

Genes essential for morphological development and antibiotic production in *Streptomyces coelicolor* are targets of BldD during vegetative growth

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Summary

BldD is a transcriptional regulator essential for morphological development and antibiotic production in *Streptomyces coelicolor*. Here we identify the BldD regulon by means of chromatin immunoprecipitation-microarray analysis (ChIP-chip). The BldD regulon encompasses ~167 transcriptional units, of which more than 20 are known to play important roles in development (e.g. *bldA*, *bldC*, *bldH/adpA*, *bldM*, *bldN*, *ssgA*, *ssgB*, *ftsZ*, *whiB*, *whiG*, *smeA-ssfA*) and/or secondary metabolism (e.g. *nsdA*, *cvn9*, *bldA*, *bldC*, *leuA*). Strikingly, 42 BldD target genes (~25% of the regulon) encode regulatory proteins, stressing the central, pleiotropic role of BldD. Almost all BldD binding sites identified by ChIP-chip are present in the promoters of the target genes. An exception is the tRNA gene *bldA*, where BldD binds within the region encoding the primary transcript, immediately downstream of the position corresponding to the processed, mature 3' end of the tRNA. Through gene overexpression, we identified a novel BldD target gene (*cdgA*) that influences differentiation and antibiotic production. *cdgA* encodes a GGDEF domain protein, implicating c-di-GMP in the regulation of *Streptomyces* development. Sequence analysis of the upstream regions of the complete regulon identified a 15 bp inverted repeat that functions as a high-affinity binding site for BldD, as was shown by electrophoretic mobility shift assays and DNase I footprint-

ing analysis. High-scoring copies of the BldD binding site were found at relevant positions in the genomes of other bacteria containing a BldD homologue, suggesting the role of BldD is conserved in sporulating actinomycetes.

Introduction

Streptomyces spp. are Gram-positive soil bacteria that grow as a vegetative mycelium of branching hyphae. For dispersion, spores are formed on specialized reproductive structures called aerial hyphae, which emerge from the colony surface into the air. Genetic analysis of differentiation in *Streptomyces coelicolor* has identified two classes of regulatory mutants, blocked in distinct stages of differentiation. White (*whi*) mutants form aerial hyphae in the normal way but these hyphae are unable to complete the developmental process to form mature chains of spores (Flårdh and Buttner, 2009). They appear white when grown on solid media because they fail to produce the grey polyketide pigment associated with mature, wild-type spores. *bld* mutants are blocked at an earlier stage of development; they are unable to erect aerial hyphae and therefore appear 'bald', lacking the characteristic fuzzy morphology of the wild type (Flårdh and Buttner, 2009). Streptomycetes are renowned for their ability to produce clinically important antibiotics and other bioactive compounds, and the production of these molecules is temporally and genetically co-ordinated with the developmental programme. Thus, in addition to causing loss of aerial mycelium formation, mutations in many *bld* loci, including *bldD*, the focus of this report, pleiotropically block antibiotic production.

One of the *bld* genes relevant to the work described here is *bldA*. Unlike almost all the other developmental regulatory genes in *Streptomyces*, *bldA* does not encode a transcription factor. Instead, *bldA* encodes the only tRNA that can efficiently translate the leucine codon UUA, a codon rarely used in streptomycete genomes, which typically have ~73% G+C content (Lawlor *et al.*, 1987). In *S. coelicolor*, only ~2% of the genes contain a TTA codon (Chandra and Chater, 2008) and these depend on *bldA* for translation (Leskiw *et al.*, 1991). No essential genes

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contain a TTA codon and so *bldA* null mutants are viable, but they are blocked in morphological differentiation and the production of the four antibiotics made by the wild-type strain: actinorhodin, the prodigionines, methylenomycin and calcium-dependent antibiotic (CDA) (Chater and Chandra, 2008). Recently, a fifth antibiotic produced by *S. coelicolor* was described, originating from the *cpk* gene cluster (Gottelt *et al.*, 2010). The requirement for the BldA tRNA in the production of actinorhodin and the prodigionines can be attributed to the presence of TTA codons in the genes encoding pathway-specific regulators of the corresponding antibiotic biosynthetic gene clusters, *actII-ORF4* and *redZ* respectively. The role of BldA in differentiation is mediated, at least in large part, by the presence of a TTA codon in the global regulator BldH (also called AdpA), as changing the codon to CTC or TTG substantially suppresses the morphological defect of *bldA* mutants (Nguyen *et al.*, 2003; Takano *et al.*, 2003).

The principal focus of this work is BldD. BldD is a small (18 kDa) DNA-binding protein that exists in solution predominantly as a homodimer (Elliot *et al.*, 2003b). BldD has two distinct domains, a C-terminal domain of unknown function and an N-terminal domain that mediates DNA binding and dimerization (Lee *et al.*, 2007). The crystal structure of the N-terminal domain has been determined and shows similarity with lambda repressor, a major-groove binding transcriptional regulator containing a classical helix–turn–helix DNA-binding motif (Kim *et al.*, 2006). Interestingly, BldD shows homology to *Bacillus subtilis* SinR, which functions as a transition-state regulator that represses sporulation and biofilm formation during vegetative growth, and promotes competence and motility functions (Bai *et al.*, 1993; Chu *et al.*, 2006). Like *bldA* mutants, *S. coelicolor bldD* mutants are blocked at the earliest stage of morphological differentiation and also fail to produce actinorhodin, the prodigionines, methylenomycin and CDA (Elliot *et al.*, 1998). Expression of *bldD* is temporally regulated and BldD has been shown to directly repress four developmental genes, as well as its own expression, during vegetative growth by binding to sequences overlapping the promoter and/or the transcriptional start site (Elliot and Leskiw, 1999; Elliot *et al.*, 2001; 2003b). Among the known BldD-regulated targets are two genes encoding sigma factors (σ^{BldN} and σ^{WhiG}) that, in turn, play crucial roles in *Streptomyces* development (Chater *et al.*, 1989; Bibb *et al.*, 2000; Elliot *et al.*, 2001). It has also been suggested that BldD links differentiation to stress, as it regulates expression of a third sigma factor gene, *sigH*, encoding σ^{H} , involved in the regulation of stress responses (Kelemen *et al.*, 2001).

Through the work of many laboratories, significant progress is being made in understanding the cell biological processes underlying morphogenesis in *Streptomyces* (Flårdh and Buttner, 2009), and all of the *bld* and

whi developmental master regulators defined by classical mutant hunts have been cloned and characterized. However, in total, only a handful of direct targets of these regulators has been identified. As a consequence, what is strikingly needed is an understanding of the regulatory networks that connect the developmental cell biological processes to the master regulators. Here we apply global approaches to identify the complete regulon of genes targeted by BldD, which reveal unexpected connections between BldD and a multitude of well-characterized genes involved in developmentally co-ordinated processes.

Results and discussion

Characterizing the regulatory networks that govern morphological development in *S. coelicolor* poses certain logistical problems. *S. coelicolor* sporulates only on solid medium and the differentiating part of the colony (the aerial mycelium) constitutes just ~10% of the total biomass (Flårdh and Buttner, 2009), making the application of global techniques like chromatin immunoprecipitation-microarray analysis (ChIP-chip) to development-specific transcription factors problematic. Characterizing the regulon of BldD was feasible, however, because previous work suggested that BldD functions to repress developmental genes during vegetative growth, and BldD is therefore active in vegetative cells grown in liquid culture where ChIP-chip can readily be applied. However, transcriptional profiling under these conditions has drawbacks because genes that are repressed by BldD but also activated by a developmental regulator, absent from liquid cultures, are unlikely to be differentially expressed between wild type and a *bldD* mutant. Finally, the highly pleiotropic role of BldD leads to a significantly reduced growth rate for *bldD* mutants on solid and in liquid media, with the potential to cause many indirect effects on the transcriptome. With these issues in mind, we attempted to characterize the full extent of the BldD regulon.

Effects of bldD inactivation on the S. coelicolor transcriptome

To examine the effects of BldD on genome-wide transcription, DNA microarray experiments were performed, comparing the transcriptional profile of *S. coelicolor* M600 (a plasmid-free derivative of the wild type) with that of a congenic $\Delta bldD$ null mutant in rapidly growing cultures in liquid-rich medium. RNA samples were prepared from both strains and, following cDNA synthesis and labelling, were hybridized to DNA microarrays. Analysis of the DNA microarray data from three independent experiments revealed that the expression of 359 genes (located in 261 putative transcription units) was changed over twofold as

Table 1. Transcriptome comparison of *S. coelicolor* M600 $\Delta bldD$ and *S. coelicolor* M600.

Transcriptional unit ^a	Expression ratio ^{b,c}	Description ^d
<i>rpmG</i>	121	Probable 50S ribosomal protein
SCO2505 to SCO2507	68	Putative ABC-transporter metal-binding lipoprotein
<i>leuB</i>	52	Probable 3-isopropylmalate dehydrogenase
<i>leuC</i>	52	3-Isopropylmalate dehydratase
<i>chpH</i>	45	Putative small membrane protein
SCO0475, SCO4772	36	ABC transporter system
SCO2492	26	Putative membrane protein
<i>bldN</i>	25	Putative RNA polymerase sigma factor
SCO1860	25	Putative secreted protein
SCO4677	24	Anti-sigma
SCO3097	19	Putative secreted protein
SCO4175 to SCO4172	17	Hypothetical protein
SCO3429 to SCO3432	16	Putative 50S ribosomal protein L28
SCO5970	15	Hypothetical protein
SCO3560	15	Putative ATP-binding protein
SCO3835	13	Putative dehydrogenase
<i>paa</i>	13	Putative phenylacetic acid degradation proteins
SCO4302	12	Putative secreted protein
SCO2529	10	Putative metalloprotease.
<i>katA</i>	10	Conserved hypothetical protein
SCO4768	10	<i>bldM</i> , putative two-component regulator
<i>cvnB10</i>	-10	Hypothetical protein
SCO4260, SCO4259	-12	Hypothetical protein
SCO4248 to SCO4244	-13	Hypothetical protein
SCO5519, SCO5520	-13	Hypothetical protein
<i>sacA</i>	-13	Aconitase
SCO4187	-18	Putative membrane protein
SCO6197	-18	Putative secreted protein
SCO0247	-19	Conserved hypothetical protein
SCO4261	-19	Putative response regulator
<i>eno2</i>	-21	Enolase
SCO1118	-24	Putative integral membrane protein
<i>tktB</i>	-26	Transketolase B
SCO4253 to SCO4251	-35	Hypothetical protein
<i>sti</i>	-41	Protease inhibitor precursor
SCO7657, SCO7658	-44	Putative secreted protein
SCO0268	-97	Hypothetical protein

a. Listed are genes transcription of which is affected 10-fold or more by the *bldD* mutation.

b. Shown is the relative expression for the gene (in an operon) that shows the highest ratio.

c. Level of expression in *S. coelicolor* M600 $\Delta bldD$ compared with that in *S. coelicolor* M600.

d. (Possible) gene function (see Bentley *et al.*, 2002 or text for references).

a result of the *bldD* mutation (Table S1). Of these transcription units, 118 were upregulated in the mutant, whereas 143 of them were downregulated in the mutant.

Among the genes that were most severely affected by *bldD* (Table 1), several are involved in primary metabolism. Expression of *leuB* and *leuC*, genes required for branched-chain amino acid biosynthesis, was increased 52-fold (see also below). Genes belonging to central pathways of primary carbon metabolism (*eno2*, glycolysis/gluconeogenesis; *tktB*, pentose phosphate pathway; *sacA*, citric acid cycle) were downregulated in the *bldD* null mutant. This is likely to be an indirect consequence of the slow growth rate of the $\Delta bldD$ strain, which is apparent from its reduced colony size, compared with the wild-type, when grown on plates (Elliot *et al.*, 2003b). Reduced expression of several

ABC transport systems and amino acid permeases could also reflect the slow growth rate of the mutant. The genes encoding putative 50S and 30S ribosomal proteins, *rpmEGB* and *rpsN*, were strongly upregulated in the *bldD* mutant, which could again be an indirect effect, indicative of a global change in physiology associated with *bldD* disruption.

One of the established roles of BldD in morphological differentiation is to repress *bldN* expression during vegetative growth (Elliot *et al.*, 2001). *bldN* encodes a developmentally regulated σ factor, σ^{BldN} , required for aerial mycelium formation (Bibb *et al.*, 2000). Consistent with this, the expression of *bldN* was 25-fold derepressed in the *bldD* mutant (Table 1). As part of the developmental cascade, σ^{BldN} in turn directly activates expression of *bldM*, which encodes a response regulator also required

Fig. 1. The *S. coelicolor* BldD regulon.

A. Chromosome-wide distribution of BldD binding sites identified by ChIP-chip analysis. DNA obtained from immunoprecipitation of BldD was labelled with Cy3 and hybridized to DNA microarrays together with a total-DNA control that was labelled with Cy5. Data are plotted as Cy3/Cy5 ratios (*y*-axis), as a function of chromosome location (*x*-axis).

B. ChIP-chip data for selected targets in wild-type *S. coelicolor* and the *S. coelicolor* $\Delta bldD$ mutant (black and white dots respectively). Peaks indicate the presence of BldD binding sites in regions near eight newly identified targets (*pepA/pepA2*, *ssgA*, *nsdA*, *cvnA9*, *slpD*, *bldA*, *smeA* and SCO4677), and upstream region of four known targets (*prs/sigH*, *whiG*, *bldD* and *cdgA*). Plots span approximately 8 kb of DNA sequence. Gene names or identifiers (SCO numbers) are indicated above the arrows, which indicate gene orientation.

for aerial mycelium formation (Bibb *et al.*, 2000; Molle and Buttner, 2000). *bldM* expression was 10-fold derepressed in the *bldD* mutant (Table 1), probably reflecting a combination of increased σ^{BldD} expression and the fact that *bldM*, like *bldN*, is directly repressed by BldD (see below). Expression of the *prsH-sigH* operon, encoding an anti-sigma factor/sigma factor pair directly regulated by BldD, was increased sixfold in the mutant. Differential expression of two other previously identified targets of BldD (*whiG* and *bdtA*) could not be detected, however.

The chaplins are a family of eight hydrophobic cell wall-associated proteins that confer hydrophobicity on aerial hyphae and spores, and constructed strains lacking most or all of the chaplin (*chp*) genes fail to form aerial hyphae on most media (Claessen *et al.*, 2003; Elliot *et al.*, 2003a). The *chpE* and *chpH* genes are strongly derepressed in the *bldD* mutant (Table 1 and Table S1). Of the chaplin genes, expression of *chpA*, *chpB*, *chpC*, *chpD*, *chpF* and *chpG* is strictly correlated with formation of aerial hyphae, whereas *chpE* and *chpH* are expressed in submerged hyphae as well (Claessen *et al.*, 2003), perhaps explaining why only the latter two were upregulated in the *bldD* mutant under the conditions of our transcriptome analysis.

The BldD regulon contains ~167 genes, including 42 genes that encode DNA-binding proteins

To distinguish between indirect and direct effects, BldD ChIP-chip experiments were performed. A culture of *S. coelicolor* M600 grown under the same conditions as for the transcriptional profiling experiments was subjected to formaldehyde cross-linking and sonication as described in *Experimental procedures*. After immunoprecipitation using a BldD-specific polyclonal antibody, the DNA was labelled with Cy3 and hybridized together with a total-DNA control, labelled with Cy5, to a high-density microarray representing the *S. coelicolor* genome. Figure 1 presents representative results from three independent biological experiments. Peaks in the fluorescence ratio indicate regions of the chromosome that are bound by BldD. As a control, a ChIP-chip experiment was performed using the congenic *bldD* null mutant to eliminate any signals arising from cross-reaction of the antibody with other transcription factors. A total of ~172 peaks were detected throughout the chromosome in the

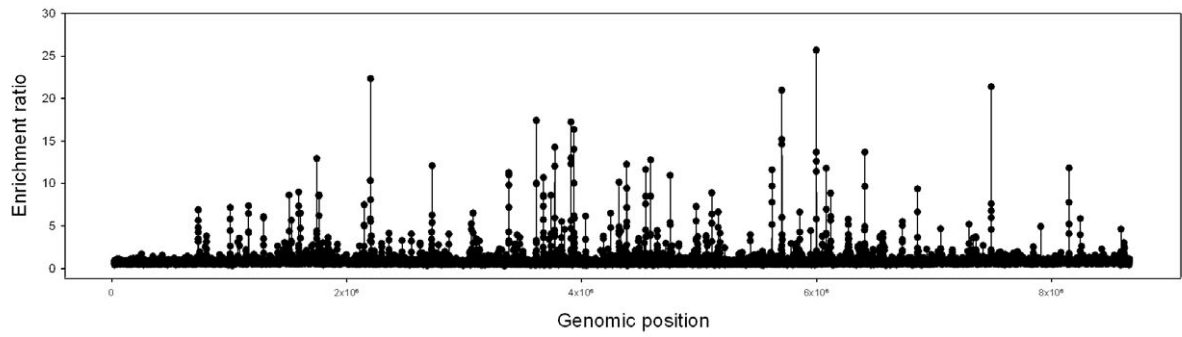
wild-type strain. Five of these peaks were also found in the *bldD* null mutant control experiment and these were removed from the data set, leaving ~167 putative BldD binding sites, of which 29 of the downstream genes were identified in the transcriptome analysis described above. BldD binding sites were found across the genome but the density of sites was greater in the core essential genome and less in the two arms of the linear chromosome (Fig. 1A), regions that can be lost without affecting viability under laboratory conditions and that contain a preponderance of conditionally adaptive genes (Bentley *et al.*, 2002).

Strikingly, 42 of the BldD target genes (~25% of the regulon) encode regulatory proteins themselves, stressing the central, highly pleiotropic role of BldD. The BldD targets include genes involved in morphological differentiation, energy storage, cell division, cell wall modification, signalling pathways, antibiotic production, and proteolytic and regulatory functions. Of the BldD targets, ~19% encode proteins of unknown function.

BldD targets involved in morphological differentiation

All of the five previously identified BldD-regulated genes (*bldN*, *sigH*, *bdtA*, *whiG* and *bldD*) were among the enriched targets. In addition, strong signals for BldD binding were found in the upstream regions of 16 other genes or operons that are known to be involved in morphological differentiation and/or antibiotic production (Table 2 and Fig. 1B). Among these are the genes *ftsZ*, *ssgA*, *ssgB* and *smeA-ssfA*. Each of these genes is known to be under developmental control, but none of the direct regulators involved has previously been identified, except that *ssgA* is activated by SsgR (Traag *et al.*, 2004). Sporogenic aerial hyphae undergo a synchronous round of cell division, initiated by the polymerization of a ladder of 50 or more FtsZ rings, and FtsZ ring placement is strongly influenced by SsgA and SsgB (Schwedock *et al.*, 1997; Flårdh *et al.*, 2000; van Wezel *et al.*, 2000; Keijser *et al.*, 2003; Grantcharova *et al.*, 2005; Noens *et al.*, 2005). The *smeA-ssfA* operon encodes a DNA translocase (SffA), involved in chromosome segregation into spores, which is specifically targeted to sporulation septa by the small membrane protein SmeA (Ausmees *et al.*, 2007). BldD also binds near SCO2525, a recently identified gene that encodes a putative methyltransferase that has effects

A



B

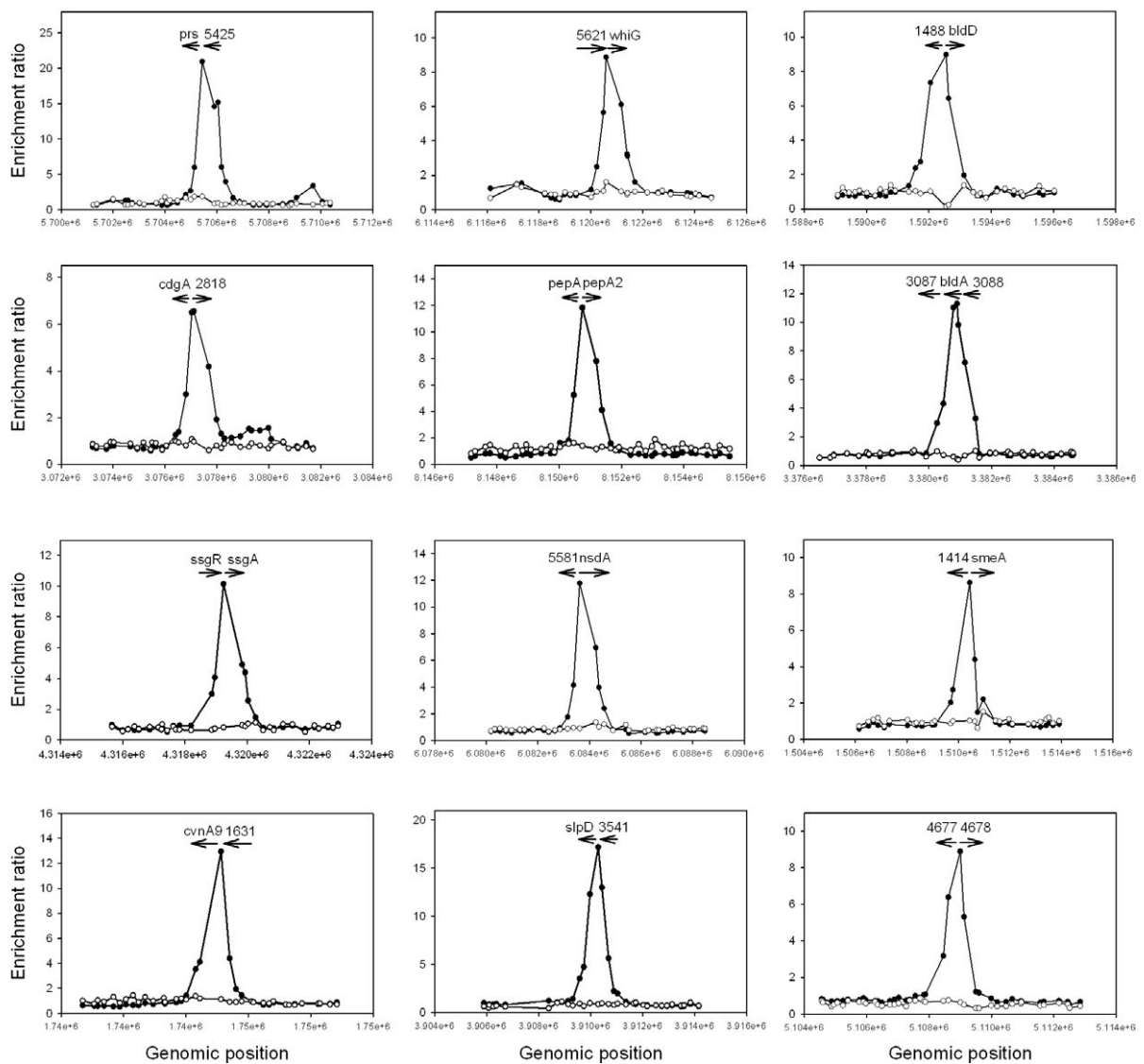


Table 2. BldD binding sites upstream of genes involved in sporulation and/or antibiotic production.

Flanking genes ^a	Enrichment ^b	Putative BldD binding site(s) ^c	Distance ^d	(Proposed) function ^e	
<i>whiD</i>	<i>bldM</i>	7.8	GGCACTCTACGTGAG	170	Regulatory protein; two-component regulator
<i>whiB</i>	SCO3035	4.2	TTCACCTGCGGAAC	540	Regulatory protein; hypothetical protein
SCO1414	<i>smeA</i>	8.4	AGTACTCATGGTGAC	26	Membrane protein
–	<i>ssgA</i>	12.5	CTCACTCCTGTGTGAT	109	Cell division-related protein
SCO1540	<i>ssgB</i>	2.6	AACACTCAGAGGGGT	83	Cell division-related protein
–	<i>ftsZ</i>	2.2	TTCACCCTATGTGAT	226	Cell division protein
–	<i>hrdB</i>	2.1	CTTACGGGGTGTGAC	208	Major vegetative sigma factor
–	<i>whiG</i>	5.7	ATCACCCAGAGCGAT	198	Polymerase sigma factor
			GTCACGCTACGCTCA	101	
SCO4677	SCO4678	14.0	GCCACGCTGAGTGAC	97	Antagonistic regulator of sigma(F); hypothetical protein
–	<i>prs1</i>	16.8	TTTACTATGAGTGAC	128	Antagonistic regulator of sigma(H)
–	<i>bldN</i>	11.3	TGCACGAAGCGTTAT	353	RNA polymerase sigma factor
			CGTACTGCACGTGAT	285	
<i>pyrR</i>	<i>bldD</i>	61.9	GTAACGCTGCGTAAC	71	Pyrimidine operon regulatory protein; regulatory protein
			CTCACAGTGAGTTAC	56	
–	<i>bldC</i>	5.6	GTGACTGATCGTCAC	241	Possible DNA-binding protein
–	<i>osaB</i>	3.0	GCCACGCGAAGTCGC	247	Two-component regulator
SCO5581	<i>nsdA</i>	13.0	TGAACCTCGCGGTGGC	234	Hypothetical protein
<i>cvnA9</i>	–	11.4	ATGACTCACCGTGAC	270	Putative integral membrane protein
–	<i>slpD</i>	21.2	GTAACCTCCTCCGCGAC	9	Proteinase
<i>pepA</i>	<i>pepA2</i>	8.5	ACGACGCTGCGTGGG	112	Glycogen branching, <i>glgBII</i> locus
<i>sti</i>	SCO0763	3.5	AATACGCAAGGTTAC	225	Protease inhibitor precursor; putative oxidoreductase
<i>bldA</i>	–	25.7	GTCACGCTGCGTGAC	+92	tRNA-Leu
–	<i>bldH</i>	2.5	GCAACGCTTCGTGAT	194	Transcriptional regulator

a. Genes immediately downstream of the ChIP-chip peak are listed.

b. Enrichment ratio (wild type/mutant) of the mean fluorescence ratio of a triplicate ChIP-chip experiment.

c. Motif was found using the *Virtual Footprint* software tool (Munch *et al.*, 2005).

d. Distance to the predicted start codon of downstream gene.

e. (Possible) gene function (see Bentley *et al.*, 2002 or text for references).

on several aspects of colony growth (Gehring *et al.*, 2004).

Our analysis also identified *bldC* as a member of the BldD regulon. *bldC*, which is required for normal morphological differentiation and antibiotic production in *S. coelicolor* (Hunt *et al.*, 2005), encodes a member of a family of small DNA-binding proteins that are related to the DNA-binding domains of the MerR family of transcriptional activators. The *S. coelicolor* genome carries one homologue of *bldC*, *bdtA* (Hunt *et al.*, 2005), which is also a target for BldD (Elliot *et al.*, 2001). We constructed a *bdtA* deletion mutant, but it did not display any apparent phenotype (data not shown).

BldD binds the region lying upstream of *bldM* (Table 2), which encodes a response regulator required for aerial mycelium formation (Molle and Buttner, 2000). *bldM* transcription was 10-fold derepressed in the *bldD* mutant, suggesting that BldD functions to repress *bldM* expression during vegetative growth. Consistent with this, a bioinformatically predicted BldD binding site (see below) was found overlapping the –10 region of the *bldMp2* promoter (Table 2). BldD also binds the upstream region of *whiB* (Table 2), which is required for the early stages of sporulation in aerial hyphae (Davis and Chater, 1992). Although the role of the actinomycete-specific *WhiB*-like (*Wbl*) family of proteins has been controversial (den Hengst and Buttner, 2008), recent biochemical experiments suggest

they function as transcription factors (Guo *et al.*, 2009; Singh *et al.*, 2009).

σ^F is required for spore maturation in *Streptomyces* (Potuckova *et al.*, 1995; Sun *et al.*, 1999). One of the strongest BldD targets, both in our ChIP-chip and in our transcriptome analysis, is SCO4677, which encodes a protein that was recently found to interact with σ^F as a potential antagonistic regulator (anti-sigma) (Kim *et al.*, 2008b). In turn, the SCO4677 anti- σ^F interacts with SCO0869 and SCO0781, which have similarity with anti-anti-sigma factors such as SpoIIAA of *B. subtilis* (Kim *et al.*, 2008b). Thus, although BldD does not directly regulate expression of *sigF*, their regulons are connected through SCO4677.

BldD targets involved in antibiotic production

BldD is essential for the production of secondary metabolites in different species of actinomycetes (Elliot *et al.*, 1998; Chng *et al.*, 2008). Our ChIP-chip analysis pinpointed several genes and operons, in addition to *bldC* discussed above, that are known to influence antibiotic production in *S. coelicolor* (Table 2 and Fig. 1B). A strong ChIP-chip signal was detected upstream of the five-gene 'conservon', *cvn9*, disruption of which causes precocious aerial mycelium formation and conditional overproduction of actinorhodin (Komatsu *et al.*, 2006). The *cvn9* operon is

likely to encode a membrane-associated heterocomplex, resembling the eukaryotic G-protein-coupled receptor system, and is therefore likely to function in signal transduction (Komatsu *et al.*, 2006). BldD binds to the upstream region of *nsdA*, a gene that negatively influences antibiotic production in multiple species of actinomycetes. Disruption of *nsdA* in *S. coelicolor* causes hypersporulation and overproduction of the antibiotics actinorhodin, methylenomycin and CDA (Li *et al.*, 2006; Wang *et al.*, 2009). The genes *leuB* and *leuC*, required for branched-chain amino acid biosynthesis, are strongly derepressed in the *bldD* mutant and BldD binds upstream of *leuA*. Recently it was shown that 50% of the acetate for synthesis of the type II polyketide actinorhodin is derived from the catabolism of branched amino acids (Sprusansky *et al.*, 2005; Stirrett *et al.*, 2009). Additionally, antibiotic production might be influenced by BldD through regulation of the mobilization of triacylglycerol (TAG) during the stationary phase of growth in submerged liquid cultures. TAG has been proposed to serve as a carbon source for antibiotic biosynthesis in *S. coelicolor* (Olukoshi and Packter, 1994; Packter and Olukoshi, 1995). The product of SCO0958, which carries out the esterification of diacylglycerol with a fatty acid molecule, is largely responsible for TAG production (Arabolaza *et al.*, 2008) and SCO0958 showed a strong signal in the BldD ChIP-chip experiment.

BldD targets involved in polysaccharide metabolism

Besides neutral lipid storage compounds such as TAG, *S. coelicolor* synthesizes polysaccharides, the interconversion of which may also be influenced by BldD. BldD targets the region between *pepA* and *pepA2* (SCO7336 and SCO7337), the most upstream genes of two divergent operons (*glgBII* locus) that are involved in the deposition of glycogen as carbon reserves in the apical compartments of the aerial mycelium (Schneider *et al.*, 2000; Yeo and Chater, 2005). BldD could also influence utilization of cellulose as it might bind to the intergenic region of the divergently transcribed genes SCO6546 and SCO6548, encoding secreted cellulases, and to the upstream region of *msiK*, a gene required for growth on minimal medium containing cellulose or other polysaccharides (Schlosser *et al.*, 1999; Saito *et al.*, 2008). Recently, an important additional role for cellulose in *S. coelicolor* has been established. It was shown that extracellular cellulose fibrils mediate the attachment of hyphae to hydrophobic surfaces, and that the enzyme responsible for its production, cellulose synthase, localizes to the tip of growing hyphae and interacts with DivIVA, the essential cytoskeletal protein that directs apical growth in *Streptomyces* (Hempel *et al.*, 2008; Xu *et al.*, 2008; de Jong *et al.*, 2009b).

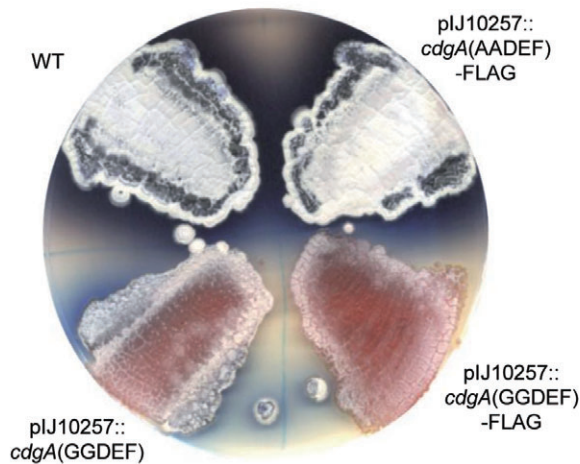
Overexpression of a novel BldD target gene implicates c-di-GMP in morphological differentiation and antibiotic production

Cyclic-di-GMP (c-di-GMP) has been shown to be a second messenger primarily involved in the developmental switch to biofilm formation in a wide variety of bacteria (Jenal and Malone, 2006). c-di-GMP levels are controlled through enzymes containing GGDEF domains, needed for synthesis, and EAL domains, needed for hydrolysis of the molecule. In *Streptomyces*, a role for c-di-GMP has not been established, but the *S. coelicolor* genome encodes several proteins with GGDEF and/or EAL domains. Interestingly, the upstream regions of three of these genes (SCO2817, SCO5511 and SCO4281) are bound by BldD. Although expression of SCO2817, here designated *cdgA* (c-di-GMP), is unaffected by BldD in liquid cultures (this work), transcriptome analysis of wild-type *S. coelicolor* cells, grown on solid medium, showed that *cdgA* transcription is developmentally regulated (Hesketh *et al.*, 2007). We overexpressed *cdgA* in *S. coelicolor* M600, and found that the resulting strain was unable to form aerial hyphae (the bald phenotype) (Fig. 2A). Moreover, this strain displayed reduced production of the blue-pigmented antibiotic actinorhodin.

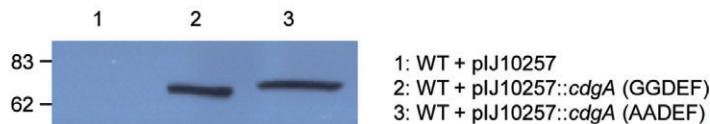
To address whether overexpression of *cdgA* is associated with an increase in c-di-GMP levels, site-directed mutagenesis was carried out. First, we repeated the *cdgA* overexpression experiment with a C-terminally FLAG-tagged version of the wild-type allele and obtained exactly the same results, showing that the FLAG tag does not interfere with CdgA activity (Fig. 2A). Then we introduced a GG to AA mutation into the GGDEF motif of the FLAG-tagged *cdgA* overexpression construct and introduced it into *S. coelicolor*. This mutation has been shown to abolish the c-di-GMP biosynthetic activity of several diguanylate cyclases in other organisms (e.g. Paul *et al.*, 2004). The substitutions abolished the bald phenotype caused by overexpression of the wild-type *cdgA* allele and restored normal levels of production of the blue-pigmented antibiotic actinorhodin (Fig. 2A). Immunoblot analysis, using anti-FLAG antibody, showed that the levels of CdgA protein produced in the strains expressing the wild-type allele and the GG to AA mutant allele were the same, showing that the GG to AA mutation did not cause CdgA instability (Fig. 2B). From this we conclude that the block to aerial mycelium formation caused by CdgA overexpression results from c-di-GMP synthesis.

cdgA is a predicted 67.2 kDa cytoplasmic protein that contains a PAS domain in addition to its GGDEF diguanylate cyclase and EAL domains. PAS domains are often associated with GGDEF and EAL domain proteins and can be involved in signal transduction, because of its ability to bind a wide variety of cofactors that can modu-

A



B



late the catalytic activity of the protein in response to environmental stimuli. The data presented here suggest for the first time a role for c-di-GMP in *Streptomyces* development, and the involvement of BldD in the regulation of c-di-GMP metabolism.

Identification of a consensus BldD binding site

The oligonucleotides on the DNA microarrays used in this study have a dense coverage of the genome, but do not precisely define the location of each of the BldD binding sites. To validate the ChIP-chip data, and to establish a consensus BldD operator site, a computational analysis of the targeted DNA regions was performed. Defining the exact position of the BldD binding site with respect to the transcriptional start site could also help to explain the role of BldD as a transcriptional repressor or activator. Previously, BldD binding to the promoters of *bldN*, *sigH*, *bdtA*, *whiG* and *bldD* itself was examined by DNase I footprinting analysis (Elliot and Leskiw, 1999; Elliot *et al.*, 2001; Kelemen *et al.*, 2001). These experiments clearly showed that the BldD binding sites overlap the promoters or the transcriptional start sites, explaining how BldD functions as a repressor of these genes. Alignment of the BldD-bound sequences identified an imperfect inverted repeat sequence, AGTgA N_(m) TCACc, that might function as the consensus BldD binding site (Elliot *et al.*, 2001). This sequence has similarity with a motif, [TA]GTGAN_(18,20)TN₍₂₎C, that was found

Fig. 2. Overproduction of *cdgA* blocks the formation of aerial hyphae.

A. Overexpression of *cdgA* from the *ermEp** promoter in *S. coelicolor* M600 using the vector pIJ10257 causes loss of aerial mycelium formation and reduced actinorhodin production. However, introduction of a GG to AA mutation in the GGDEF motif abolishes the bald phenotype and restores normal levels of actinorhodin production. Strains were grown on R5.

B. Substitution of GGDEF by AADEF in CdgA does not cause protein instability. FLAG-tagged CdgA (GGDEF) and CdgA (AADEF) were constitutively expressed from pIJ10257 in *S. coelicolor* M600 and subjected to immunoblot analysis using ANTI-FLAG antibodies. Cells were grown in liquid YEME/TSB medium for 16 h.

using the SIGffRid algorithm, employed to compare sequences from related genomes (Touzain *et al.*, 2008). However, both of these proposed motifs implied the length of the spacer separating the inverted repeat sequences was variable. The ChIP-chip experiments conducted here identified 162 potential new direct targets of BldD, creating a much larger data set that allowed us to identify a consensus BldD binding site.

Because operator sites of regulatory proteins in bacteria are usually located close to their target promoters, fragments of up to 200 bp were chosen such that they encompassed the known or predicted promoter sequences of their cognate genes. The data set, comprised of all genes directly targeted by BldD, was examined using the *MEME* algorithm (Bailey and Elkan, 1994). Over 80% of the DNA sequences contains a well-conserved copy of a 15 bp palindromic sequence, 5'-nTnACnC(A/T)GnGTnAn-3', for which the sequence logo is shown in Fig. 3. To validate this sequence (designated the BldD box), and to confirm and extend the ChIP-chip analysis, DNase I footprinting analysis using purified histidine-tagged BldD was carried out on the intergenic region between *pepA* and *pepA2*, involved in glycogen branching (Schneider *et al.*, 2000), which was 8.5-fold enriched in the ChIP-chip experiment (Fig. 1 and Table 2). Addition of BldD protein resulted in protection of a region containing a well-conserved copy of the BldD box (Fig. 4 and Table 2), consistent with this sequence serving as a high-affinity binding site for BldD. Similarly, DNase I footprinting showed that BldD protected a region

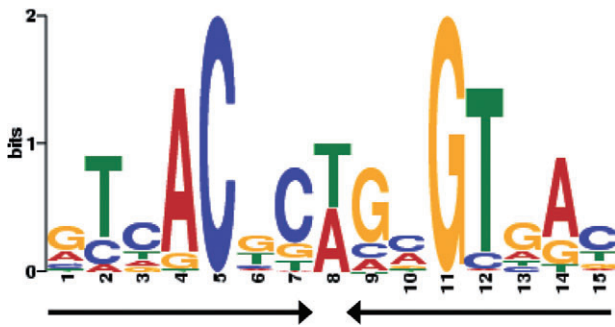


Fig. 3. Presence of an over-represented motif upstream of BldD target genes. A conserved motif was identified using the *MEME* algorithm (Bailey and Elkan, 1994) by analysing the DNA sequences that were enriched for BldD binding in the ChIP-chip experiment. The height of the letters in the sequence logo, in bits, is proportional to the frequency of the A, C, T or G nucleotides at each position of the motif. Black arrows indicate the sequence palindrome.

containing a predicted BldD box upstream of *cdgA* and of *ftsZ* (Fig. 4).

Using the new BldD-binding consensus sequence, it can be explained why mutagenesis of the *bldD* promoter in a previous study (Elliot and Leskiw, 1999) did not have a clear effect on BldD binding, since the introduced mutations were located just outside the BldD box identified here, or at position 3 (Fig. 3), which is not conserved. In some cases (e.g. SCO5887, *whiG*, *bldN*, and *bldD* itself), two copies of the BldD box could be identified bioinformatically within a single promoter region (Table 2). For *whiG*, *bldN*, and *bldD*, these predicted binding sites correlate well with DNase I footprinting analyses (Elliot and Leskiw, 1999; Elliot *et al.*, 2001), where, in each case, two separate BldD binding sites were found, and with our *in vivo* data, where very strong ChIP-chip signals for these targets were found, suggesting particularly tight binding by BldD (Fig. 1). Stringent regulation of the regulons of σ^{BldN} and σ^{WhiG} by BldD also becomes apparent from the fact that their downstream targets, *bldM* and the *glgBII* locus (Bibb *et al.*, 2000; Ye

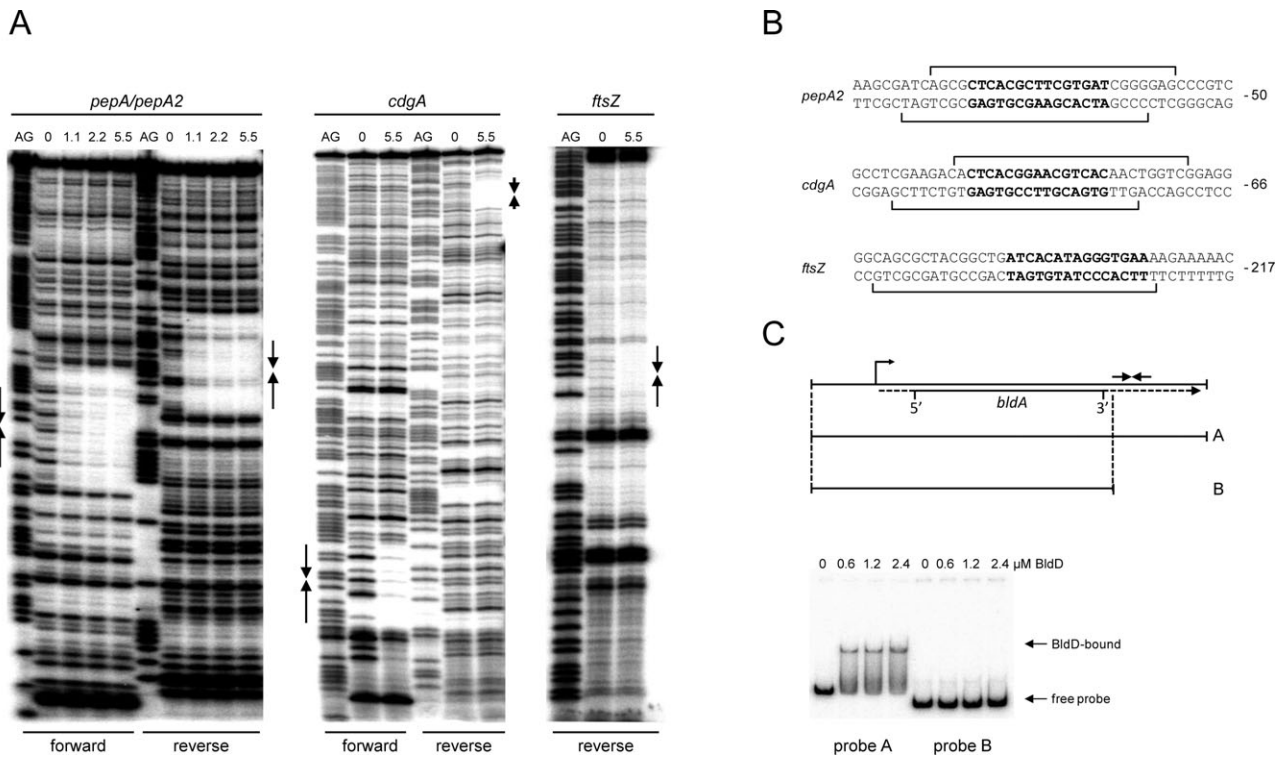


Fig. 4. Confirmation of the predicted BldD binding site.

A. DNase I footprinting analysis of BldD binding to the intergenic region between *pepA* and *pepA2*, the *cdgA* promoter region, and the *ftsZ* promoter region. 5' end-labelled probes were incubated with increasing amounts of BldD (indicated in μM above the lanes) and subjected to DNase I footprinting analysis as described in *Experimental procedures*. Footprints are flanked by Maxam and Gilbert sequence ladders (AG). Black arrows indicate the position of the bioinformatically predicted BldD binding site.

B. Summary of DNase I footprinting results presented in Fig. 4A. Brackets indicate the protected regions, bioinformatically predicted BldD binding sites are depicted in bold, and the number indicates the distance to the putative start codon of the downstream gene.

C. Deletion analysis of the potential BldD binding site located within the *bldA* gene. Two radioactively labelled DNA probes were generated and tested for the ability to interact with increasing amounts of purified BldD in an EMSA as described in *Experimental procedures*. Probe A contains a well-conserved copy of a putative BldD binding site (inverted arrows), whereas probe B was truncated from the 3' end, and lacked this sequence. The positions of the free and retarded probe are indicated by arrows.

Fig. 5. Connections between the regulons of BldD, BldH and *bldA*.

A. Involvement of BldD in the 'regulon' of the BldA tRNA. The genome of *S. coelicolor* encodes 146 genes containing a TTA^{Leu} codon, which depend on *bldA* for efficient translation. Of the potential BldD targets that were identified by ChIP-chip, 11 contain a TTA codon (Venn diagram). The BldA tRNA influences *Streptomyces* development and antibiotic production in a manner depicted schematically on the right (adapted from Chater and Chandra, 2008). Genes co-regulated by BldD are indicated in bold. The dashed arrow indicates a connection in the homologous system of *S. griseus* in which the BldH orthologue AdpA activates transcription of the σ factor gene *bldN/adsA*.

B. DNase I footprinting analysis of BldD binding to DNA fragments located within *bldA* and upstream of *sti* and *bldH/adpA*. 5' end-labelled probes were incubated with increasing amounts of BldD (indicated in μ M above the lanes) and subjected to DNase I footprinting analysis as described in *Experimental procedures*. Footprints are flanked by Maxam and Gilbert sequence ladders (AG). Black arrows indicate the position of the bioinformatically predicted BldD binding site. DNase I footprinting results are summarized below. Brackets indicate the protected regions, bioinformatically predicted BldD binding sites are depicted in bold, and the number indicates the distance to the putative start codon of the downstream gene.

and Chater, 2005), respectively, are also targets of BldD (Fig. 1), although direct regulation of *glgBII* by σ^{WhiG} has not been verified experimentally.

To test the predictive power of the derived consensus sequence, the genome of *S. coelicolor* was searched for the occurrence of BldD boxes using *Virtual Footprint* (Munch *et al.*, 2005). When only the intergenic regions were scanned, hundreds of well-conserved BldD binding sites were detected (Table S3). These were ranked according to their similarity with the consensus. Obviously, high-scoring copies were found in the promoter sequences that were used to build the weight matrix. However, the BldD box was also found upstream of genes that were not identified as BldD targets in the ChIP-chip experiment, and these might constitute additional targets for BldD. This could happen if they were subject to complex regulation involving other transcription factors; such promoters might appear as BldD targets if the ChIP-chip experiments were performed under different growth conditions.

Strikingly, when the entire genome was scanned, hundreds of BldD boxes were found within coding regions, although these intragenic sites were not bound by BldD *in vivo*, as determined by our ChIP-chip experiment. Topological constraints of the DNA, or the presence of nucleoid proteins (McArthur and Bibb, 2006), might prevent BldD from interacting with predicted binding sites present within ORFs.

Conversely, a well-conserved BldD box could not be distinguished, by the bioinformatic tool used in this study, in the upstream regions of approximately 20% of the target genes identified in the ChIP-chip experiment. We cannot therefore rule out the possibility that other sequences play a role in the recognition and regulation of these genes by BldD. Alternatively, it could be that interaction with other DNA-binding proteins is important for recruitment of BldD to these promoters.

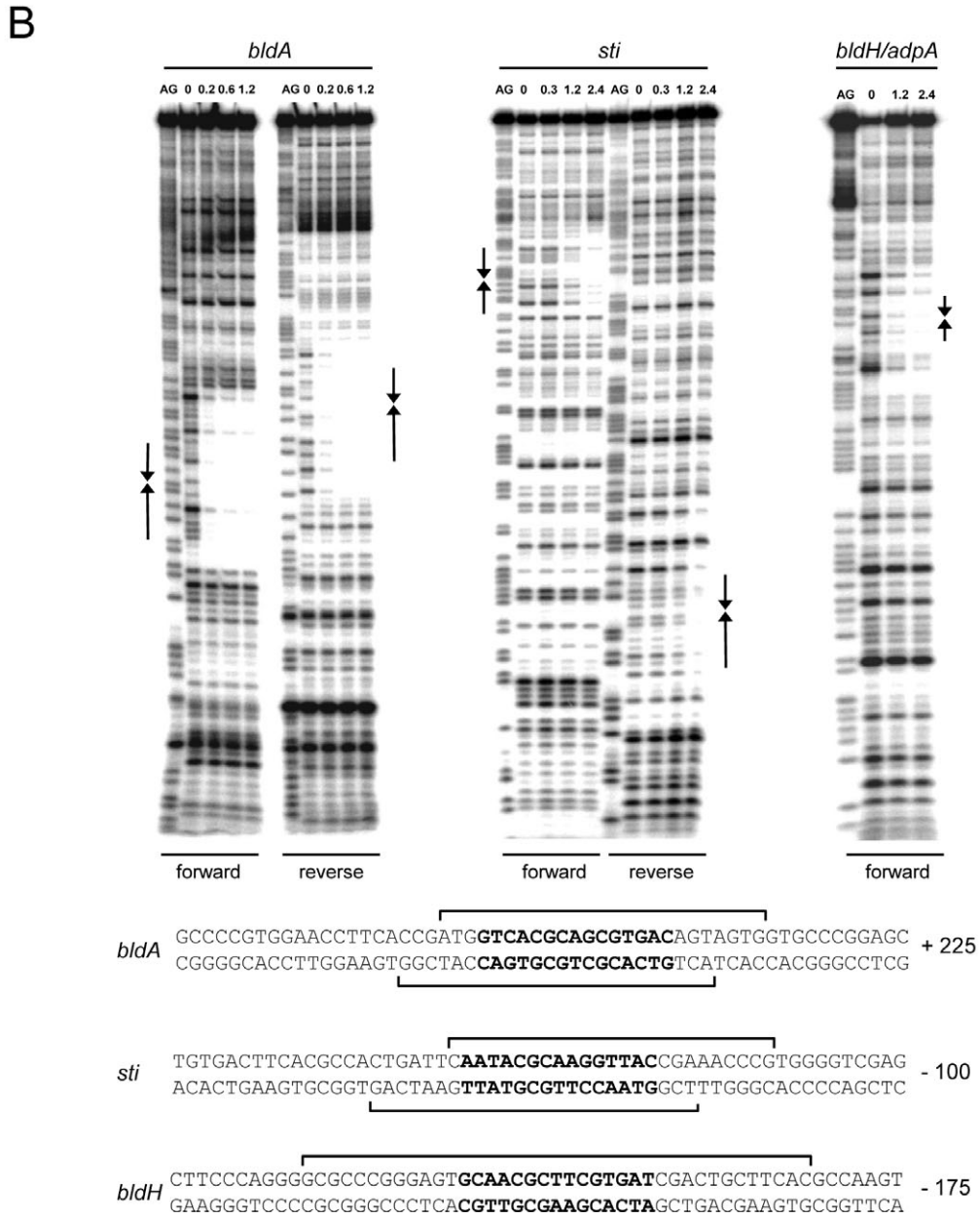
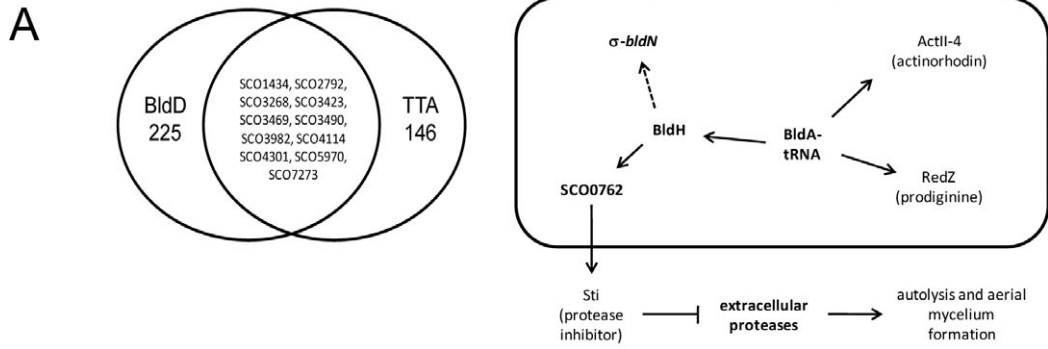
As judged by ChIP-chip analysis, BldD binding is restricted to promoter regions in all but three cases. Two of these exceptions correspond to the BldD binding sites in the intergenic regions downstream of the convergently transcribed genes SCO2445/SCO2446, and the conver-

gently transcribed genes SCO5809/SCO5810. The third exception is BldD binding to the *bldA* gene.

The bldA tRNA gene is a BldD target

A very strong ChIP-chip signal was detected for *bldA* (Fig. 1) and *bldA* contains one of the bioinformatically highest scoring copies of a BldD box (Table 2), suggesting a good correlation between the level of sequence conservation in a BldD box and the affinity of BldD for its target. Electrophoretic mobility shift assays (EMSAs) demonstrated binding of BldD to a DNA fragment spanning the bioinformatically identified BldD box in *bldA* (Fig. 4B), and deletion of the BldD box from the fragment abolished BldD binding (Fig. 4B). This result was extended by DNase I footprinting, showing that this region in *bldA* indeed serves as a high-affinity binding site for BldD (Fig. 5B). Intriguingly, the BldD box in *bldA* is not present in the promoter but within a region encoding the primary transcript of *bldA*, immediately downstream of the position corresponding to the site where the primary transcript would be processed to give rise to the mature 3' end of the tRNA (Fig. 4B).

The regulatory significance of BldD binding to *bldA* will be the subject of future investigation. It is not excluded that BldD binding could influence transcription initiation at the *bldA* promoter. However, because of the location of the BldD binding site, another speculative possibility is that BldD affects transcriptional termination, and thus influences the post-transcriptional processing of the *bldA* primary transcript. Regardless of mechanism, the fact that BldD targets *bldA* has important implications for our understanding of the regulatory network that controls *Streptomyces* development. Ever since *bldA* was first shown to encode a tRNA, there has been extensive debate as to whether BldA constitutes a true regulatory device. In other words, although it is apparent that *bldA* null mutants cannot differentiate, it has never been clear if BldA availability regulates (limits) translation of TTA-containing genes during development of wild-type *S. coelicolor* (Leskiw *et al.*, 1991; 1993; Gramajo *et al.*, 1993; Rebets *et al.*, 2006). The fact that *bldA* is a BldD



target suggests that BldA is integrated into the regulatory network that controls *Streptomyces* development, consistent with BldA tRNA functioning as a true regulatory device. This suggestion is further supported by the evident integration of the BldD, BldH and BldA regulons.

The regulons of BldD, BldH and the BldA tRNA overlap

Conserved BldD binding sites were detected upstream of 11 genes containing a TTA codon (Fig. 5A). As only 146 out of the 7825 (< 2%) predicted genes in the *S. coelicolor* genome contain a TTA codon, TTA genes are overrepresented in the BldD regulon (11 out of ~167 targets). In both *Streptomyces griseus* and *S. coelicolor* the *bldA* 'regulon' is extended through AdpA (also called BldH in *S. coelicolor*), a global regulator containing a TTA codon (Nguyen *et al.*, 2003; Takano *et al.*, 2003; Kim *et al.*, 2005; Ohnishi *et al.*, 2005). In *S. griseus*, AdpA directly regulates at least 37 transcriptional units, several of which are involved in morphological development and secondary metabolism (Yamazaki *et al.*, 2003; 2004; Ohnishi *et al.*, 2005; Akanuma *et al.*, 2009). Among the *S. coelicolor* BldD-regulated genes (Table 2) is the *S. coelicolor* *adpA* orthologue (*bldH*) itself, as was confirmed by DNase I footprinting (Fig. 5B). These results suggest that BldD is intertwined with the BldH regulon, both directly, by regulating transcription of *bldH*, and indirectly, by regulating *bldA*, on which BldH depends for translation. In addition to these genes, BldD binds upstream of the BldH targets SCO5821, a SprU-like serine protease, and SCO0762, encoding STI (Table 2), as was also confirmed by DNase I footprinting (Fig. 5B). STI is a protease inhibitor that forms part of a complex cascade (Kim *et al.*, 2008a; Chater *et al.*, 2010) involving multiple extracellular proteases (Fig. 5A). STI activity, and its subsequent degradation by an unidentified protease, are important for differentiation, at least under certain conditions (Kato *et al.*, 2005; Kim *et al.*, 2008a).

Expression of *sti* is significantly downregulated in a *chp* null mutant that forms few aerial structures (de Jong *et al.*, 2009a) as is SCO1978, encoding a predicted secreted hydrolase, which was enriched in the BldD ChIP-chip experiment. In addition, ChIP-chip peaks and conserved BldD binding sites were detected upstream of several other genes encoding putatively secreted proteins (SCO2818, SCO3268 and SCO4132) and putative intra- and extracellular proteases (SCO3373, SCO3540, SCO0752 and SCO7226). The intracellular signals and regulatory pathways leading to the initiation of extracellular events involved in developmental and catabolic processes are largely unknown (Chater *et al.*, 2010), but our results strongly implicate BldD in regulating the composition of the extracellular proteome.

The *S. coelicolor* BldD binding site is conserved among sporulating actinomycetes

Although the BldH/AdpA regulons in *S. coelicolor* and *S. griseus* overlap significantly, there are strain-specific differences. For example, in contrast to *S. griseus*, *S. coelicolor* BldH does not depend on the bacterial hormone A-factor for its expression to become derepressed (Takano *et al.*, 2003). *S. griseus* contains an orthologue of BldD (Ohnishi *et al.*, 2008), but, thus far, only one target gene has been identified (Ueda *et al.*, 2005). In this organism, BldD targets *ramCSAB*, a gene cluster required for the formation of AmfS, which acts as an extracellular surfactant peptide that stimulates aerial growth. In contrast, the orthologous system in *S. coelicolor*, *ramCSAB*, encoding the SapB surfactant peptide, is probably not targeted by BldD (Ueda *et al.*, 2005). We did not identify *ramCSAB* by ChIP-chip with BldD, and sequence inspection of its regulatory region revealed no apparent BldD box.

Using BLAST, we found that BldD is present throughout the sporulating actinomycetes, but also in the actinomycete *Acidothermus*, which is not known to form spores, and species of *Kribella*, *Nakamurella* and *Stackebrandtia* (Table S2). *S. coelicolor* BldD is 97–98% identical to BldD from other sequenced *Streptomyces* species, and more distantly related to orthologues from other sporulating actinomycete genera (e.g. 77% identical to *Saccharopolyspora erythraea* BldD). To assess the extent to which the BldD regulatory network is conserved in sporulating actinomycetes, several available genomes were searched for potential BldD binding sites, using the weight matrix obtained from the *S. coelicolor* data set (Fig. 3). The results of these searches are available through Table S3. Among the highest-scoring hits in *S. griseus* were sites located upstream of genes orthologous to the *S. coelicolor* developmental loci *whiB*, *bldH*, *bldM*, *ftsZ*, *ssgA*, *rarA*, *bldA* and *bldD* itself, all of which were identified as BldD targets in the current study. Potential BldD binding sites in *S. griseus* were also found upstream of the cytoskeletal protein gene *mreB*, and *sprV*, encoding a putative trypsin-like protease. In *Streptomyces avermitilis*, conserved binding sites were found upstream of, among others, *minD2*, *leuA*, *ftsZ*, *whiG*, *sprB*, *ssgA*, *wblB*, *cvnA5*, *bldK* and *whiP*. *whiP* is the orthologue of *S. coelicolor* *crpA*, important for co-ordinating growth and cell division in sporogenic hyphae (Del Sol *et al.*, 2006). Similarly, high-scoring copies of the BldD binding site were found at relevant positions in *Salinispora tropica* and *Frankia*. These data suggest the function of BldD is conserved in sporulating actinomycetes.

We also searched the genome of *S. erythraea*. A constructed *bldD* null mutant of *S. erythraea* has a bald phenotype and fails to make erythromycin (Chng *et al.*, 2008).

The erythromycin (*ery*) biosynthetic cluster lacks a pathway-specific regulatory gene and *in vitro* DNA-binding assays suggest that BldD binds to five promoter regions in the *ery* cluster (Chng *et al.*, 2008). Given that a *S. erythraea* *bldD* mutant fails to make erythromycin, these results were interpreted to imply that BldD functions as an activator of the *ery* gene cluster (Chng *et al.*, 2008). However, the only DNase I footprinting analysis performed, on *eryBVI*, showed that BldD protects a region covering the -35, the -10 and the transcription start site (Chng *et al.*, 2008), which is perhaps more consistent with BldD acting as a repressor of *eryBVI*. This is strengthened by the presence of a BldD box (TTTCCCCGCCGTGAC) located between the -35 and -10 sequences of the *eryBVI* promoter. We would like to raise the speculative possibility that BldD functions to repress the *ery* cluster during vegetative growth and the inability of *S. erythraea* *bldD* mutants to make erythromycin arises indirectly.

For those BldD targets whose transcriptional start sites have been determined experimentally in *S. coelicolor*, we examined the positions of the predicted BldD binding sites with respect to the promoters. In most cases, BldD binding sites overlapped with the promoter elements or transcriptional start sites, consistent with BldD acting as a repressor by preventing RNA polymerase from binding DNA and/or initiating transcription. In contrast, in the case of *sti*, we found that BldD binds to a BldD box centred 63 bp upstream of the transcriptional start site (Fig. 5), suggesting that BldD might function as activator of *sti*. Consistent with this suggestion, *sti* expression was 41-fold downregulated in the *bldD* mutant (Table 1). This gene is co-regulated by BldH (AdpA), for which predicted binding sites are located 84 bp and 159 bp upstream of the transcriptional start site (Kato *et al.*, 2005). Therefore, activation of *sti* might result from interplay between BldH, BldD and the *sti* promoter, but such a model is further complicated by the fact that the *bldH* is also a BldD target.

BldD acts as a repressor of key developmental genes during vegetative growth

Our data suggest that the main function of BldD is to repress, during vegetative growth, genes that are needed for morphological differentiation and antibiotic production. BldD transcript levels are highest during this stage of the *S. coelicolor* life cycle (Elliot *et al.*, 1998), and immunoblot analysis of BldD protein during development of *S. coelicolor* on solid medium shows that BldD protein levels correlate with the *bldD* transcription pattern (Fig. 6). BldD protein is present predominantly during vegetative growth, although some BldD could still be detected even after onset of aerial mycelium formation and initiation of antibiotic production (Fig. 6).

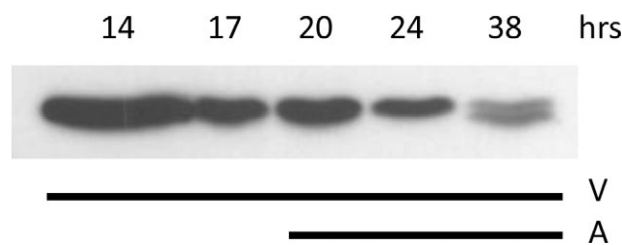


Fig. 6. Abundance of BldD protein during growth of wild-type *S. coelicolor* M600. Immunoblot analysis of BldD protein levels in cells that were harvested at various time points, as indicated in hours, after inoculation on R5 solid growth medium. Bars indicate the presence of vegetative mycelium (V) and aerial mycelium (A) as judged by microscopic examination.

The proposed role of BldD in *Streptomyces* is reminiscent of the role the *B. subtilis* transition state regulator AbrB, which functions to prevent premature expression of genes required for developmental processes during vegetative growth (Strauch, 1993). Like BldD, expression of *abrB* decreases during entry into stationary phase, but this is not a complete explanation of how AbrB targets become derepressed. In addition, AbrB is inactivated by AbrA, which acts as an anti-repressor, interacting with AbrB to disable DNA binding (Banse *et al.*, 2008). Transcription factor inactivation has also been shown for another *B. subtilis* transition state regulator, SinR, which controls biofilm formation. SinR is antagonized through hetero-dimerization with an anti-repressor, SinI (Bai *et al.*, 1993). Interestingly, the N-terminal domain of BldD has similarity with the Xre family of transcriptional repressors, which includes SinR (Kelemen *et al.*, 2001). However, our attempts to identify a BldD partner protein have, thus far, been unsuccessful.

In summary, we show that BldD is a key regulator of morphological differentiation and antibiotic production and that it connects the regulons of several other regulators that play pivotal roles in these two central aspects of *Streptomyces* biology (Fig. 7). Our ChIP-chip approach allowed the identification of many new candidate developmental genes. Overexpression of one of these genes (*cdgA*) influences both differentiation and antibiotic production and implies a role for c-di-GMP in *Streptomyces* development. Given the conservation of BldD and the presence of predicted BldD binding sites at relevant positions in other genomes, it is likely that BldD plays a similarly important role across sporulating actinomycetes.

Experimental procedures

Bacterial strains, plasmids, oligonucleotides and growth conditions

Strains, plasmids and oligonucleotides, used in this study, are described in Table 3. *Escherichia coli* strains were grown in

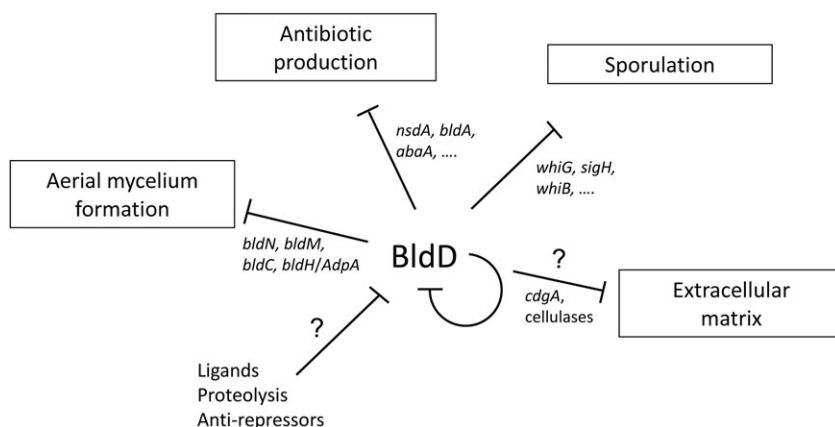


Fig. 7. Involvement of BldD in developmentally co-ordinated processes.

Luria–Bertani medium for routine purposes. *Streptomyces* were grown on R2YE or R5 solid media (Kieser *et al.*, 2000). Strains used for transcriptional profiling, and ChIP-chip analysis, were grown in a 1:1 mixture of YEME and TSB liquid media (Kieser *et al.*, 2000) at 30°C and 250 r.p.m. For immunoblot detection, suspensions of 6×10^8 spores from wild-type or *bldD* null mutant strains were grown on R2YE solid medium, overlain with sterile cellophane, and incubated at 30°C. Samples of mycelium were harvested after 14, 17, 20, 24 and 38 h, by using a spatula, and immediately flash-frozen in liquid nitrogen.

Construction of overproducing strains

For overproduction in *S. coelicolor*, SCO2817 was amplified by PCR using oligonucleotides SCO2817OVERFW and SCO2817OVERRV, carrying NdeI and HindIII restriction sites respectively. The resulting fragments were first cloned into SmaI-cut PUC19, and transferred to NdeI–HindIII-cut pIJ10257 to generate pIJ10350. The plasmid was introduced into *E. coli* ET12567 (pUZ8002) to allow transfer into *S. coelicolor* M600 by conjugation (Kieser *et al.*, 2000). Similarly, a strain containing a C-terminally FLAG-tagged version of the

Table 3. Strains and plasmids used in this study.

	Relevant genotype/comments	Source or reference
Strains		
<i>S. coelicolor</i>		
M600	Prototroph, SCP1 ⁻ , SCP2 ⁻	Kieser <i>et al.</i> (2000)
M600 Δ <i>bldD</i>	In-frame deletion mutant of <i>bldD</i>	Elliot <i>et al.</i> (2003b)
<i>E. coli</i>		
ET12567 (pUZ8002)	ET12567 containing helper plasmid pUZ8002	Kieser <i>et al.</i> (2000)
Plasmids		
pIJ10257	Integrative expression vector based on constitutive ermE* promoter	Hong <i>et al.</i> (2005)
pIJ10351	pIJ10257 ermEp*–SCO2817	This study
pIJ10354	pIJ10257 ermEp* carrying FLAG-tagged derivative of SCO2817	This study
pIJ10366	pIJ10257 ermEp* carrying SCO2817 GGDEF replaced by AADEF	This study
Primers		
SCO2817OVERFW	CATATGGTGAACGGAACCTCCGAAG	
SCO2817OVERRV	AAGCTTACGAGCTGATCGCCGACG	
SCO2817OVERFLAGRV	AAGCTTCTACTTATCGTTCATCCTTGTAGTCCGGTTGCCGACGCCGCTG	
SCO2817AADEFW	GGTGGCCCGGCTCGCGCGGACGAGTTCGCC	
SCO2817AADEFRV	GGGCGAACTCGTCCGCCGAGCCGGGCCACC	
bldA_F1	GGCAGGACGAAAGCCCATAC	
bldA_R1	GTTCAAGTCCGGCTCCGGGC	
bldA_R2	CATCGGTGAAGGTTCCACGG	
7773_F1	CTGCTCGTGTTCACGAATG	
7773_R1	GCACTGCGGTCTTCATCGTC	
0792_F1	CAGGCCTATTGGCGAAGGTG	
0792_R1	GAGCGCGAGTCTGCCGAGTC	
2792_F1	CTGCACGGACAGGGTCTCGG	
2792_R1	GTTCCGGCACTTGGGACATTG	
bldA_F3	GCCGCTGAGTCGCAACCAG	
bldA_R3	GACACGGCGAGCTTAAACC	
2082_F1	GACGTGAGTGTTGCCACCGC	
2082_R1	GACTTCCGAACAGGACAC	

wild-type allele (pIJ10354) was obtained using oligonucleotides SCO2817OVERFW and SCO2817OVERFLAGRV. The QuikChange® Site-Directed Mutagenesis Kit (Stratagene) was used to replace the GGDEF motif in *cdgA* by a AADEF sequence to generate pIJ10366.

Immunoblot detection

Frozen mycelium was ground in liquid nitrogen with a pre-cooled mortar and pestle and resuspended in lysis buffer (0.005% KH₂PO₄, 0.37% CaCl₂, 0.57% TES) containing 2 mg ml⁻¹ lysozyme (Sigma). Samples were incubated for 1 h on ice and sonicated at 10 microns for four cycles of 15 s with 1 min intervals. Cell debris was removed by centrifugation at 14 000 r.p.m. for 15 min at 4°C, after which the protein concentration of the cleared extract was determined with Bradford reagent (Bio-Rad). Equal amounts (25 µg) of protein from each sample were loaded onto a 15% polyacrylamide gel and, after electrophoresis, transferred to a Hybond-C Extra nylon membrane (Amersham Pharmacia Biotech), and probed with a 1:10 000 dilution of anti-BldD antibody that was raised against His₆-tagged BldD in rabbits and affinity purified. BldD was detected by chemiluminescence with ECL Western blotting detection reagents following the manufacturer's instruction (Amersham Pharmacia Biotech) using horseradish peroxidase-coupled secondary antibody. Similarly, FLAG-CdgA fusion proteins were detected in liquid-grown cells using ANTI-FLAG® antibodies (Sigma) that were raised in rabbits.

RNA isolation and DNA microarray analysis

RNA isolation and DNA microarray analysis were essentially carried out as described previously (Hesketh *et al.*, 2009). In short, *S. coelicolor* M600 and a *S. coelicolor* M600 *bldD* null mutant were grown in triplicate for 15 h in YEME/TSB liquid medium. Total RNA was isolated using the RNeasy midi kit (Qiagen) according to the manufacturer's instructions, with modifications as described (Hesketh *et al.*, 2009). Subsequently, single-strand reverse transcription (amplification) and indirect labelling of 10 µg of total RNA were performed for hybridization to *Streptomyces* diS_div712a GeneChip arrays according to the manufacturer's published protocol (Affymetrix). The GeneChips were washed and stained using a GeneChip fluidics workstation model 450, and then scanned with a Gene Array Scanner. After preprocessing, the data were imported into *GeneSpring* 9.0 (Agilent Technologies), converted to log² values and normalized per gene to the median. Error models based on replicate values were implemented and statistical calculations, on the filtered data, were performed in *GeneSpring* by two-way ANOVA as described previously (Hesketh *et al.*, 2009). Transcriptome data have been deposited at the MIAME-compliant ArrayExpress database under accession number E-MEXP-2853.

ChIP-chip analysis

ChIP-chip culture conditions were exactly the same as those for transcriptional profiling and were done in triplicate for the wild-type strain. Formaldehyde was added to cultures at a

final concentration of 1% (v/v) and incubation was continued for 30 min. Glycine was then added to a concentration of 125 mM to stop the cross-linking. The sample was left at room temperature for 5 min and washed twice in PBS buffer (pH 7.4). The pellet was resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl pH 8, 50 mM NaCl), containing 15 mg ml⁻¹ lysozyme and protease inhibitor (Roche Applied Science), and incubated at 25°C for 1 h. Subsequently, 0.5 ml of IP buffer (100 mM Tris-HCl pH 8, 250 mM NaCl, 0.5% Triton X-100, 0.1% SDS), containing protease inhibitor, was added and chilled on ice. The sample was sonicated for seven cycles of 15 s each at 10 microns to shear chromosomal DNA into fragments ranging from 300 to 1000 bp on average. The sample was then centrifuged twice at 13 000 r.p.m. at 4°C for 15 min to clear the cell extract, after which 10 µl of cell extract was set aside for total-DNA extraction. The remainder (900 µl) was incubated with 45 µl of protein A-sepharose (Sigma) for 1 h on a rotating wheel to clear from non-specifically binding proteins. Samples were then centrifuged for 15 min at 4°C and 13 000 r.p.m. to remove the beads. The supernatant was incubated with 50 µl of anti-BldD antibody overnight at 4°C with rotation. Subsequently, 90 µl of protein A-sepharose was added to precipitate BldD and incubation was continued for 4 h. The sample was centrifuged at 3500 r.p.m. for 5 min and the pellet was washed twice with 0.5× IP buffer, then twice with 1× IP buffer, and transferred to a new tube after the first washing step. The pellet, and 10 µl of total cell extract, were eluted overnight at 65°C in 150 µl of IP elution buffer (50 mM Tris-HCl pH 7.6, 10 mM EDTA, 1% SDS) to reverse cross-links. The sample was centrifuged at 13 000 r.p.m. for 5 min to remove the beads. The pellet was re-extracted with 50 µl of TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA) and incubated with 0.2 mg ml⁻¹ Proteinase K (Roche) for 2 h at 55°C. The samples were extracted with phenol-chloroform and further purified using QiaQuick columns (Qiagen). DNA was eluted in 50 µl of EB buffer and quantified using a NanoDrop spectrophotometer (Thermo Scientific).

DNA labelling and hybridization to DNA microarrays were performed by Oxford Gene Technology (OGT) and were essentially carried out as described previously (Bucca *et al.*, 2009). Briefly, using the Bio-Prime kit (Invitrogen), 800 ng of the total and immunoprecipitated DNA was labelled with Cy5-dCTP and Cy3-dCTP respectively. Labelled DNA was hybridized, in an Agilent Technologies hybridization oven, to high-density DNA microarrays, representing the genome of *S. coelicolor* A3(2) (Bentley *et al.*, 2002), that were manufactured by OGT. Following washing, the arrays were read out using an Agilent Technologies scanner and Cy5 and Cy3 signals were quantified using Agilent's Feature Extraction software. Data were analysed using a combination of bespoke Perl and R programs. Essentially, the following steps were carried out. Columns containing the positions of probes in the genome sequence, the green median signals and the red median signals were extracted from the data files received from OGT. Ratios of green median signals to the red median signals were calculated. For the *bldD* mutant control and for each of the three replicates of the wild type, the signal ratios were read into a data frame in R and scaled to make the mean as well as the standard deviation for each fall within the range of 0 to 1. For each probe, the mean of the three

(scaled) *bldD* ratios was calculated and from it the control ratio was subtracted to arrive at a vector of the differences between the *bldD* means and the control. For each of the difference so calculated, a *P*-value was calculated assuming a population mean equal to the mean of all differences and a population standard deviation equal to the standard deviation of all the differences. These *P*-values were adjusted for multiple testing by the method of Benjamini and Hochberg to arrive at adjusted *P*-values. Data were ordered by increasing (adjusted) *P*-values and all probes having an adjusted *P*-value < 5e⁻³ were manually inspected to determine whether they were a part of a signal peak or not. ChIP-chip data have been deposited at the MIAME-compliant Gene Expression Omnibus database under accession number GSE23401.

Identification of DNA motifs

To identify conserved DNA motifs, DNA sequences encompassing up to 200 bp of the upstream regions of the genes, found in the ChIP-chip experiment, were collected from the genome sequence of *S. coelicolor* (Bentley *et al.*, 2002). This data set was used as input for the *MEME* software tool (Bailey and Elkan, 1994) to search for over-represented sequences. A search for the occurrence of identified motifs was performed using *Virtual Footprint* software suite (Munch *et al.*, 2005) in the genome of *S. coelicolor*. Alternatively, the entire genomes of *S. griseus* NBRC 13350, *S. avermitilis* NRRL 8165, *S. erythraea* NRRL 23338, *S. tropica* CNB-440 and *Frankia* sp. EAN1 were used as templates. Results of all motif searches are available in Table S3.

In vitro DNA-binding assays

Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (Elliot and Leskiw, 1999). In short, DNA fragments containing a predicted BldD binding site downstream of *bldA*, and a truncated derivative, were generated by PCR using Expand DNA polymerase (Roche) with combinations of oligonucleotides *bldA_F1* and *bldA_R1*, and *bldA_F1* and *bldA_R2* respectively. Radioactive probes were generated from these fragments by end-labelling and incubated with various amounts of histidine-tagged BldD, purified as described previously (Elliot and Leskiw, 1999), at 30°C in a 20 µl volume containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM dithiothreitol, 1 µg of poly[dl-dC] (Roche) and 10% glycerol. Protein-DNA complexes were separated in 4% polyacrylamide gels, run in TBE buffer at 100 V for 1 h, which were dried after electrophoresis and used for autoradiography using a FLA-7000 phosphorimager (Fujifilm).

DNase I footprinting experiments were carried out essentially as described previously (den Hengst *et al.*, 2005) and according to the description supplied with the Sure Track footprinting kit (Amersham Pharmacia Biotech). DNA fragments were prepared by PCR with combinations of oligonucleotides 7773_F1 and 7773_R1, 0792_F1 and 0792_R1, 2792_F1 and 2792_R1, 2082_F1 and 2082_R1, and *bldA_F3* and *bldA_R3* to generate DNA probes overlapping potential BldD binding sites near the *pepA*, *sti*, *bldH*, *ftsZ*

and *bldA* genes respectively. Oligonucleotides were first end-labelled with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [³²P]-ATP as described by the manufacturer. Binding reactions were identical to those used in EMSAs, but in a total volume of 40 µl, and in the presence of approximately 110 000 cpm of the DNA probe.

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