mutants, encodes a five-subunit oligopeptide permease. Uptake of signal 1 by this permease is proposed to lead, via unknown genes, to production of signal 2. Since *bldA* and *bldH* mutants stimulate *bld261* and *bldK* mutants, but no others, their deficiency is a failure to respond to signal 2 by producing signal 3, i.e. they must be defective in the recognition or uptake of signal 2, or the subsequent signal transduction, or the actual production of signal 3. Similar logic implicates *bldG* at some stage between signals 3 and 4, *bldC* between signals 4 and 5, and *bldD* between signal 5 and the production of SapB. Nodwell *et al.* (1996) also pointed out that much of the apparent signalling cascade might alternatively be explained if the extracellular signals were precursors in a biosynthetic pathway, either for SapB itself or for a signal to activate SapB biosynthesis. The different precursors would be produced if the *bld* mutants were blocked at sequential steps in the relevant biosynthetic pathway. In this case the extracellular cascade might be no more than co-synthesis of an end product via secretion of biosynthetic intermediates.

Surprisingly, this elaborate pathway and the need for SapB are apparent only on rich medium. On defined minimal medium SapB is not detectable (Willey *et al.*, 1991). Instead, a different kind of responsiveness is observed: several kinds of *bld* mutant can be induced to form normal, sporulating aerial mycelium on minimal medium if carbon sources other than glucose are used (Chater & Merrick, 1976, 1979; Champness, 1988). On the other hand, representative *bldA*, *B*, *C*, *D*, *G* and *H* mutants seem to lack glucose catabolite repression of galactose utilization, as judged by levels of catechol dioxygenase activity in colonies containing the

Fig. 4. Diagrammatic section through a *Streptomyces* colony, indicating the different physiological states of hyphae at different locations. The 'storage deposits' particularly represent glycogen, but a comparable distribution is broadly applicable to other storage compounds, such as oil and polyphosphates (Olukoshi & Packter, 1994; Plaskitt & Chater, 1995).

Fig. 5. *bld* genes of *S. coelicolor* A3(2) and their roles in extracellular signalling. The scheme is based on the pattern of interactions among different *bld* mutants, some of which exhibit the ability to cause restoration of aerial growth to others. The apparent molecular nature of some *bld* gene products is also shown. See text for further details and sources of information.

xylE reporter gene fused to the *galP1* promoter. One mutant (*bldB*) was analysed further and shown also to be catabolite derepressed for genes for glycerol utilization and agar decomposition (Pope *et al.*, 1996). Many of the *bld* genes (*bldA*, *B*, *D*, *G* and *H*) are also needed for general secondary metabolism, including production of the pigmented antibiotics actinorhodin and undecylprodigiosin referred to earlier (Champness & Chater, 1994), and for the normal occurrence of phase I glycogen (Plaskitt & Chater, 1995). The *bld* genes thus seem to operate at the interface of primary metabolism, secondary metabolism, storage metabolism and morphological development.

In addition to *bldK*, mentioned above, several *bld* loci have been cloned (Piret & Chater, 1985; Harasym *et al.*, 1990; Elliot *et al.*, 1998; B. K. Leskiw, R. Passantino & K. F. Chater, unpublished). *bldA*, *bldB*, *bldD* and *bldG* encode products that are, or may well be, associated with gene expression. Thus *bldD* encodes a transcription-factor-like protein (Elliot *et al.*, 1998), and *bldG*, a protein of a class known to interact with transcription factors (J. Warawa & B. K. Leskiw, personal communication). Likewise, *bldB* encodes a

potential transcription factor (Pope *et al.*, 1998). The *bldA* gene product, which I discuss in the next section, is the tRNA for the rare leucine codon UUA (Lawlor *et al.*, 1987; Leskiw *et al.*, 1991b). It is a curious fact that *bldA* is so far the only *S. coelicolor* *bld* gene also identified as a *bld* gene in *S. griseus* (Kwak & Kendrick, 1996).

The rare codon UUA is specifically important for morphological and physiological differentiation

In the case of *bldA*, the effect on one aspect of physiological differentiation, pigmented antibiotic production, is largely understood (Fig. 6). It is because of the presence of a UUA codon in the mRNA for a pathway-specific activator for each pathway (Fernández-Moreno *et al.*, 1991; Guthrie *et al.*, 1998; White & Bibb, 1997). This codon, the target for the leucyl tRNA determined by *bldA* (Leskiw *et al.*, 1991b), is extremely rare in *Streptomyces* genes (Wright & Bibb, 1992) and there is no known case of its occurrence in genes required for vegetative growth (Leskiw *et al.*, 1991a). This gives rise to a model in which the *bldA* tRNA, in some circumstances, is present in amounts low enough to limit translation of UUA-codon-containing mRNAs. Indeed, when Trepanier *et al.* (1997) compared the abundance of the *bldA* tRNA with that of a tRNA for the abundant leucine codon CUC during growth and differentiation on the rich medium R2YE, it was clear

Fig. 6. UUA codons in the transcripts of regulatory genes for antibiotic production (*actIIORF4* and *redZ*) provide targets for *bldA*, which encodes the tRNA needed to translate this rare codon. The diagram is based on information in Fernández-Moreno *et al.* (1991), White & Bibb (1997) and Guthrie *et al.* (1998). 'SARPs' (*Streptomyces* antibiotic regulatory proteins) are a family of regulatory proteins apparently peculiar to antibiotic production in *Streptomyces* spp. (Wietzorrek & Bibb, 1997).

that the proportion of *bldA* tRNA increased in older cultures, in extension of earlier results suggestive of increased translatability of UUA codons, and more rapid processing of primary *bldA* transcripts, at this growth stage (Leskiw *et al.*, 1993). However, clear-cut evidence that *bldA* really *regulates* translation of UUA-containing mRNAs is still lacking (see Gramajo *et al.*, 1993).

On the other hand, the nature of the involvement of *bldA* in aerial growth is unclear: the predicted TTA-containing genes through which *bldA* might influence aerial growth have proved elusive. The available *S. coelicolor* protein-encoding *bld* gene sequences (*bldB*, *bldD*, *bldG*, *bldK*) do not contain TTA codons, though the codon does occur in a few *Streptomyces* genes that are connected with aerial mycelium formation. One of these is a gene (*orf1590*) of *S. griseus* discovered by its ability, in multiple copies, to suppress the Bld phenotype of certain *S. griseus* *bld* mutants (Babcock & Kendrick, 1988, 1990; Horinouchi, 1996). *S. coelicolor* also contains an *orf1590* gene with a TTA codon (McCue *et al.*, 1992). This gene has so far resisted disruption, leading McCue *et al.* (1996) to propose that it acts to *repress* sporulation during growth (thus disruption of *orf1590* would prevent growth because of constitutive sporulation). On this view, suppression of certain *S. griseus* *bld* mutant phenotypes by multiple copies of *orf1590* may be related to the discovery that the relevant *bld* mutants (including *bldA*) actually undergo key events in sporulation prematurely, near the start of aerial growth instead of at its end (Kwak & Kendrick, 1996: a result consistent with the presence of aberrant sporulation septa in the surface mycelium of *bldA* mutants of *S. coelicolor*; Chater & Merrick, 1976, 1979). Thus premature engagement in sporulation septation on the surface of a colony prevents visible aerial growth, and excessive *orf1590* gene product may repress such premature engagement.

One might argue that *orf1590* should be nullified in a *bldA* mutant, since the UUA codon in its mRNA should render it untranslatable. It appears that some UUA codons can in fact be translated, albeit at a much reduced efficiency, in *bldA* mutants, and that multiple copies or increased transcription of the relevant genes can sometimes compensate for the inefficiency of translation [e.g. *actII* ORF4 (Passantino *et al.*, 1991; Fernández-Moreno *et al.*, 1991) and *redZ* (Guthrie *et al.*, 1998)].

A TTA codon is also present in the *S. griseus* gene *amfR*, one of a cluster of three genes that, in multiple copies, restored aerial mycelium to A-factor-deficient (*afsA*) mutants of *S. griseus* but inhibited aerial growth in the wild-type *S. griseus* (Ueda *et al.*, 1993; Horinouchi, 1996). Thus, like *orf1590*, *amfR* might also be proposed to encode a repressor of key sporulation events. [Ueda *et al.* (1993) found that multiple copies of *orf1590* also suppress the morphological deficiency of *afsA* mutants, a result that Babcock & Kendrick (1988) and McCue *et al.* (1996) could not repeat, perhaps because of differences in growth conditions or genetic background.]

Intercellular signalling and speciation

The *ramABR* gene cluster of *S. coelicolor* is homologous with *amfABR* of *S. griseus*, but the *ram* genes contain no TTA codons (Ma & Kendall, 1994). When one of the *ram* genes, *ramB*, was disrupted in *Streptomyces lividans*, the result was bald colonies with low viability, while additional copies of the *ram* cluster in *S. lividans* accelerated aerial growth. Since *ramR* and *amfR* encode response-regulator-like proteins, and *ramAB* and *amfAB* encode proteins related to peptide transporters, Ma & Kendall (1994) and Horinouchi (1996) suggested that they are involved in the production of, and response to, peptide signal molecules. It remains to be determined how, if at all, these signal molecules are related to those involved in the cascade described above. The *S. griseus amf* and *S. coelicolor ram* genes, at around 57% DNA and 40–50% protein sequence identity, differ from each other much more than appears to be typical for homologous genes among streptomycetes (e.g. the glycerol utilization operons of *S. coelicolor* and *S. griseus* are about 80–90% identical; Bolotin & Biró, 1989). This may reflect a role for cell–cell signalling in self-recognition and, hence, in species divergence: self-recognition implies discrimination between species, and therefore selection for divergence in some aspects of the molecular machinery for signalling.

The most extensively studied example of divergence in cell–cell signalling machinery concerns the production and recognition of extracellular γ -butyrolactones. Most streptomycetes produce a small range of such structures, and in several species they are known to be important signals for the onset of morphological and/or physiological differentiation (Horinouchi & Beppu, 1994; Chater & Bibb, 1997). Small, but significant, differences between profiles of such molecules produced by different species are sufficient to bring about very considerable species specificity in their effects, a specificity reflected in the marked divergence among streptomycetes of genes for γ -butyrolactone receptors (e.g. Onaka *et al.*, 1995).

A further possible example of gene divergence associated with cell–cell signalling concerns the *spaA* gene of *S. coelicolor* and the *spa2* gene of *Streptomyces ambofaciens*. *spaA* is the closest homologue of *spa2* in *S. coelicolor*. The genes encode proteins that are only about 60% identical to each other and about equally similar to the product of the *Escherichia coli* gene *rspA* (Schneider *et al.*, 1993, 1996; Huisman & Kolter, 1994). Huisman & Kolter (1994) proposed that the *rspA* product affects the abundance of a homoserine lactone molecule involved in signalling at the onset of *E. coli* stationary phase. Disruption of *spaA* in *S. coelicolor* affected the timing of antibiotic production, and possibly of sporulation, at high plating density (Schneider *et al.*, 1996), while disruption or multiple copies of *spa2* in *S. ambofaciens* reduced aerial hyphal development (Aubert *et al.*, 1997). Thus the *spa* genes may also be involved in cell–cell signalling at stationary phase, with each species perhaps employing a subtly different signal molecule.

The early *whi* genes: regulators of the developmental fate of aerial hyphae

The destiny of aerial hyphae is to produce spores. Therefore, once aerial hyphae have begun to grow, some signal(s) should exist to determine whether growth will continue or whether it will stop, to provide conditions necessary for sporulation to be initiated. The hypothetical signal(s) might also directly activate multiple sporulation septation, or alternatively sporulation septation might require one or more additional regulatory elements. At least some of the genes contributing to these decisions have been identified by studying *whi* mutants, whose aerial mycelium remains white on prolonged incubation because mature, grey spores are not formed (Hopwood *et al.*, 1970; Chater, 1972). Some *whi* mutants are directly defective in pigment biosynthesis. These define the complex *whiE* gene cluster, which specifies a type II polyketide synthase, indicating that the spore pigment is an aromatic polyketide like actinorhodin and many other antibiotics (Davis & Chater, 1990; Hopwood & Sherman, 1990; Hopwood, 1997). However, many of the *whi* mutants are also defective in producing unigenomic prespore compartments (Chater, 1972; McVittie, 1974). These mutants appear to define genes involved in a regulatory cascade governing the initial stages of sporulation. These genes are the 'early' *whi* genes.

Genetic analysis had suggested that the early *whi* mutants defined six loci: *whiA*, *B*, *C* (= *J*), *G*, *H* and *I* (Chater, 1972). [The mutant *whiC193* (Chater, 1972), originally used to define *whiC*, proved unrecoverable from lyophils, but other mutations with similar phenotypes and map location (*whi-53*, *whi-77*; Chater & Merrick, 1976) could be recovered. Because we do not know how they are related to *whiC193*, these mutations have been named *whiJ*.] The phenotypes of early *whi* mutants in relation to the wild-type developmental cycle are shown in Fig. 7. Molecular characterization has shown that *whiG* encodes a sigma factor (Chater *et al.*, 1989; Tan *et al.*, 1998) and that *whiB* encodes an unusual protein with transcription-factor-like features (Davis & Chater, 1992). More recently, Ryding (1995) used large-scale complementation experiments to clone *whiA*, *whiH* and *whiJ*; and *whiI* has been cloned by J. A. Aínsa & H. D. Parry (personal communication), in a map-based cloning strategy using the ordered *S. coelicolor* cosmid library of Redenbach *et al.* (1996). Molecular characterization of *whiA*, *I* and *J* is still at an early stage, and here the main focus is on *whiG*, *whiH* and *whiB*.

Activation of early- and late-sporulation-specific sigma factors

A critical factor for initiating sporulation is the level of the σ^{WhiG} form of RNA polymerase ($E\sigma^{\text{WhiG}}$) (Chater *et al.*, 1989). Premature and/or substrate-mycelium-associated ectopic sporulation can be elicited by multiple copies of *whiG* (Chater *et al.*, 1989). [Such ectopic

Fig. 7. Genes important in the life cycle of *S. coelicolor* A3(2). The terminal phenotypes of aerial hyphae in *whi* mutants differ somewhat from natural differentiation stages. This is shown by arrows diverging from the normal life cycle immediately before the relevant gene designations.

sporulation can also result from certain mutations mapping in or near *glkA*, which encodes a glucose kinase implicated in glucose catabolite repression (Kelemen *et al.*, 1995; Hodgson, 1982).] Moreover, multiple copies of certain σ^{WhiG} -dependent promoters elicit a leaky *whiG* mutant phenocopy, implying that the number of $E\sigma^{\text{WhiG}}$ molecules is so low that they can readily be reduced to a level insufficient to activate sporulation when sequestered by the supernumerary promoters (Tan & Chater, 1993). Considering the critical role of $E\sigma^{\text{WhiG}}$ in initiating sporulation, it was surprising to find that *whiG* transcription appears to be at a more or less constant level during the whole life cycle, even long before aerial hyphae begin to form (Kelemen *et al.*, 1996). This suggests some kind of post-transcriptional regulation of *whiG*. Most speculation on the nature of this has focused on the close similarity of σ^{WhiG} to motility σ factors in other bacteria, which are known to be regulated by anti- σ factors whose abundance depends on the stage of flagellar development (Hughes *et al.*, 1993; Losick & Shapiro, 1993; Kelemen *et al.*, 1996). However, there are no reports of progress in detecting such an anti- σ factor, and the question of how levels of σ^{WhiG} activity are determined in relation to the progression of morphological differentiation remains entirely unsolved.

Some later stages of sporulation (notably full pigmentation and spore wall thickening) require the activity of a different RNA polymerase holoenzyme, containing σ^{F}

($E\sigma^{\text{F}}$) (Potůčková *et al.*, 1995). Studies of mRNA abundance indicate that *sigF*, the gene encoding σ^{F} , is not transcribed until about the time when sporulation septa become detectable in aerial hyphae, and that *sigF* mRNA is absent from *whiG* mutants (Kelemen *et al.*, 1996). Could $E\sigma^{\text{WhiG}}$ interact directly with the *sigF* promoter? Almost certainly not, because the *sigF* promoter contains no sequences similar to the consensus sequence for promoter recognition by $E\sigma^{\text{WhiG}}$, which has been defined by *in vitro* and *in vivo* transcription experiments using promoters from *S. coelicolor* and *Bacillus subtilis* (Tan & Chater, 1993; Tan *et al.*, 1998). Presumably, therefore, some other σ factor directs RNA polymerase to transcribe *sigF*. None of the other known early *whi* genes (*whiA*, *B*, *I* and *J*) appears to encode an alternative σ factor, so it is at least possible that the σ factor concerned may also play a significant role outside of sporulation, with its activity on the *sigF* promoter being dependent on some additional factor(s). The other early *whi* gene products are candidates as such transcription factors, since they are all needed for efficient and appropriately timed *sigF* transcription (Kelemen *et al.*, 1996).

What regulatory role does WhiH play?

The sequence of *whiH* shows that it encodes a member of a large family of regulatory proteins found in many

Fig. 8. Some homologues of *WhiH* are regulated by organic acids at the carbon catabolism/anabolism interface. *FadR*, *LctR* and *PdhR* are *E. coli* homologues of *WhiH*. *FadR* regulates fatty acid metabolism and is sensitive to fatty acids in the form of long-chain acyl CoA (DiRusso & Nyström, 1998); *LctR* probably regulates lactic acid utilization in response to lactate (Dong *et al.*, 1993); and *PdhR* regulates genes for the pyruvate dehydrogenase complex, in response to pyruvate (Quail & Guest, 1995). The three co-regulatory carbon metabolism intermediates are all readily converted to PEP (phosphoenolpyruvate), allowing them to enter gluconeogenesis and thence glycogen synthesis; they can also readily be converted to storage oil.

different bacteria (Ryding *et al.*, 1998). The family is often named after one of its best-characterized members, *GntR* of *B. subtilis*. Many *GntR*-like proteins, including those most similar to *WhiH*, are repressors of some genes involved in carbon metabolism. Such repressors appear to bind to cognate sequences in target promoters via a typical helix–turn–helix motif located near their N-terminus (Haydon & Guest, 1991). The resulting repression can be alleviated by the presence of appropriate inducers, such as gluconate, lactate and CoA esters of fatty acids. Several of these repressors also repress their own production, and indeed *whiH* transcription was markedly increased in a *whiH* point mutant (Ryding *et al.*, 1998). Based on these observations and analogies, we hypothesize that *WhiH* can repress certain target promoters, and that this repression can be relieved by interaction with an unknown carbon metabolite whose concentration increases during the development of aerial hyphae (Ryding *et al.*, 1998). It may be relevant that several of the carboxylic acids that regulate *WhiH*-like proteins in *E. coli* are close to the anapleurotic interface whose modulation plays an important role in determining carbon flux to alternative sinks such as growth-associated biomass or storage metabolites (Fig. 8).

How could a repressor be *necessary* for full *sigF* expression and sporulation? Possibly, *WhiH* may have transcriptional activator properties as well as repressor properties, and activate the promoter of a gene needed for *sigF* expression (perhaps *sigF* itself). Alternatively,

one of the genes repressed by *WhiH* could itself encode a repressor of genes for further steps in sporulation. In this case, *sigF*, or a gene needed for its expression, would be constitutively repressed by this second repressor in a *whiH* mutant.

Interestingly, *whiH* is transcribed *in vivo* and *in vitro* by the σ^{WhiG} form of RNA polymerase (Ryding *et al.*, 1998). Thus to some degree the effect of *whiG* on late gene expression may be exerted via *whiH*. The finding that *whiH* transcription is significantly delayed compared with that of *whiG* appears to lend support to the idea of post-transcriptional regulation of *whiG* (see above). However, there is a caveat: some of the difference in transcription pattern of *whiG* and *whiH* disappeared in a *whiH* mutant, which showed earlier and increased *whiH* transcription compared with the wild-type, presumably reflecting a direct or indirect autoregulatory circuit for *whiH* (Ryding *et al.*, 1998).

The regulation and role of *whiB*

At the time when *whiB* was sequenced (Davis & Chater, 1992) there were no *WhiB*-like deduced gene products in databases. However, examples have recently come to light in mycobacteria and streptomycetes (W. Bishai & J. Gomez, personal communication; Chater, 1993; J. Soliveri, personal communication), suggesting that these (and therefore probably many other) actinomycetes may possess more than one *whiB*-like gene. Alignment of the first examples of these has revealed several conserved features, including small size (approx. 80–120 amino acids), four cysteine residues in a particular array, and a glycine-rich motif preceding a predicted basic α -helix near the C-terminus.

whiB is transcribed from a weak constitutive promoter (P1) and a strong developmentally regulated one (P2). Transcription from either *whiB* promoter is independent of *whiG* and vice versa (Soliveri *et al.*, 1992; Kelemen *et al.*, 1996), whereas the promoter of the late-sporulation gene *sigF* is inactive in *whiG* and *whiB* mutants. It therefore seems that *whiB* and *whiG* exert their effects on sporulation through converging pathways, rather than being components of the same linear dependence pathway (Fig. 9). At present, a reasonable working hypothesis is that *WhiB* protein is cytoplasmically located (it has a high proportion of charged residues and no extensive hydrophobic patches), and that its four cysteine residues may either interact with a metal or form inter- or intra-molecular disulphide bridges. Conceivably, the protein may act as a sensor of changes in the cytoplasmic redox state resulting either from aerial growth per se or from metabolic changes that take place as the growth of aerial hyphae stops. Structure predictions are consistent with the idea that a C-terminal helix–loop–helix of *WhiB* interacts with DNA, hence the notion that *WhiB* may be a transcription factor (Davis & Chater, 1992). Unfortunately, progress towards testing this model has been minimal: candidate target genes have not yet been discovered, and the *WhiB*

Fig. 9. Outline of the roles of *whi* genes during sporulation of *S. coelicolor* A3(2).

protein has not proved convenient to handle when overexpressed in *E. coli*.

Comparison of the regulation of sporulation in *S. coelicolor* and *B. subtilis*

Although consecutive activation of sigma factors is involved in both sporulation in *S. coelicolor* and endospore formation in *B. subtilis* (Stragier & Losick, 1996), there are considerable differences between the early stages of the two processes. The first indication of this is the role of σ^{WhiG} , whose homologue in *B. subtilis* (σ^D) is involved in flagellum biogenesis, chemotaxis and autolysis but not in sporulation (Helmann, 1991). The differences are very marked in the ways in which physiological information relevant to the decision to sporulate is gathered. In *B. subtilis*, this decision mainly reflects the phosphorylation state of the Spo0A protein (see Grossman, 1995 and Hoch, 1995 for recent reviews). This is determined by the sum total of phosphotransfer along a sporulation-specific phosphorelay system that culminates in Spo0A phosphate formation. Several

physiological signals cause autophosphorylation of certain protein kinases, which then phosphorylate the protein Spo0F. Its phosphate is passed to Spo0A via an intermediary protein, Spo0B. Spo0F phosphate and Spo0A phosphate are susceptible to dephosphorylation by phosphatases whose activity is also sensitive to signals relevant to the decision to sporulate. Most *spo0* mutations, which specifically prevent sporulation septation, affect components of this cascade, which seems to have evolved to permit the non-replicating stationary-phase *B. subtilis* cell to sporulate only if a number of internal and external parameters indicate that no alternative physiological state is appropriate (if all else fails, sporulate!). In these circumstances, Spo0A phosphate activates transcription of genes encoding the first two components of the famous sigma factor cascade controlling the orderly course of spore formation (Stragier & Losick, 1996).

In *S. coelicolor*, the sequences of the early *whi* genes do not reveal evidence of a similar phosphorelay. Moreover, the terminal morphologies of aerial hyphae of most of the early *whi* mutants are different from each other, whereas *spo0* mutants of *B. subtilis* are more or less indistinguishable from each other by phenotype. This indicates that in addition to their contribution to the regulation of sporulation via *sigF*, the early *whi* genes control their own regulons of genes, rather than contributing solely to the activation (via Spo0A) of a single regulon. This may reflect the fact that in streptomycetes, the decisions leading to sporulation are taken by cells (more precisely, aerial hyphae) that are growing. This contrasts with the situation in *B. subtilis*, in which the decision to sporulate is made by stationary-phase cells. [However, protein phosphorylation is important in initiating aerial growth from presumptively stationary phase substrate mycelium in *S. griseus* (see above). Horinouchi (1996) drew attention to the analogy of these observations to the sporulation phosphorelay of *B. subtilis*.]

The growth of aerial hyphae is unusual in that the necessary nutrients are supplied, at least partially, by self-cannibalization (Méndez *et al.*, 1985). This should provide a relatively controllable source of nutrients, permitting growth into a very uniform environment – the air. If these premises are correct, the regulatory proteins needed for sporulation septation might respond to a sequence of comparatively well-defined signals, such as changes in the levels of particular metabolites. In this model, each of the regulatory proteins would not only contribute to the regulatory cascade(s) needed to activate later sporulation processes, but also more directly activate changes in the physiology of the aerial hyphae (i.e. through regulation of metabolic pathways, cell wall growth, DNA replication, cell division components etc.). In *S. coelicolor* sporulation, the earliest known point by which all the regulatory signals are known to have converged is in the transcription of *sigF* (Kelemen *et al.*, 1996). The phenotype of a *sigF* mutant [chains of thin-walled, poorly pigmented spores

(Potůčková *et al.*, 1995)] shows that *sigF* acts later in *Streptomyces* sporulation than *spo0A* does in endospore formation. Interestingly, the closest known homologues of σ^F are the *B. subtilis* forespore-specific σ factors (σ^F and σ^G) and their relative σ^B (involved in stress responses) (Potůčková *et al.*, 1995). It therefore remains possible that there is an underlying common origin for the later stages of sporulation in *B. subtilis* and *S. coelicolor*.

What aspects of sporulation-associated cell division are defective in early *whi* mutants?

The tubulin-like protein FtsZ plays a crucial role in bacterial cell division (Bramhill, 1997). This is true in *Streptomyces*: disruption of *ftsZ* prevents all septation, whether the comparatively infrequent kind found in vegetative mycelium, or the kind that gives rise to spore compartments in aerial hyphae (McCormick *et al.*, 1994). [Remarkably, the latter study and its sequel (McCormick & Losick, 1996) revealed that vegetative and aerial growth can still take place in the absence of any septation!] FtsZ assembles in the cytoplasm as a peripheral ring at sites where cell division is to take place; the ring then contracts, leading the ingrowth of the septum (Bramhill, 1997). Recently, Schwedock *et al.* (1997) have shown by immunofluorescence that sporulation septation of aerial hyphae involves the formation of long 'ladders' of FtsZ rings, which disappear as the sporulation septa form. When *whiB*, *whiG* or *whiH* (or *whiA*; K. Flärdh & E. Angert, personal communication) mutants were used, ladders were not detected. Thus either the production of FtsZ or its assembly into sporulation-specific rings depends on these genes. The attractive possibility that this may be a transcriptional dependence has not been easy to test, because only a very small proportion of hyphae in a colony are at the appropriate developmental stage at any one time. However, recent studies of *S. griseus*, in which synchronous, large-scale sporulation can be induced in liquid medium, have shown that greatly increased expression of *ftsZ* from a sporulation-specific promoter precedes sporulation septation (K. E. Kendrick, personal communication). Bearing in mind that in *B. subtilis* sporulation-specific septation is crucially involved in the activation of sporulation-specific programmes of gene expression (Stragier & Losick, 1996), it will be interesting to investigate whether *sigF* expression is morphologically coupled to sporulation septation, in which case the dependence of *sigF* on early *whi* genes could be indirect.

The use of food storage depots in colonies

As mentioned above, glycogen deposits are found in two locations in colonies. Phase I deposits appear to be suitably located to permit their use as a source of carbon and energy for aerial growth, while phase II deposits in prespore compartments presumably provide much of the carbon for abundant components of mature spores

such as spore wall, spore pigment and trehalose (Braña *et al.*, 1986; Plaskitt & Chater, 1995; Karandikar *et al.*, 1997). Mature spores contain comparatively little glycogen (Braña *et al.*, 1986).

In general, a minimal set of enzymes needed for glycogen synthesis comprises: ADPglucose pyrophosphorylase, which provides activated glucose monomers as ADPglucose; glycogen synthase, which transfers glycosyl residues from ADPglucose to the growing 1,4- α -polyglucan chain; and branching enzyme, which takes the tips from such chains and relocates them as 1,6- α -linked branches. Two *glgB* (branching enzyme) genes have been discovered in *S. coelicolor*, and phenotypic analysis of disruptants showed that each branching enzyme isoform is responsible for glycogen branching in one of the two locations (Bruton *et al.*, 1995). This situation appears to be general among streptomycetes (Bruton *et al.*, 1995; Homerová *et al.*, 1996). Although only one *glgC* (ADPglucose pyrophosphorylase) gene has been identified in *S. coelicolor*, the phenotype of *glgC* disruptants showed that this gene is responsible only for phase I glycogen synthesis, and that a second *glgC* gene must also exist (Martín *et al.*, 1997). No *glgA* (glycogen synthase) gene has yet been unambiguously identified.

Recent unpublished results of D. Schneider & C. J. Bruton (personal communication) have uncovered at least three more duplicated genes linked to the *glgB* genes, and presumptively involved in the two localized phases of glycogen metabolism: the deduced products of two of them have features in common with various glucan-hydrolysing enzymes, though it remains uncertain whether they are involved in the tailoring of glycogen during biosynthesis, or in degradation. The analysis of how the biosynthetic genes are activated in a spatially specific manner, and what tips the balance of glycogen metabolism towards degradation at a developmentally appropriate time, will be a fascinating task during the next few years.

Some physical problems of aerial growth

Growth into the air provides a mechanical problem: how can sufficient turgor pressure be generated to drive hyphal extension into the air? I have previously suggested that the degradation of osmotically inactive polymers such as glycogen, oil and polyphosphate to give osmotically active monomers might contribute to the generation of osmotic pressure (Chater, 1989). The isolation of *glgCI* mutants, described in the previous section, provided the first test for the hypothesis: would the mutants show normal aerial growth if they could make no phase I glycogen? In fact, the mutants had no obvious morphological abnormality, ruling out an essential role for glycogen in aerial growth (Martín *et al.*, 1997). However, this did not rule out the general hypothesis, since other storage systems were still active and potentially able to compensate for any deficiency caused by lack of glycogen metabolism.

In most bacteria, the ability to grow in a hypertonic environment involves the accumulation of compatible solutes such as glycine betaine, which is very abundant in many natural environments. Recently, the first evidence has come to light that may indicate a developmental role for osmotically balancing compatible solutes during aerial growth. It has turned out that a promoter (P_{TH4}) discovered during a search for promoters dependent on $E\sigma^{WhiG}$ (Tan & Chater, 1993) drives transcription of a gene (*orfTH4*) encoding a homologue of ProX proteins (Tan *et al.*, 1998). In *B. subtilis* and *E. coli*, ProX is part of a system for the uptake of glycine betaine: in *E. coli* it is a periplasmically located glycine-betaine-binding protein, and it probably fulfils the equivalent role in *B. subtilis* by being attached to the outer face of the cytoplasmic membrane (Lin & Hansen, 1995). During *S. coelicolor* aerial growth the ProX-like protein may perhaps capture glycine betaine or a related compound diffusing up from the substrate in the aqueous layer trapped between the cell membrane and the hydrophobic outer layer of the aerial hyphae, and present it to a system for transport into the cytoplasm, where it would help to generate turgor to drive growth. However, just as with *glgCI* mutants, *orfTH4* mutants are morphologically normal (Tan *et al.*, 1998), again ruling out an *obligatory* role in morphogenesis, but leaving open the possibility that there are multiple systems to provide osmotic pressure. [Previous studies have shown that several other substances could act as compatible solutes at least during vegetative growth of streptomycetes (Killham & Firestone, 1984; Malin & Lapidot, 1996), and some other bacteria – notably *B. subtilis* – are known to have multiple systems for the uptake of glycine betaine and other compatible solutes (see Knappes *et al.*, 1996).]

My interpretation of the absence of morphological effects of *glgC* and *orfTH4* mutations raises a general point about the genes regulated by developmental regulatory genes. In general, we should not expect mutations in such target genes to give simple, readily observable blocks in the developmental process, because if they did, they should often have been recognized in the same way as the developmental regulatory genes themselves were identified. Moreover, the experience with similar studies in fungi was that mutations in many genes activated during conidiation had no readily observable phenotype (e.g. Aramayo *et al.*, 1989). On the other hand, the fact that the target genes are specifically activated by developmental regulatory genes makes it highly likely that they fulfil a role that, in some circumstances, confers more fitness on the developmental process during Darwinian selection. The absence of a phenotype in laboratory tests on mutants does not necessarily make the genes less interesting or important – merely less convenient! If a responsive physiological process (such as turgor control) is of great importance to an organism, it should not be surprising if the organism can bring it about by multiple routes, any one of which may assume a more dominant role in different environmental or developmental circumstances. No doubt

the increasing availability of complete genome sequences will help in teasing out the complexities of such multiple buffering systems in diverse organisms and processes.

What is to come?

I hope that this review has conveyed the sense that a skeletal integrated view of *Streptomyces* colony development is getting closer. Most of the known *whi* and *bld* genes have been cloned, and the nature of their gene products is becoming apparent. The first clear-cut regulatory interactions have begun to emerge, and some of the potential targets of the regulatory genes are being characterized. These include genes for some developmentally significant aspects of cell biology and metabolism. Although the intracellular signals that are perceived by some of the regulatory gene products remain a mystery, progress is being made in understanding several extracellular autoregulatory signals. The apparent spatial/cellular mutual exclusivity of sporulation and secondary metabolism/antibiotic production illustrated in Fig. 2, and analysed by Yu & Hopwood (1995), suggests the involvement of reciprocal regulatory systems [it is interesting to note that over-expression of *whiG* or *whiB* can lead to reduced transcription of genes involved in actinorhodin biosynthesis (J. Soliveri, personal communication)]. A fuller understanding of sporulation is likely to increase our knowledge of antibiotic production and vice versa. Most of these prospects will be greatly enhanced by the information coming from the on-going project at the Sanger Centre, Cambridge, to sequence the entire chromosome of *S. coelicolor* A3(2) (information available at ftp://ftp.sanger.ac.uk/pub/s_coelicolor/sequences).

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