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BldD is a direct regulator of key developmental genes in *Streptomyces coelicolor* A3(2)

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Summary

BldD is a transcription factor required for aerial hyphae formation in the filamentous bacterium *Streptomyces coelicolor*. Three targets of BldD regulation were discovered by a number of means, including examination of *bld* gene interdependence, selective enrichment of chromosomal DNA fragments bound by BldD and searching the promoter regions of known developmental genes for matches to a previously characterized BldD binding site. The three BldD targets identified were the developmental sigma factor genes, *whiG* and *bldN*, and a previously uncharacterized gene, designated *bdtA*, encoding a putative transcription factor. In each target gene, the sequences bound by BldD were characterized by electrophoretic mobility shift and DNase I footprinting assays, and their alignment suggested AGTgA $(n)_m$ TCACc as a consensus BldD operator. The *in vivo* effect of mutation in *bldD* on the expression of these three target genes was assessed using S1 nuclease protection assays. In each case, target gene expression was upregulated during early colony development in the *bldD* background, suggesting that, in the wild type, BldD acts to repress premature expression of *whiG*, *bldN* and *bdtA* during vegetative growth.

Introduction

The life cycle of streptomycetes is remarkably complex for a prokaryote, encompassing a number of structurally differentiated states and physiological changes. Growth initiates with spore germination and continues through a mycelial outgrowth to form a mat of branched hyphae. In response to an as yet undetermined signal(s), probably the sensing of nutritional deficiency, vegetative growth ceases and aerial hyphae begin to form. Maturation of these aerial filaments proceeds through a synchronous septation event, leading to the formation of unigenomic spores. Coincident with the onset of aerial hyphae formation is a shift to secondary metabolism, which results in the production of antibiotics and a variety of other compounds. Both the morphological and the physiological changes are tightly regulated and require the integration of environmental stimuli and extracellular signalling. The events that lead up to and allow differentiation to proceed are slowly being pieced together; however, the connections between the many signals and responses are not well understood, and it is likely that many of the genes involved have yet to be identified.

Studies of sporulation in *Streptomyces coelicolor* have revealed a class of genes, the *whi* genes (named for the white colony colour, which stems from a lack of characteristic grey spore pigment when mutant), to be essential for the formation of mature spores. Understanding of the *whi* gene regulatory cascade is advancing rapidly, and it has been shown to involve at least two different sigma factors, σ^{WhiG} (*whiG*; Chater *et al*., 1989) and σ^{F} (*sigF*; Potúčková *et al*., 1995), and a number of proposed DNA-binding transcription factors (reviewed by Chater, 1998). A separate group of genes appears to be required for the erection of aerial hyphae. The *bld* genes were initially identified by mutations that caused the loss of white fuzzy aerial hyphae, resulting in colonies with a smooth, shiny, 'bald' appearance. The *bld* genes encode a diverse range of products, including a tRNA (*bldA*; Lawlor *et al*., 1987), an ATP-binding cassette (ABC) membrane-spanning transporter (*bldK*; Nodwell *et al*., 1996), a sigma factor (*bldN*; Bibb *et al*. 2000) and an unrelated anti-anti-sigma factor (*bldG*; Bignell *et al*., 2000), as well as numerous transcription factors [*bldB* (Pope *et al*., 1998), *bldD* (Elliot *et al*., 1998) *and bldM* (Molle and Buttner, 2000)]. Interestingly, a number of the *bld* genes also appear to influence antibiotic production, and mutations in some *bld* genes have been found to affect the regulation of carbon utilization (Pope *et al*., 1996), suggesting a global regulatory role for the *bld* genes in colony development. Metabolic defects have also been implicated in blocking differentiation through studies on the adenylate cyclase gene, *cya*, which catalyses the formation of cAMP. Disruption of *cya* results

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in a classical 'bald' phenotype, with the mutants showing deficiencies in both aerial hyphae formation and pigmented antibiotic production when grown on unbuffered media (Susstrunk *et al*., 1998). As neutralization of the medium or growth on buffered media allowed the developmental defects to be overcome, this conditional bald phenotype appears to result from acidification of the medium, perhaps suggesting a connection between metabolism, the resulting generation of organic acids and development.

Assessment of environmental conditions and communication between different colony compartments is accomplished, at least in part, by extracellular signalling molecules, such as γ-butyrolactones and oligopeptides, which are involved in the control of development in *Streptomyces*. In *S. coelicolor*, *bldK* has been implicated in signal uptake. *bldK* is a complex locus that encodes an ABC transporter, and *bldK* mutants are resistant to the toxic tripeptide bialaphos, implying that BldK is an oligopeptide importer (Nodwell *et al*., 1996). A small extracellular signalling peptide has been identified as a potential import target for BldK (Nodwell and Losick, 1998). BldK is one of the early players in an apparent *bld* gene extracellular signalling cascade, which is thought to culminate in the formation of SapB, a surfactant that contributes to the erection of aerial hyphae on rich media by reducing the surface tension at the colony surface (Tillotson *et al*., 1998). Studies carried out by Willey *et al*. (1993) revealed that, when certain pairs of *bld* mutants were grown in close proximity, aerial hyphae formation was restored to one member of each pair. By examining all possible pairings of the *bld* mutants, a hierarchy of genes was constructed, based upon the ability of each *bld* mutant to restore aerial hyphae production to all others (Willey *et al*., 1993; Nodwell *et al*., 1999; Molle and Buttner, 2000).

bldJ (formerly *bld*261) < *bldK*/*bldL* < *bldA*/*bldH* < *bldG* < *bldC* < *bldD*/*bldM*

Each mutant strain is able to complement all those shown to the left, presumably through the provision of some signal that the others lack or through the inactivation of an extracellular inhibitor, and is complemented by all those to the right. Those strains that share complementation groups show the same complementation profile, yet have no effect on the hyphal production of each other. The *bld* gene effect is not likely to be as simple as a linear cascade, however, as *bldB* and *bldN* do not conform to any one complementation group. Despite these anomalies, the number of genes in each defined complementation group has recently been expanded through a comprehensive *bld* gene screen by Nodwell *et al*. (1999). A large number of these *bld* genes have been found to fall into the *bldD/bldM* complementation class and, in some cases, may represent BldD targets.

BldD was predicted to be a DNA-binding protein (Elliot *et al*., 1998) and was shown to bind to its own promoter, consistent with the marked upregulation of *bldD* transcription in a *bldD* mutant (Elliot and Leskiw, 1999). Here, we identify three additional chromosomal targets for BldD, which show that BldD is a regulator of key genes associated with development.

Results

BldD regulates expression of whiG

Previous examination of BldD binding, using its own promoter as a target, revealed an inverted repeat sequence between the −10 and −35 regions of the *bldD* promoter (Elliot and Leskiw, 1999). In an attempt to identify other BldD targets in *S. coelicolor*, the promoter regions of genes known to be involved in differentiation (other *bld* genes, *whi* genes and antibiotic regulatory genes) were examined for the presence of similar sequences. A related sequence was found in the promoter region of *whiG*, which encodes a sigma factor that plays a critical role in triggering sporulation in the aerial hyphae (Chater *et al*., 1989). To assess the ability of BldD to bind to the *whiG* promoter region, electrophoretic mobility shift assays (EMSAs) were carried out using purified, N-terminally His-tagged BldD in the presence of excess, non-specific competitor DNA [poly-(dI–dC)]. Upon the addition of 0.5 μM BldD, the 354 bp fragment, spanning the *whiG* promoter from −144 to +210 (relative to the transcription start point) shifted to three different positions on an 8% polyacrylamide gel; however, all labelled DNA shifted to the upper position upon the addition of 2.0 μM BldD (Fig. 1A), possibly suggesting the existence of multiple BldD binding sites within this DNA fragment. Specificity of binding was examined through competition gel shift assays, involving both the addition of 500-fold excess non-specific competitor DNA (BKL41-MAE5; Table 1), which had no influence on BldD binding, and the addition of 500-fold excess unlabelled probe, which abolished BldD binding to labelled fragments (Fig. 1A).

Binding of BldD to the *whiG* promoter was examined further using DNase I footprinting assays. The same 354 bp fragment used for the gel shift assays was end labelled on the template strand and incubated with 2–4 μM BldD. BldD binding to the non-template strand was examined in the same way, only using a 479 bp probe, to permit full resolution of the BldD protected region(s). Two protected sites were observed on both template and non-template strands: the first site spanned the *whiG* promoter region, stretching from +13 to −45 relative to the

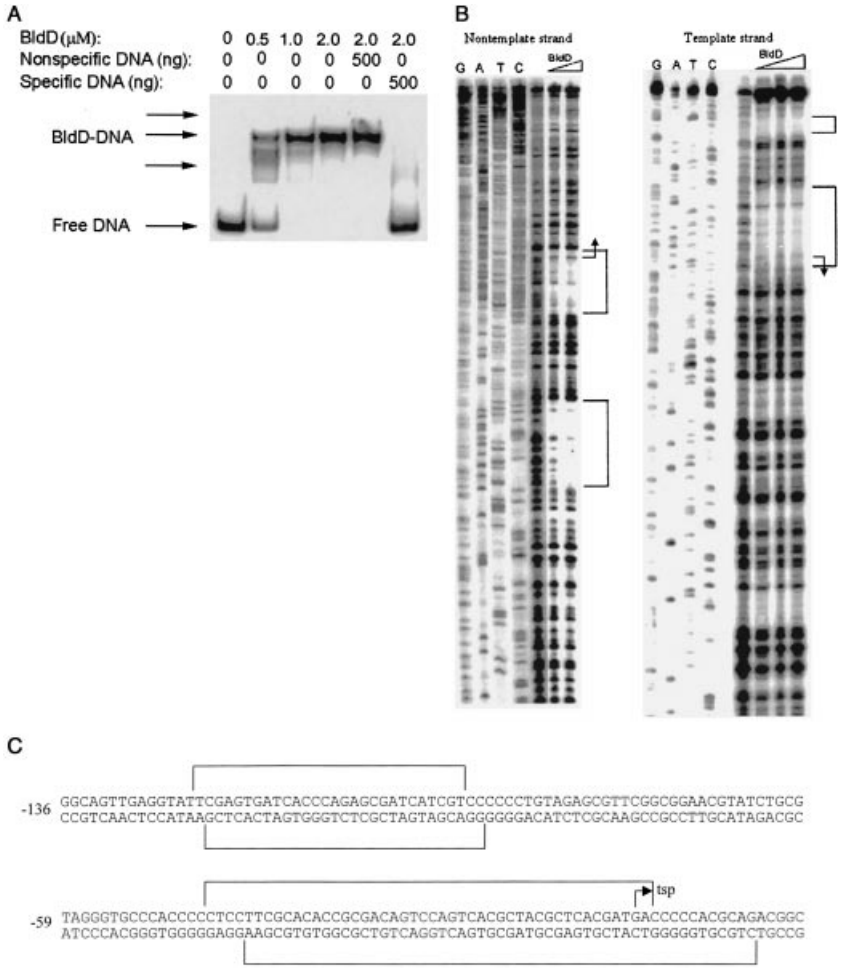

Fig. 1. BldD binding to the *whiG* operator.
A. EMSA of BldD binding to a 354 bp region of the *whiG* promoter. Free DNA and BldD-complexed DNA are indicated with arrows. BldD binding specificity was demonstrated by the addition of ≈ 500 ng of unlabelled, non-specific competitor DNA (BKL41-MAE5; Table 1) to the reactions loaded into the fifth reaction lane and ≈ 500 ng of unlabelled probe (specific competitor; MAE44-45) to the reactions separated in the final reaction lane.
B. DNase I footprinting of the template strand and non-template strand of the *whiG* probe. The 354 bp (MAE44-45) probe DNA was end labelled specifically on the template strand, whereas the 478 bp (MAE76-45) probe DNA was end labelled specifically on the non-template strand. Areas protected by BldD are bracketed, and the transcription start point is indicated by an arrow. The sequencing ladders, as indicated by G A T C, were generated using the oligonucleotide primers MAE76 or MAE45 for examination of the template or non-template strands respectively.
C. The *whiG* operator region. The areas protected from DNase I cleavage by BldD binding are bracketed, and the transcription start site (tsp) is indicated.

transcription start point, whereas the second site was significantly upstream, between nucleotides −122 and −92 (Fig. 1B and C). This experiment was repeated three times with representative results shown.

The influence of BldD on *whiG* expression *in vivo* was investigated by comparing the transcription of *whiG* in the *S. coelicolor bldD* mutant 1169 and its congenic parent, 916, using S1 nuclease protection assays. RNA was

Table 1. Oligonucleotides used in this work.

Oligonucleotide	Purpose	Sequence (5′ to 3′)
BKL41	Non-specific gel shift probe (PCR)	CGCCGTCATCTACGACC
BKL54	Probe for 16S rRNA	CCGCCTTCGCCACCGGT
MAE5	Non-specific gel shift probe (PCR) *bldD* coding sequence for BldD overexpression (PCR)	GGTAAGCTTTCAGAGCTCGTCGTGGGAC
MAE6	*bldD* coding sequence for BldD overexpression (PCR)	CGCGGATCCTCCAGCGAATACGCCAAAC
MAE42	SELEX linker	CCTACTGTACTATGGTTAGC
MAE43	SELEX linker	P-**GATC**GCTAACCATAGTACAGTAGG[a]
MAE44	*whiG* gel shift, footprinting and S1 mapping probe (PCR; sequencing)	GGATTTCTGGCAGTTGAGG
MAE45	*whiG* gel shift, footprinting and S1 mapping probe (PCR; sequencing)	TACGACCGCCACAGCTCG
MAE65	*bdta* gel shift, footprinting and S1 mapping probe (PCR; sequencing)	GCATCACCGAGGCGACC
MAE68	*bldN* gel shift, footprinting and S1 mapping probe (PCR; sequencing)	CGTAGCCAGGCCCGAGG
MAE71	*bldN* gel shift, footprinting, and S1 mapping probe (PCR; sequencing)	GTCAATCGGGCACAGAAGC
MAE72	*bdta* gel shift and footprinting probe (PCR; sequencing)	GCAGAATTCCGTGTCCGTCGCCGTTCC
Reverse primer	*bdta* S1 mapping probe (PCR; sequencing)	AACAGCTATGACCATG
Universal primer	Cycle sequencing of SELEX fragments (sequencing)	GTAAAACGACGGCCAGT

a. P, phosphate.
Underscore, engineered restriction enzyme site.
Bold, *Sau*3A1 overhang.

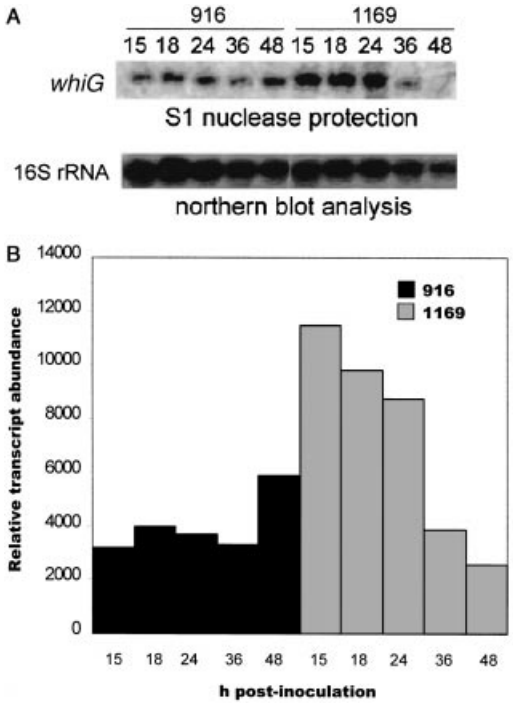

Fig. 2. A. S1 nuclease protection analysis of *whiG* in the wild-type (916) and *bldD* mutant (1169) strains over a 48 h time course. As a control for RNA levels, Northern blot analysis of the same RNA samples is shown below.
B. Comparison of *whiG* expression in the wild-type (916; black) and *bldD* mutant strain (1169; grey) corrected for RNA loading levels.

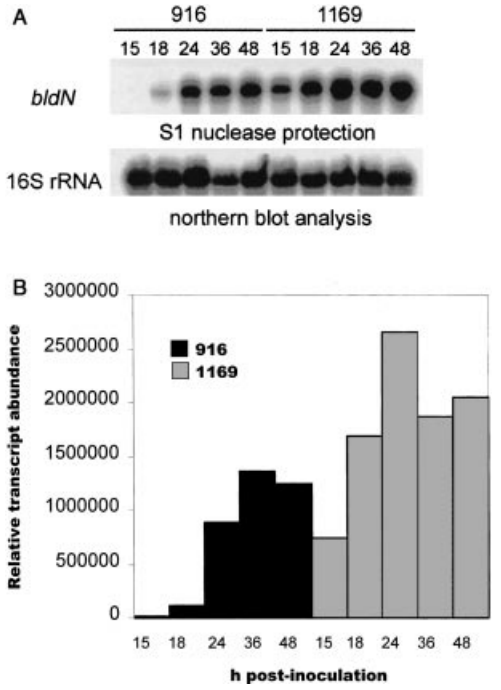

Fig. 3. A. S1 nuclease protection assay for *bldN* transcripts using RNA isolated from surface-grown cultures of the *S. coelicolor* strains 916 (wild type) and 1169 (*bldD*). The RNA samples were isolated at five different time points over a 48 h period of colony development (indicated as hours after inoculation). Northern blot analysis of the same RNA samples is shown below as a control for RNA levels.
B. Comparison of *bldN* expression in the wild-type strain 916 (black) and the *bldD* mutant strain 1169 (grey) using standardized RNA amounts.

isolated from plate-grown cultures, allowing the level of transcription to be correlated with the stage of development. In 916, the *whiG* transcript was present at relatively constant levels at all time points examined (Fig. 2A and B), as has been observed previously in the wild type (Kelemen *et al*., 1996). However, transcription of *whiG* in the *bldD* mutant 1169 followed a different pattern (Fig. 2A and B). In the RNA samples isolated from vegetative mycelium (up to 24 h after inoculation), the *whiG* transcripts were present at two- to threefold higher concentration in the *bldD* mutant than in 916. In addition, by 36 h and 48 h, when aerial hyphae become abundant and spore formation had begun, the level of *whiG* transcripts had dropped off significantly to barely detectable levels in the 48 h samples. To verify these results, the experiments were repeated using RNA isolated from a separate time course, and the results were very similar to those seen in Fig. 2A. As an internal control, relative RNA abundance was compared for each time point using Northern blot analysis. RNA samples from the same preparations used for the S1 nuclease protection assays were hybridized with a labelled oligonucleotide specific for 16S rRNA, showing that approximately equivalent amounts of RNA were present in each sample aliquot (Fig. 2A). The RNA levels were standardized, and the resulting *whiG* expression profiles for 916 and 1169 are shown in Fig. 2B.

BldD regulates the expression of bldN

A second BldD target was discovered when known *bld* mutants were screened for their effects on the expression of *bldN*, a gene encoding a sigma factor required for aerial hyphae formation in *S. coelicolor* (Bibb *et al*., 2000). Using S1 nuclease protection assays, *bldN* expression was shown to be significantly different in the *bldD* mutant strain 1169 when compared with its parent 916 (Fig. 3A and B). Transcription of *bldN* in *S. coelicolor* 916 appeared to be temporally regulated, with very low levels of transcript observed in RNA samples isolated from vegetative mycelium (virtually undetectable at 15 h), and increasing levels seen upon the initiation of aerial mycelium formation at $\approx$24 h, as has been observed previously in other morphologically wild-type strains (Bibb *et al*., 2000). In contrast, the *bldN* transcript was easily detectable at all time points in RNA samples isolated from the *bldD* mutant. In this case, the *bldN* transcripts were present in low, but

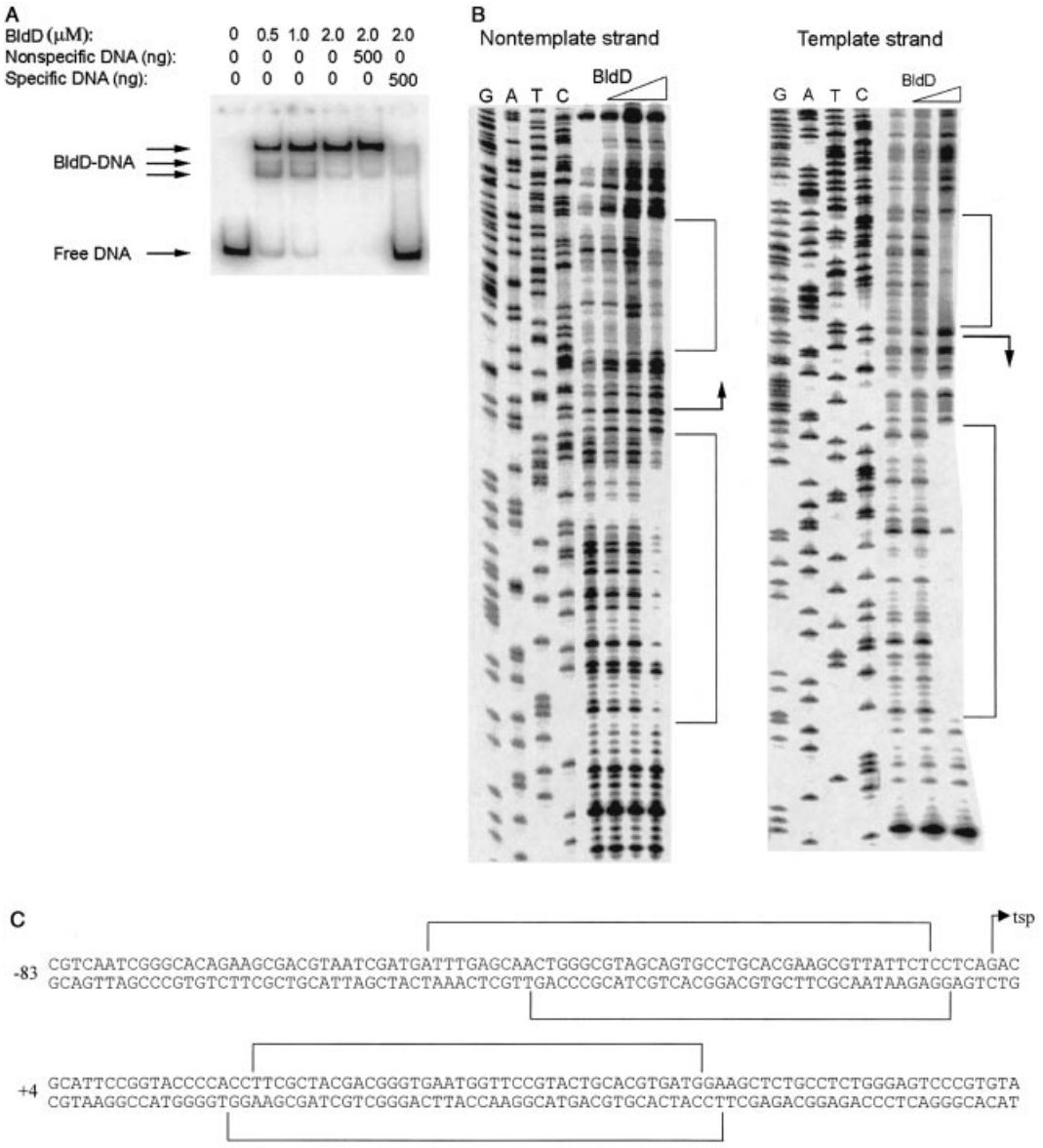

Fig. 4. BldD binding to the *bldN* operator.
A. EMSA of BldD binding to a 208 bp DNA fragment spanning the *bldN* promoter region. The separated products, free DNA and BldD-bound DNA are indicated by arrows. Binding specificity controls included the addition of ≈ 500 ng of unlabelled non-specific DNA (BKL41-MAE5; internal to the *bldD* coding sequence) in the penultimate lane and the inclusion of ≈ 500 ng of unlabelled specific probe (MAE71-68) in the final lane.
B. DNase I footprinting of the *bldN* promoter region in the presence of increasing amounts of BldD. The 208 bp gel shift probe fragment, MAE71-68, was specifically labelled on either the template or the non-template strand and incubated with increasing amounts of BldD (0, 0.8, 1.6 and 3.3 μM for the template strand and 0, 1.6 and 3.2 μM for the non-template strand). Regions of BldD protection are bracketed, and the transcription start point is marked by a vertical arrow. The sequencing reactions, labelled G A T C, were performed using the oligonucleotide primers MAE71 or MAE68 for analysis of the non-template and template strands respectively.
C. The *bldN* operator region. The BldD footprinted regions are bracketed, and the transcription start site (tsp) is shown.

readily detectable, amounts in RNA samples from the earliest examinable time point, after which they increased to a maximum at 24 h and remained high until 48 h after inoculation (Fig. 3A and B). In comparison with the parental strain, *bldN* transcripts were significantly more abundant in the RNA samples from the *bldD* mutant, ranging from > 10-fold higher concentration in the 15 h and 18 h samples to twofold higher concentration in the later samples. Relative RNA abundance was compared using Northern blot analysis as a control for RNA levels, and the variance in RNA levels was standardized for (Fig. 3B). The pattern of *bldN* expression was verified using RNA from a separate time course, and a very similar expression profile was observed (data not shown).

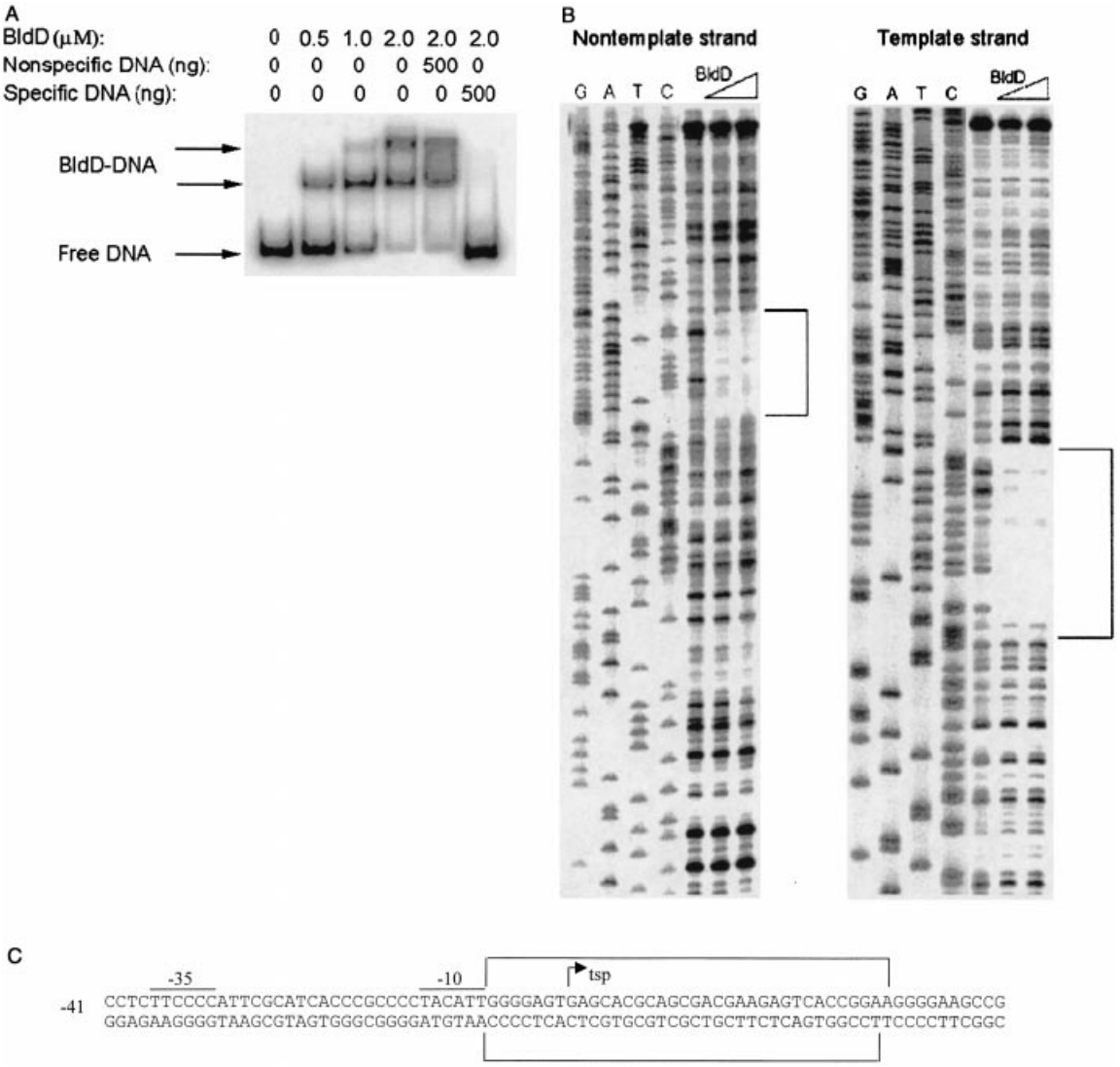

Fig. 5. BldD binding upstream from *bdta* on cosmid SCE68.
A. EMSA of BldD binding to a 308 bp DNA fragment upstream from *bdta*. The free probe and probe complexed with BldD are indicated. Binding specificity was established using unlabelled probe (≈500 ng of MAE65-72) as a competitor in the final (sixth) reaction lane and using unlabelled, non-specific competitor DNA (≈500 ng of BKL41-MAE5) in the fifth reaction lane.
B. DNase I footprinting of BldD bound upstream from *bdta*. The 308 bp probe, MAE65-72, was end labelled on either the template or the non-template strand and incubated with BldD (0, 1.6 or 3.2 μM). The BldD protected area is bracketed. The sequencing reactions (G A T C) were generated using MAE65 or MAE72 as oligonucleotide primers for examination of the non-template strand or the template strand respectively.
C. The *bdtA* operator region. The BldD footprint is bracketed, and the transcription start site (tsp) is shown together with the −10 and −35 promoter regions.

In an attempt to determine whether BldD influenced *bldN* expression directly or indirectly, EMSAs were conducted using a 208 bp fragment containing the *bldN* promoter region (extending from −83 to +126 relative to the transcription start site) as a target for binding by purified BldD protein. Upon the addition of 0.5 μM BldD, three different retarded species were observed, and virtually all the probe was found in the most retarded species upon the addition of 2.0 μM BldD (Fig. 4A). These results suggest the existence of multiple binding sites for BldD in the promoter region of *bldN*. As with *whi*G, 500 ng of non-specific DNA had no effect on the ability of BldD to shift the *bldN* probe fragment, but the addition of 500 ng of unlabelled *bldN* probe abolished the binding of BldD to the labelled probe (Fig. 4A).

To define the BldD binding sites in the *bldN* promoter region, DNase I footprinting assays were conducted using the same 208 bp gel fragment, labelled on either the template or the non-template strands. Protection from DNase I was conferred by BldD at two separate sites:

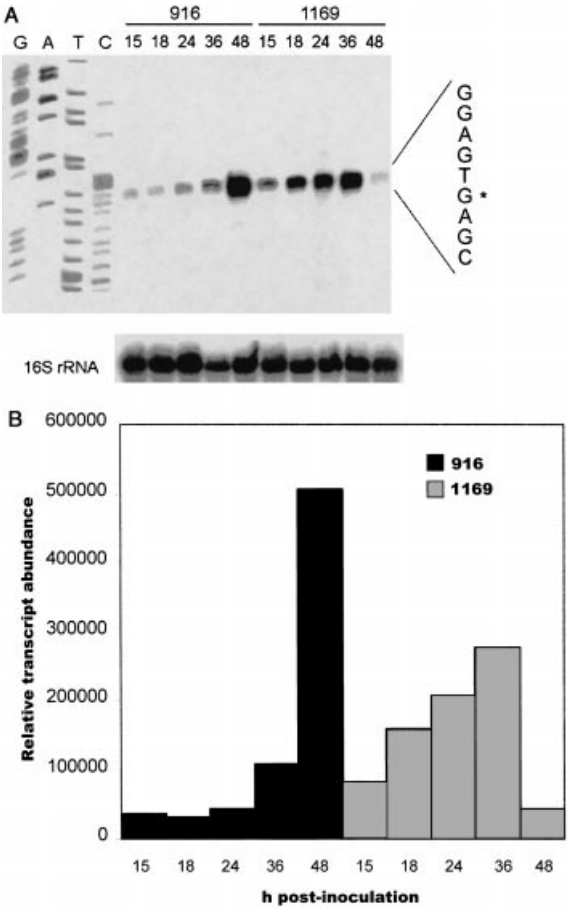

Fig. 6. A. High-resolution S1 nuclease mapping of the *bdtA* transcription start site using RNA isolated at various time points as indicated from *S. coelicolor* strains 916 (wild type) and 1169 (*bldD*). MAE65 was used as the oligonucleotide primer to generate the sequence ladder, shown as G A T C. The asterisk (*) indicates the most probable transcription start site in the sequence corresponding to the template strand. RNA levels in the samples were assessed by Northern blot analysis and are shown below the S1 nuclease protection assay.
B. *bdtA* expression compared between the wild-type strain 916 (black) and the *bldD* mutant strain (grey) corrected for RNA loading levels.

from −3 to −49 and from +19 to +63 relative to the transcription start site (Fig. 4B and C).

Use of systematic evolution of ligands by exponential enrichment (SELEX) identifies a further transcription factor under BldD control

In an effort to identify further BldD chromosomal targets, a modified SELEX approach was undertaken. Selection of sequences bound by BldD was carried out using a library of size-fractionated (300–600 bp) *Sau*3AI fragments of *S. coelicolor* chromosomal DNA, to which linkers containing polymerase chain reaction (PCR) primer sequences had been ligated. EMSAs were carried out using 10–20 ng DNA fragments and 1 μM of His-tagged BldD. Retarded DNA fragments were excised from the gel, purified, subjected to PCR amplification, and the resulting products were end labelled and cycled through the gel shift selection procedure at least twice more. The final products were cloned, sequenced and compared with the *S. coelicolor* genome sequence database (http://www.sanger.ac.uk/Projects/S.coelicolor). The *bldD* promoter region served as a positive internal control; it was found to be present in the final ligation mixture for each selection experiment conducted, as determined by PCR, and was also identified as a positive clone by sequencing.

One particular fragment appeared with overwhelming frequency (> 50% of all positive clones), and it corresponded to a region upstream from ORF 26.c, a small open reading frame on cosmid SCE68. ORF 26.c was predicted to encode a protein of 70 amino acids and was found to be 68.3% identical over 63 of its 70 amino acids to an 87-amino-acid protein from *Mycobacterium leprae*. Although a function has not been assigned to either of these proteins, ORF 26.c contains a strongly predicted helix–turn–helix motif, suggesting DNA-binding potential. In accordance with these and further results (see below), ORF 26.c was designated *bdtA* (= BldD target).

Several additional putative targets appeared with varying frequencies, including fragments corresponding to regions from cosmids SCE2 (position 18 151–18 555; upstream from a potential transposase), SCD78 (2063–2524; within a putative plasmid replication initiator protein), SC66T3 (7121–7613; within a hypothetical protein) and St6F2 (cosmid sequencing unfinished; within a cytochrome P_{450}). Use of these fragments as probes for gel shift assays revealed that they shifted poorly (data not shown), with less than 10% of the labelled DNA present in the shifted bands even after the addition of 2.0 μM BldD. This observation, combined with the position of most of these selected fragments within ORFs, made it unlikely that these were *bona fide* BldD targets. The selection experiments were repeated using glutathione *S*-transferase (GST)-tagged BldD to ensure that the His-tag did not play a role in non-specific binding by BldD, and the results obtained were very similar to those seen using His-tagged BldD. *bldN* and *whiG* were not cloned from the selection mix, indicating that perhaps the conditions were not optimal for BldD target selection.

*Analysis of BldD–*bdtA *interactions*

To define the BldD binding site(s) in the promoter region of the previously uncharacterized gene *bdtA*, gel shift assays were conducted using probe DNA spanning the *bdtA* translation start site (from +74 to −226 relative to the translation start site). Separation of the free DNA from the BldD-bound DNA revealed two shifted fragments, with

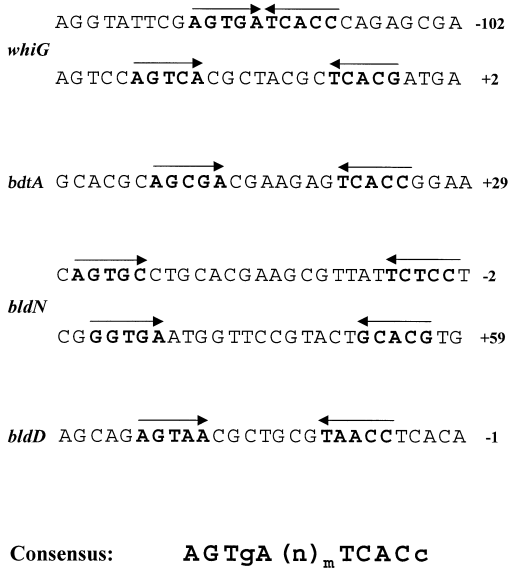

Fig. 7. Compilation of six known BldD binding sites. The nucleotide sequences of four BldD targets are shown with putative BldD recognition sequences in bold. The arrowheads indicate the inverted repeats within each sequence, corresponding to the proposed BldD recognition sequence for each BldD binding site. The nucleotide position shown to the right of each sequence is relative to the transcription start site for the representative gene. A BldD binding consensus sequence is shown at the bottom with capital letters representing extremely well-conserved residues, whereas the lower case letters indicate residues with more variance associated at those positions. The (n) represents the intervening sequence between the consensus halves and is of a variable length.

the labelled DNA found to be distributed evenly between these two products upon the addition of 2.0 μM protein (Fig. 5A). As before, 500 ng of unlabelled specific competitor abolished BldD binding to the labelled probe, whereas the addition of 500 ng of unlabelled non-specific competitor did not influence BldD binding (Fig. 5A).

To define further the BldD binding sites within the *bdtA* promoter region, footprinting assays were carried out using the same 308 bp probe (MAE65–MAE72; Table 1) as used in the gel mobility shift assays, labelled on the template or the non-template strand. The DNase I protection analysis revealed one BldD binding site (Fig. 5B and C), spanning 36 bp from −43 to −79. The fact that only one binding site was seen in the DNase I footprints, but two species were seen in the gel shift assays, suggests that a conformational change can occur upon binding of BldD to the *bdtA* promoter region.

To investigate the influence of BldD on *bdtA* expression *in vivo* and to identify the transcription start point, high-resolution S1 nuclease mapping was used to examine transcript levels over a 48 h time course in the parent (916) and *bldD* mutant (1169) strains. A single protected fragment of 145 nucleotides (nt) was detected (Fig. 6A), corresponding to a transcriptional start site at the G residue 72 nt upstream from the ATG start codon. The sequence upstream from the transcription initiation point was examined, and putative −10 and −35 sequences, separated by an 18 bp spacer and bearing similarity to σ^{HrdB}-dependent promoters (σ^{70}-like promoters; see Fig. 5C), were identified. Defining the transcription start site showed that BldD protected the *bdtA* operator from +29 to −7 (Fig. 5C).

Examination of transcript abundance over the 2 day time course revealed temporal regulation of *bdtA* expression (Fig. 6A and B). In the wild-type strain, a low level of transcript was seen in the 15 h sample, and this was observed to increase to maximal levels between 36 h and 48 h. This transcript abundance pattern would be consistent with a role for *bdtA* in differentiation, as aerial mycelia were first visible between 24 h and 36 h, were abundant by 36 h and were undergoing sporulation by 48 h. In the *bldD* mutant, as in the parent, transcript levels increased from 15 h to 36 h; however, the amount of transcript present at each time point was more than threefold greater in the *bldD* mutant compared with the wild type. Between 36 h and 48 h, when substantial upregulation of *bdtA* expression was observed in the parent strain, transcription in the mutant strain fell off significantly, to ≈15-fold less than the amount present in the wild type.

As before, an internal control comparing relative RNA abundance for each time point was performed using Northern blot analysis. Any variation in RNA levels was taken into account when comparing the profiles of *bdtA* expression in the *bldD* mutant and its parent strain (Fig. 6B). To ensure that these results were reproducible, the experiments were repeated using RNA isolated from a separate time course, and a very similar pattern of expression was seen (data not shown).

Discussion

BldD binds to an inverted repeat with variable spacing

Alignment of BldD binding sequences (two sites for each of *whiG* and *bldN* and one site for each of *bldD* and *bdtA*) suggests AGTgA $(n)_m$ TCACc as the consensus sequence for BldD binding (Fig. 7). Although this imperfect inverted repeat sequence resembles the binding site for many known DNA-binding proteins, it is unusual in that the spacer length is variable. The ability to bind operator half-sites separated by varying distances has been demonstrated for the *Escherichia coli* arabinose catabolism regulator AraC (Carra and Schleif, 1993) and for CytR, a LacI family member in *E. coli* (Jorgensen *et al.*, 1998), both of which possess a flexible connection between dimerization and DNA-binding domains. BldD structural predictions suggested that it has two domains,

with a long, disordered linker region (Elliot *et al*., 1998), which might allow BldD to bind sequences with a relaxed spacer requirement.

In the SELEX experiments, *bldN*, *whiG and sigH* (another BldD target; see below) were not cloned from the selected fragments; however, it was possible to amplify *bldN* and *sigH* from the selected fragments by PCR. The reason for the inability to clone fragments containing the *bldN* and *sigH* operator regions is unknown. Digestion of the chromosomal library with *Pst*I before beginning the gel shift selection, to eliminate *bdtA* and increase the likelihood of isolating other targets, did not result in the cloning of *bldN* and *sigH*. *whiG* was not detectable by PCR in the selected fragments, probably because there are four *Sau*3AI sites within the *whiG* operator.

BldD is a regulator of key genes associated with development

In the *bld* gene extracellular signalling cascade, *bldD* is at the end, restoring aerial mycelium formation to all other *bld* mutants, whereas none of the other *bld* mutants restores aerial mycelium formation to *bldD* (Willey *et al*., 1993; Nodwell *et al*., 1999). Additional screens, conducted since the cascade model was first proposed, have identified several new *bld* loci that fall into the *bldD* complementation group (Nodwell *et al*., 1999; Molle and Buttner, 2000). To date, *bldM*, which encodes a response regulator, is the only one of these loci to be characterized (Molle and Buttner, 2000). *bldM* is transcribed from two promoters, one of which is a direct biochemical target for σ^{BldN}-containing RNA polymerase holoenzyme (Bibb *et al*., 2000), and *bldN*, in turn, is a direct regulatory target for BldD. *bldN* is transcribed prematurely and to a greater extent in the *bldD* mutant relative to the wild type, suggesting that BldD functions as a repressor of *bldN* transcription, consistent with the positions of the two BldD binding sites in *bldN*. The resulting regulatory cascade, in which BldD represses *bldN* transcription, and σ^{BldN} directs the transcription of one of the two *bldM* promoters, takes us one step closer to understanding the interplay between the different *bld* genes in directing aerial hyphae formation.

In addition to influencing aerial hyphae formation through its control of *bldN*, BldD also exerts influence over the differentiation of aerial hyphae into spore chains through its regulation of *whiG*, which plays a critical role in the initiation of sporulation. *whiG* mutants form aerial hyphae in the normal way, but these fail to initiate sporulation septation. In contrast, overexpression of *whiG* leads to hypersporulation, even within the substrate mycelia, which are normally fated to lyse (Chater *et al*., 1989). Two known σ^{WhiG} targets, *whiH and whiI*, are not transcribed until aerial hyphae formation has begun, despite the fact that *whiG* transcription occurs during vegetative growth (Ryding *et al*., 1998; Aínsa *et al*., 1999). It was therefore proposed that σ^{WhiG} might be regulated post-translationally by an anti-sigma factor, as is the case for the closely related σ^{FliA} in *Salmonella typhimurium* (Kelemen *et al*., 1996). The discovery that *whiG* is subject to transcriptional regulation by BldD was therefore somewhat unexpected. In the *bldD* mutant, *whiG* expression was upregulated during the early stages of growth, suggesting that BldD represses *whiG* during vegetative growth in the wild type. At later time points, when sporulation was beginning in the wild type, *whiG* transcript levels dropped to virtually undetectable levels in the *bldD* mutant. This latter effect could reflect a direct positive influence of BldD on *whiG* transcription, but could also be indirect (for example, continued transcription might be dependent on the appearance of aerial hyphae).

BldD also regulates transcription of the previously unknown gene, *bdtA*, on cosmid SCE68. The function of *bdtA* is not known; however, its transcription is developmentally regulated, and it may encode a putative transcription factor, possessing a strongly predicted helix–turn–helix motif at its amino-terminal end. In the *bldD* mutant, *bdtA* is expressed prematurely. This, combined with the location of the BldD binding site downstream from the transcription start site, again suggests that BldD normally functions to repress the transcription of these genes during vegetative growth.

An additional BldD target, the stress response sigma factor, *sigH*, has been identified independently (Kelemen *et al*., 2001). *sigH* is transcribed from two promoters, *p1* and *p2*, which are temporally and spatially regulated, with *p2* activity being restricted to sporulating aerial hyphae in the wild type. BldD binds to the *sigH* promoter and, in the *bldD* mutant, transcription of *sigH* and levels of the σ^{H} protein are substantially upregulated, suggesting that BldD represses *sigH*. Thus, *sigH* is one of three sigma factor genes known to be controlled by BldD, each with different roles during colony development.

Our data suggest that *bldD* acts as a repressor of key developmental genes during vegetative growth. This is reminiscent of the control exerted by the transition state regulators of *Bacillus subtilis*, which also function to prevent premature expression of genes required for subsequent developmental processes (Strauch and Hoch, 1993). Among these transition state regulators are AbrB, which controls such diverse cellular functions as sporulation, antibiotic synthesis, catabolite repression, ribose transport and cellular competence, and SinR, which regulates sporulation, competence and motility. BldD appears to share several characteristics with these transition state regulators. BldD regulation is mediated, in part, through the control of σ^{BldN}, σ^{WhiG} and σ^{H}, whereas regulation by AbrB and SinR is also partly mediated

through the control of the *B. subtilis* sporulation sigma factors σ^H, σ^F and σ^E (Strauch and Hoch, 1993). Like BldD, AbrB negatively autoregulates its own expression (Strauch *et al*., 1989) and appears to bind its target operators with a degree of sequence flexibility (Xu and Strauch, 1996). In addition, PSI-BLAST and PFAM searches indicate that the N-terminal 69-amino-acid residues of BldD show significant similarity to the HTH-3 DNA-binding domain of the Xre family of repressors, which includes SinR (Kelemen *et al*., 2001). However, mutation in *sinR* or *abrA* does not lead to a significant sporulation defect (Strauch and Hoch, 1993), whereas the *bldD* point mutation abolishes aerial hyphae development and sporulation.

How BldD repression of its various targets is relieved during development is unknown. The repression exerted by the *B. subtilis* transition state regulators is alleviated in different ways: SpoOA~P represses *abrB* expression (Strauch *et al*., 1990), whereas SinR repression is relieved through interaction with an antagonist, SinI (Bai *et al*., 1993). It is tempting to speculate that, as for SinR, BldD repression is alleviated by interaction with another protein or small molecule cofactor. In the case of the *bldN* orthologue of *Streptomyces griseus*, its transcription is activated by AdpA, which, unusually, binds a site downstream from the *bldN* transcription start site in a position similar to the BldD binding site in *S. coelicolor* (Yamazaki *et al*., 2000). It is not yet clear, however, whether *S. coelicolor bldN* and its *S. griseus* orthologue are regulated in the same way.

The involvement of BldD in regulating *bldN*, *whiG* and *sigH* suggests that it is an important regulator of biological processes associated with development, acting as a repressor of developmental genes during vegetative growth. Repeated attempts to make a *bldD* null mutant have failed (M. Elliot, unpublished), suggesting that *bldD* might be essential, consistent with a role for BldD in repressing differentiation-associated genes during vegetative growth. It also implies that the product of the *bldD* mutant allele (*bldD53*), carrying a Tyr to Cys mutation (Elliot *et al*., 1998) close to the C-terminal end of the proposed SinR-related DNA-binding domain (position 62), may retain some function.

Experimental procedures

Bacterial strains

Streptomyces coelicolor strains used in this study include J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) (Chater *et al*., 1982), 916 [*hisA1 mthB2 pheA1 strA1* SCP1^{NF} (SCP2*)] (Merrick, 1976) and 1169 [*hisA1 mth32 pheA1 strA1 bldD53* SCP1^{NF} (SCP2*)] (Merrick, 1976). Media, culture conditions and chromosomal DNA isolation for *Streptomyces* were carried out as described previously (Hopwood *et al*., 1985). *E. coli* DH5α [F′ *supE44* Δ*ladU149* (Φ*80lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Gibco BRL) was used as host for plasmid construction and isolation, as was *E. coli* One Shot TOP10 [Invitrogen; used specifically with pCR-TOPO (Invitrogen)-based plasmids]. *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gurA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB* + *lacIq LacZ* Δ*M15*)] (Yanisch-Perron *et al*., 1985) was used for the overexpression of both the His-tagged and the GST-tagged BldD fusion proteins. Media, culture conditions and DNA manipulations for *E. coli* were carried out as described by Sambrook *et al*. (1989).

Protein purification

His-tagged BldD fusion protein was purified as outlined by Elliot and Leskiw (1999). Construction of the GST–BldD expression plasmid involved PCR amplification, using Expand polymerase (Roche) and conditions recommended by the manufacturer, of a 520 bp fragment containing the *bldD* coding sequence from chromosomal DNA (1 μg) of *S. coelicolor* strain J1501. Primers MAE5 and MAE6 (40 pmol) (Table 1) were used, where MAE5 contained a *Bam*H1 site engineered onto the 5′ end, replacing the ATG at the beginning of the *bldD* coding sequence, and MAE6 possessed a *Hin*dIII site immediately after the *bldD* TGA stop codon at its 5′ end. The resulting amplification products were recovered from a 5% polyacrylamide gel by crushing and soaking (Sambrook *et al*., 1989), blunt ended by Klenow fill in and digested with *Bam*HI, before being cloned into the pGEX-2T vector digested with *Bam*HI and *Sma*I. After transformation of *E. coli* JM109 and selection of ampicillin-resistant transformants, the integrity of the recombinant plasmids was verified by DNA sequence analysis.

For overexpression and purification of the GST–BldD fusion protein, a 10 ml overnight culture was used to inoculate 1 l of 2× YT broth (Sambrook *et al*., 1989). This culture was then grown to an optical density (OD_{600}) of 0.7–0.9. IPTG was added to a final concentration of 1.0 mM, and growth was allowed to continue for another 3–5 h at 37°C. Cells were pelleted from 500 ml aliquots of the culture and stored at −80°C. The cell pellet was thawed on ice, resuspended in 1× PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 2 mg ml^{-1} lysozyme and incubated for 30 min on ice. The cell suspension was then subjected to sonication on ice for four 1 min intervals using a 2.5 mm probe, after which the cell lysate was centrifuged at 10 000 *g* for 15 min at 4°C. The resulting supernatant, containing soluble GST-tagged BldD, was applied to a column containing 3.5 ml of glutathione agarose (Sigma). The column was washed with 80 ml of 1× PBS, and GST–BldD was eluted from the column using 8 ml of 10 mM reduced glutathione (Sigma) dissolved in 1× PBS. Purified GST–BldD was dialysed against a solution of 20 mM Tris-HCl (pH 8.5) and 150 mM NaCl and concentrated using a Millipore Ultrafree-4 centrifugal filter. The protein was stored in 5 μl aliquots at −80°C.

Electrophoretic mobility shift assays

Gel mobility shift assays were performed according to previously published procedures (Elliot and Leskiw, 1999).

Probes were generated by PCR amplification using either chromosomal DNA (for *whiG and bldN*) or plasmid DNA (for the SELEX fragments) as template (see Table 1 for primers). The probe DNAs were then end labelled using [γ-^{32}P]-ATP and incubated individually with His-tagged BldD (0–40 pmol) at 30°C for 20 min in a buffer of 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM dithiothreitol (DTT), 1 μg of poly-(dI–dC) and 10% glycerol in a total volume of 20 μl. For competition gel shift assays, $\approx$500 ng of unlabelled probe (specific competitor) or $\approx$500 ng of unlabelled, non-specific target [BKL41-MAE5; a 118 bp fragment internal to the *bldD* coding sequence, amplified using the pAU171 (Elliot *et al*., 1998) as template and oligonucleotide primers BKL41 and MAE5 (see Table 1)] were also added to the reactions. Protein-bound and free DNA were separated by electrophoresis on a 1$\times$ TBE-buffered, 1.5% glycerol-containing 8% polyacrylamide gel. Gels were dried and exposed to a phosphorscreen before analysis using a phosphorimager (Molecular Dynamics model 445 SI) and IMAGEQUANT software to quantify the bands.

DNase I footprinting assays

DNase I footprint analyses were carried out using the reaction conditions described previously (Elliot and Leskiw, 1999). DNase I (Roche), diluted in binding buffer to 0.05 U μl^{-1} (or 0.025 U μl^{-1} for *whiG* footprinting), was added to the samples (1 μl per reaction tube), and the reactions were stopped after 10 s by extraction with 50 μl of neutral phenol, followed by phenol–chloroform and then chloroform. The samples were precipitated in siliconized tubes, resuspended in 4 μl of sequencing stop solution (Amersham), heated to 80°C for 10 min and resolved on a 6% polyacrylamide sequencing gel. Analysis was conducted as for the EMSAs.

Modified SELEX

Chromosomal library. To isolate potential chromosomal targets for BldD binding, a modified SELEX (Ochsner and Vasil, 1996) protocol was used. *S. coelicolor* J1501 chromosomal DNA was partially digested using 0.21 U of *Sau*3AI μg^{-1} DNA. The partial digests were separated by electrophoresis on a 5% polyacrylamide gel, and fragments of $\approx$300–600 bp were excised from the gel and purified by crushing and soaking. Complementary oligonucleotides MAE42 and P-MAE43 (Table 1), with MAE43 possessing a 4 bp *Sau*3AI complementary overhang, were annealed in a 300 μl reaction containing 300 pmol of each oligonucleotide in an annealing buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl (Zhu, 1996). The annealing mixture was heated to 100°C for 5–10 min and then slowly cooled over 45 min to 30–37°C. The reactions were precipitated and redissolved in 100 μl of milli-Q H_2O. Four micrograms of the annealed linkers were then ligated to 2.5 μg of the *Sau*3AI-digested chromosomal DNA in a 300 μl reaction volume. After overnight incubation at 15°C, the DNA was precipitated and electrophoresed on a 5% polyacrylamide gel to remove free linkers. The ligation products were excised either together as a group or in three sections of fragments in the 300–450 bp range, the 450–550 bp range and the 550–650 bp range. After excision, the DNA fragments were purified as above and redissolved in 20–30 μl of milli-Q H_2O.

Target selection strategy. The library of chromosomal fragments to which linkers were added was end labelled using [γ-^{32}P]-ATP (ICN) and polynucleotide kinase. The labelled DNA was precipitated and subjected to scintillation counting before redissolution in milli-Q H_2O to give $\approx$1–2 ng μl^{-1} (50 000–200 000 c.p.m. μl^{-1}). EMSAs were then carried out using the conditions outlined above, except that 10–20 ng of DNA and only 20 pmol of His-tagged BldD were used per reaction, and the total reaction volume was adjusted to 25 μl. After loading onto an 8% polyacrylamide gel, the samples were electrophoresed for 3–4 h at 180 V, after which the gel was wrapped in Saran wrap and exposed to X-ray film for 48 h. The developed film was used as a template to cut the shifted bands from the gel, and the shifted DNA fragments were then eluted from the gel by crushing and soaking. Up to half of the resulting purified DNA was used as a template for subsequent PCR amplification. Amplification was accomplished using 80 pmol of MAE42 as primer, 0.125 mM dNTPs and commercial PCR buffer with detergents (Expand buffer 3; Roche) in conjunction with Expand DNA polymerase (Roche) in a total reaction volume of 100 μl. After an initial 5 min denaturation, the reactions were subjected to 30 cycles of denaturing (95°C for 45 s), annealing (52°C for 45 s) and extension for 1 min at 68°C. The amplified DNA was precipitated and purified on a 5% polyacrylamide gel as described above. The purified products were end labelled and the gel shift–PCR cycle was repeated. After at least three rounds of selection, the PCR-amplified targets were either blunt ended by Klenow fill in and ligated to *Sma*I-digested pUC119 before being transformed into *E. coli* DH5α or ligated without blunt ending into pCR-TOPO and transformed into One Shot TOP10. Insert-containing transformants were selected among the ampicillin (100 μg μl^{-1})-resistant colonies using blue/white selection. The presence of an insert was confirmed by digesting the DNA of individual clones with *Xba*I and *Eco*RI and running the digestion products on a 2% agarose gel.

S1 nuclease mapping

S1 nuclease mapping of mRNA 5′ ends was conducted using the method outlined by Hopwood *et al*. (1985), except that glycogen (Roche) replaced the carrier tRNA in the final precipitation step. RNA (40 μg) was used for each S1 nuclease reaction, and the RNA samples were incubated with probes for *whiG*, *bldN and bdtA*. The 317 bp *whiG* probe was generated by end labelling the 354 bp DNA fragment used in the *whiG*-specific EMSA (MAE44-45) using [γ-^{32}P]-ATP and polynucleotide kinase, followed by digestion with *Bcl*I to remove the external label from the template strand. Annealing of the RNA to this probe took place at 58°C for 3–4 h after a 30 min denaturation at 80°C. The *bldN* probe was created by digestion of a 208 bp end-labelled fragment (MAE71-68; Table 1) with *Cla*I to give a 181 bp probe labelled on one end that was then subjected to annealing with the RNA samples at 59°C. The 391 bp probe for *bdtA*

was generated by PCR amplification using the MAE65 oligonucleotide primer, and the reverse primer (which would bind to pUC119 vector DNA to provide a non-homologous tail, allowing differentiation between full-length protection and probe–probe reannealing) was end labelled and incubated with RNA at 52°C. The samples were run under standard conditions on a 6% polyacrylamide sequencing gel, along with sequencing reactions prepared according to the dideoxy-chain termination method (Sanger *et al*., 1977) using the oligonucleotides MAE45, MAE68 and MAE65 as primers for the sequencing of *whiG*, *bldN* and *bdtA* respectively.

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