Response Regulator Heterodimer Formation Controls a Key Stage in *Streptomyces* Development

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Abstract

The orphan, atypical response regulators BldM and WhiI each play critical roles in *Streptomyces* differentiation. BldM is required for the formation of aerial hyphae, and WhiI is required for the differentiation of these reproductive structures into mature spores. To gain insight into BldM function, we defined the genome-wide BldM regulon using ChIP-Seq and transcriptional profiling. BldM target genes clustered into two groups based on their *whi* gene dependency. Expression of Group I genes depended on *bldM* but was independent of all the *whi* genes, and biochemical experiments showed that Group I promoters were controlled by a BldM homodimer. In contrast, Group II genes were expressed later than Group I genes and their expression depended not only on *bldM* but also on *whi* and *whiG* (encoding the sigma factor that activates *whiI*). Additional ChIP-Seq analysis showed that BldM Group II genes were also direct targets of WhiI and that in vivo binding of WhiI to these promoters depended on BldM and vice versa. We go on to demonstrate that BldM and WhiI form a functional heterodimer that controls Group II promoters, serving to integrate signals from two distinct developmental pathways. The BldM-WhiI system thus exemplifies the potential of response regulator heterodimer formation as a mechanism to expand the signaling capabilities of bacterial cells.

Introduction

Two-component signal transduction systems are of central importance in regulating gene expression in bacteria. Canonically they consist of a response regulator, which functions as a homodimer, and a cognate sensor histidine kinase (which may also function as a cognate phosphatase). The activity of the kinase/phosphatase is modulated in response to a perceived stimulus. The sensor kinase autophosphorylates on a conserved histidine residue, and the phosphoryl group is transferred to a receiver domain on the response regulator. The addition of the phosphoryl group autophosphorylates on a conserved histidine residue, and the activity of the kinase/phosphatase is modulated in response to a perceived stimulus. The sensor kinase autophosphorylates on a conserved histidine residue, and the phosphoryl group is then transferred to a conserved aspartate in the response regulator. The addition of the phosphoryl group stabilizes a conformation of the response regulator that drives an output response, most often the activation of gene expression. However, the intrinsic modularity of these systems has allowed bacteria to evolve variations on this basic theme, including more complex multicomponent phosphorylases, and changes in the nature of the response regulator effector domain such that the output can be, for example, an enzymatic activity rather than DNA binding [1–3]. Recognizing the diversity of mechanisms associated with these systems is therefore critical to understanding the full potential of the signaling capabilities of bacterial cells. This study concerns the behavior of two response regulators required for morphological development in the filamentous bacteria *Streptomyces*.

When *Streptomyces* spores germinate, one or two germ tubes emerge and grow by tip extension and branching to form an extensive, multicellular vegetative mycelium [4–6]. *Streptomyces* differentiate by forming specialized reproductive structures called aerial hyphae, which emerge from the colony surface into the air. The formation of aerial hyphae requires the activity of a class of developmental master regulators encoded by the *bldM* (bald) genes [4–6]. Subsequently, in the most dramatic event of the lifecycle, each multigenomic aerial hypha arrests tip growth and undergoes a massive, synchronous septation event, giving rise to ~50–100 unigenomic prespore compartments that ultimately develop into mature, pigmented exospores [4–6]. The differentiation of aerial hyphae into mature spores is coordinated by the activity of a second class of developmental master regulators encoded by the *whi* (white) genes. The focus of this work is the interaction between two of these global regulators, BldM and WhiI.

BldM and WhiI are both atypical response regulators (ARRs). In canonical response regulators, the aspartate residue that is subject to phosphorylation sits in a highly conserved pocket within the N-terminal receiver domain. ARRs usually lack essential residues within this phosphorylation pocket, suggesting that their activity is not controlled by phosphorylation [7–11]. WhiI has a degenerate phosphorylation pocket, lacking a universally conserved lysine and one of a pair of adjacent aspartate residues essential for binding Mg²⁺ [12,13]. Although BldM does have a conserved phosphorylation pocket, a *bldM* allele carrying a D54A substitution at the putative site of phosphorylation fully complements a *bldM* null mutant [14]. Moreover, BldM could not be phosphorylated *in vitro* [14]. Finally, BldM and WhiI are both ‘orphan’ response regulators – their genes are not
Two-component signal transduction systems are a primary means of regulating gene expression in bacteria. Recognizing the diversity of mechanisms associated with these systems is therefore critical to understanding the full signaling potential of bacterial cells. We have analyzed the behavior of two orphan, atypical response regulators that play key roles in controlling morphological differentiation in the filamentous bacteria *Streptomyces*—BldM and WhiI. We demonstrate that BldM activates its Group I target promoters as a homodimer, but that it subsequently activates its Group II target promoters by forming a functional heterodimer with WhiI. BldM-WhiI heterodimer formation thus represents an unusual mechanism for the coactivation of target genes and the integration of regulatory signals at promoters, enhancing the known repertoire of signaling capabilities associated with two-component systems.

adjacent to a sensor kinase gene, as is most often the case for canonical response regulators. Taken together, these observations strongly suggest that BldM and WhiI are not controlled by phosphorylation as part of conventional two-component systems. BldM and WhiI both belong to the NarL/FixJ subfamily of response regulators. bldM and whiI are found in all sequenced streptomycete genomes and their chromosomal context is conserved throughout. Strikingly, the amino acid sequence of BldM is 100% identical across all sequenced streptomycetes (the only response regulator that is 100% identical across all streptomycetes). WhiI is at least 93% identical, with amino acid variations found mainly in the linker region between the degenerate receiver domain and the DNA-binding domain. All sequenced WhiIs have a degenerate phosphorylation pocket.

During differentiation, the *whiI* and *bldm* genes are activated by two cognate, development-specific sigma factors, σ*WhiG* and σ*WhiI*, respectively. *whiI* expression is activated from a single σ*WhiG* target promoter, and thus *whiI* is not expressed in a *whiG* mutant [12]. σ*WhiI* directs transcription of the *pI* promoter of *bldM* (the other promoter, *bldMp2*, is σ*WhiI*-independent, and so transcription of *bldM* is developmentally activated from only one of its two promoters in a *bldN* mutant [15,16]). In addition to *bldM*, the other key targets of σ*WhiI* are the genes encoding the chaplins and rodlinns, the major proteins of the hydrophobic sheath that coats the aerial hyphae and spores in *Streptomyces* [16–19].

*Streptomyces venezuelae* has recently emerged as an attractive new model system for the analysis of *Streptomyces* development because it sporulates in liquid culture [16,20]. Here we take advantage of the *S. venezuelae* system to apply global microarray transcriptional profiling and ChIP-Seq to characterize the BldM and WhiI regulons. Through this route we go on to show that a key stage in *Streptomyces* development is controlled by response regulator heterodimer formation between BldM and WhiI, and to greatly expand our understanding of the regulatory network that controls morphological differentiation in these multicellular bacteria. The BldM-WhiI system thus exemplifies the potential of response regulator heterodimer formation as a mechanism to expand the signaling capabilities of bacterial cells.

Results

BldM directly activates the expression of key developmental genes

Having established conditions in which *S. venezuelae* sporulates abundantly in liquid culture [16,20], immunoblotting of samples taken at 14, 15 and 16 h of growth in MYM liquid sporulation medium showed that BldM was abundant at each of these time points ([Figure S1](#)). To gain greater insight into BldM function, we defined the genome-wide BldM regulon using ChIP-Seq. As described in Materials and Methods, wild-type *S. venezuelae* was subjected to formaldehyde cross-linking, lysis and sonication after 16 h of growth. After immunoprecipitation using a BldM-specific polyclonal antibody, the resulting DNA was subjected to deep sequencing. As a negative control, a ChIP-Seq experiment was performed on the congenic *bldM* null mutant. Several well-characterized developmental loci, including *sgR, rshA, sraA-offA, whiB* and whiE, were among the direct BldM targets identified.

Next, in order to determine how BldM influences the expression of its target genes, wild-type *S. venezuelae* and the congenic *AbldM* mutant were subjected to time-resolved, genome-wide transcriptional profiling during vegetative growth and sporulation. Strains were grown under the same conditions used for the ChIP-Seq experiments. RNA samples were prepared at 2-hour intervals from 8 to 20 hours, by which time sporulation was nearing completion, and following cDNA synthesis and labeling, samples were hybridized to Affymetrix DNA microarrays. Three independent biological replicates were performed for each strain, and analysis of the resulting data showed that the expression of 131 direct BldM targets was significantly down-regulated in the *AbldM* mutant (*p*<0.01) in comparison to the wild type. In contrast, only six genes were up-regulated in the *AbldM* mutant (*p*<0.01) in comparison to the wild type. These results suggest that BldM functions mainly as a transcriptional activator.

Many BldM target genes cluster into two discrete groups based on *whi* gene dependency

We next determined the time-resolved transcriptional profiles of the BldM target genes in seven constructed white mutants: *AwhiA, AwhiB, AwhiD, AwhiG, AwhiH* and *AwhiI*. Strikingly, many of the BldM target genes clustered into two well-defined groups according to their dependencies on the *whi* genes. Group I genes consisted of developmentally induced genes that depend on *bldM*, but were activated normally in all the *whi* mutants ([Figure 1A](#) and [Table S1](#)). Group II *BldM* target genes were also developmentally induced, but depended not only on *bldM*, but also on *whiG* and *whiI* ([Figure 1B](#) and [Table S2](#)).

Group I genes-identification of a consensus Group I BldM binding site

To gain further insight into Group I binding sites, we fed the sequences of Group I promoter regions into the MEME algorithm [21] to search for over-represented sequences, using as input the entire intergenic region in each case. This analysis revealed a well-conserved copy of a 16 bp palindromic sequence, 5'- TGACGcGcGcGTGTA-3', for which the sequence logo is shown in [Figure 2A](#). The palindromic nature of this sequence would be consistent with BldM binding as a homodimer to Group I promoters.

To test the validity of the MEME output, and to confirm and extend the ChIP-Seq analysis, we overexpressed and purified BldM from *E. coli* as a soluble, N-terminally His_6-tagged protein. The resulting BldM protein was used in DNase I footprinting analysis on the intergenic regions upstream of two Group I *BldM* targets, *sven1998* and *sven1150*. In both cases, BldM protected a region containing a well-conserved copy of the palindromic, MEME-predicted binding site ([Figure 2B](#)), consistent with this sequence serving as a high-affinity binding site for a BldM homodimer.

**Author Summary**

Two-component signal transduction systems are a primary means of regulating gene expression in bacteria. Recognizing the diversity of mechanisms associated with these systems is therefore critical to understanding the full signaling potential of bacterial cells. We have analyzed the behavior of two orphan, atypical response regulators that play key roles in controlling morphological differentiation in the filamentous bacteria *Streptomyces*: BldM and WhiI. We demonstrate that BldM activates its Group I target promoters as a homodimer, but that it subsequently activates its Group II target promoters by forming a functional heterodimer with WhiI. BldM-WhiI heterodimer formation thus represents an unusual mechanism for the coactivation of target genes and the integration of regulatory signals at promoters, enhancing the known repertoire of signaling capabilities associated with two-component systems.
Group II genes

Group II BldM target genes were expressed later than Group I genes (see insets in Figure 1). Further, and in contrast to Group I, the expression of Group II genes depended not only on \textit{bldM} but also on \textit{whiG} and \textit{whiI} (Figure 1 and Table S2). It is straightforward to account for the dependence of Group II gene expression on \textit{whiG}. In \textit{S. coelicolor}, \textit{whiI} expression is activated from a single \textit{sWhiG} target promoter, and thus \textit{whiI} is not expressed in a \textit{whiG} mutant [12]. This \textit{sWhiG} target promoter appears well conserved at the sequence level in \textit{S. venezuelae} (Figure S2A) and \textit{whiI} is not expressed in an \textit{S. venezuelae whiG} mutant (Figure S2B). Thus all genes that depend on \textit{whiI} must necessarily also depend on \textit{whiG}. Expression of \textit{whiI} was not significantly affected in the \textit{AbldM} mutant and vice versa (p<0.01) (Figure S2B), implying independent \textit{sWhiG-WhiI} and \textit{sBldM-BldM} regulatory pathways. The challenge then was to determine why Group II BldM target genes depend on \textit{whiI}.

Figure 1. Microarray transcription profiles of (A) Group-I and (B) Group-II genes in wild-type \textit{S. venezuelae} during submerged sporulation and in congenic \textit{DbldM}, \textit{DwhiA}, \textit{DwhiB}, \textit{DwhiD}, \textit{DwhiG}, \textit{DwhiH}, and \textit{DwhiI} null mutants grown under identical conditions. RNA samples were prepared at 2-hour intervals from 8 to 20 hours, by which time sporulation was nearing completion. For each panel, the x-axis indicates the age of the culture in hours, and the y-axis indicates the per gene normalized transcript abundance (log2). Group-I genes (blue) depend on \textit{bldM} but are independent of all the \textit{whi} genes. Group-II genes (red) depend on \textit{bldM} but also depend on \textit{whiG} and \textit{whiI}. The average expression profile is indicated by the black line. Group I genes are activated at least two hours earlier than Group-II genes (see insets). Red bars indicate vegetative growth (V), fragmentation of mycelium (F), and spore formation (S). doi:10.1371/journal.pgen.1004554.g001

Group II genes are direct \textit{in vivo} targets of WhiI

To determine if the dependence of Group II BldM target genes on WhiI was direct or indirect, we characterized the \textit{in vivo} WhiI binding sites across the \textit{S. venezuelae} genome using ChIP-Seq. Wild-type \textit{S. venezuelae} was harvested at 16 h of growth and treated as described for the BldM ChIP-Seq, except that a WhiI-specific polyclonal antibody was used. As a control, a ChIP-Seq experiment was performed using the congenic \textit{whiI} null mutant. The data showed that all of the BldM Group II targets were also direct targets of WhiI (Figure 3). As an independent confirmation, we repeated the ChIP-Seq experiment using a FLAG-tagged WhiI protein. An N-terminally 3xFLAG-tagged allele of \textit{whiI} (TF-WhiI) was constructed such that it was expressed from its native promoter and cloned into the single-copy vector pMS82, which integrates site-specifically into the chromosome at the phage \textit{F\beta}1 \textit{attB} site [22]. This construct fully complemented the phenotype of the \textit{whiI} null mutant (Figure S3), and the complemented strain was used for the ChIP-Seq experiment, now using wild-type \textit{S. venezuelae} as the negative control. The ChIP-Seq results seen using FLAG immunoprecipitation were almost identical to those obtained using WhiI polyclonal antibodies, confirming that Group II genes are directly regulated by both BldM and WhiI (Table S2).

At Group II promoters, \textit{in vivo} WhiI binding depends on BldM and \textit{vice versa}

Our data showed that expression of Group II genes depends on \textit{bldM} and \textit{whiI} and that both BldM and WhiI bind directly to the
promoters of these genes. One possible model consistent with these observations would be that BldM and WhiI co-activate Group II promoters by binding as two separate homodimers. An alternative model would be that these two proteins activate Group II promoters by binding as a functional BldM-WhiI heterodimer. To begin to differentiate between these models, we performed BldM ChiP-Seq on two Group-I promoters: sven1998 and sven4150. The BldM protein concentrations used were 0, 100, 250 and 500 nM for sven1998 and 0, 250 and 500 nM for sven4150. G+A indicates the Maxam and Gilbert sequence ladder. The protected regions are indicated by dotted lines and the positions of the MEME-predicted Group I binding motif are shown.

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Figure 2. BldM binds to the MEME-predicted Group-I consensus sequence. A. The MEME-predicted palindromic Group-I consensus sequence. 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. B. DNase I footprinting analysis of BldM on two Group-I promoters: BldM and WhiI interact in Streptomyces

To confirm and extend the BACTH analysis, we tested the interaction of BldM and WhiI in Streptomyces by coimmunoprecipitation. A C-terminally 3xFLAG-tagged allele of bldM (BldM-TF) was constructed such that it was expressed from its native promoter and cloned into the integrative vector pMS82 [22]. This construct complemented the phenotype of the \textit{AbldM} null mutant to wild-type levels of sporulation (Figure S3). This strain was used in conjunction with the \textit{AwhiI} mutant complemented with the N-terminally 3xFLAG-tagged allele of \textit{whiI} (TF-WhiI) described above. The BldM-TF and TF-WhiI proteins were immunoprecipitated directly from 16 h MYM liquid cultures using the M2 anti-FLAG monoclonal antibody. WhiI coimmunoprecipitated with BldM-TF and BldM coimmunoprecipitated with TF-WhiI, but neither was detected in the negative controls when the wild-type strain was used (Figure S4). Thus BldM and WhiI interact in Streptomyces \textit{in vivo}.

Co-expression of BldM rescues WhiI from inclusion bodies

An early frustration in the \textit{in vitro} analysis of WhiI function was that it overexpressed in an insoluble form in \textit{E. coli} under all conditions tested. The realization that WhiI might act as part of a functional BldM-WhiI heterodimer led us to try an alternative approach. Where proteins form a complex, it is often observed that individual components are insoluble when expressed in isolation but become soluble when expressed with their cognate partner protein. Two examples are the \(\sigma^{\text{A}}\) and \(\sigma^{\text{B}}\) subunits of lambda integrase [24], and \textit{Streptomyces} \(\sigma^{\text{RBBN}}\) and its cognate anti-sigma factor, RsbN [16]. Accordingly, BldM and WhiI were co-expressed in \textit{E. coli} using the pETDuet-1 system (Novagen). Initially, BldM was N-terminally His\(_6\)-tagged and WhiI was left untagged. Co-expression of BldM was found to solubilize WhiI completely. Further, when His\(_6\)-BldM was purified on a Histag Ni column, WhiI copurified with His\(_6\)-BldM in approximately equal amounts, despite the fact that WhiI was untagged (Figure 5A) (the identity of the two proteins was confirmed by tryptic mass fingerprinting). Thus BldM rescues WhiI from inclusion bodies and the two proteins copurify in approximately stoichiometric amounts via a His\(_6\)-tag present on BldM only.

This approach was extended by coexpressing BldM and WhiI carrying two compatible affinity tags. N-terminally His\(_6\)-tagged BldM and N-terminally StrepII-tagged WhiI were co-expressed from the pETDuet-1 vector. As before, both BldM and WhiI were found in the soluble fraction. The BldM-WhiI complex was then
purified over consecutive HisTrap Ni and StrepTrap HP affinity columns and the BldM and WhiI proteins were found to be present in stoichiometric amounts in the resulting preparation (Figure 5B).

In silico analysis of Group II promoters
To further understand Group II binding sites, we searched for over-represented sequences in Group II promoter regions using MEME [21], again using as input the entire intergenic region in each case. This analysis revealed a well-conserved 16 bp non-palindromic sequence, 5'-TGnnCCGnnCGGGTGA-3', for which the sequence logo is shown in Figure 6A. Strikingly, the 3' half of the Group II logo was equivalent to a half-site of the Group I palindrome, but the other half was different in sequence, potentially consistent with a BldM-WhiI heterodimer binding to Group II targets.

BldM-WhiI binds to the MEME-predicted motifs in Group II promoters but BldM or GST-WhiI do not
To directly test the model that Group II promoters are controlled by a functional BldM-WhiI heterodimer, and to validate the MEME-predicted binding motif for these promoters, the doubly-tagged BldM-WhiI that had been purified over consecutive HisTrap Ni and StrepTrap HP affinity columns was used in DNase I footprinting analysis on the intergenic regions upstream of two Group II targets, sven1263 and murA2 (sven5810). BldM-WhiI footprinted on both promoters and in each case the protection region contained a copy of the non-palindromic, MEME-predicted binding site (Figure 6B), consistent with this sequence serving as a high-affinity binding site for a BldM-WhiI heterodimer. In contrast, neither BldM alone nor WhiI (produced as a soluble GST fusion), footprinted on either promoter (Figure 6B).

Discussion
The focus of this study is to elucidate the mechanism underlying the direct co-activation by BldM and WhiI of the Group II genes, required for the late stages of development. Our data show that BldM activates transcription of these Group II genes as a BldM-WhiI heterodimer, while activating transcription of the Group I genes required for the early stages of development as a BldM homodimer. This work also significantly expands our knowledge of the regulatory network that controls morphological differentiation in Streptomyces (Figure 7), an advance made possible by exploiting S. venezuelae as a new model species for the genus.

Group I genes
BldM homodimer activates several genes known to play key roles in the differentiation of aerial hyphae into spores, including whiB and ssgR. In addition to their positive regulation by BldM homodimer, these genes are also subject to repression during vegetative growth by the master regulator BldD, as is bldM itself (Figure 7) [25].
SsgA and SsgB are homologous proteins directly involved in the positive control of cell division in Streptomyces [26]. Sporogenic aerial hyphae undergo a synchronous round of cell division, initiated by the polymerization of a ladder of 50 or more FtsZ rings. SsgA and SsgB function in the recruitment and accurate positioning of these FtsZ rings, and AaggA and AaggB mutants of S. coelicolor lack sporulation septa [26–28]. ssgR encodes an IclR-family transcriptional regulator that directly activates the expression of ssgA in S. coelicolor [29]. Here we show that ssgR is directly activated by, and is completely dependent on BldM in S. venezuelae. ssgB is developmentally induced in S. venezuelae, but despite being a direct BldM target, its expression is only weakly affected in the AblΔM mutant, suggesting complex regulation of this gene.

BldM homodimer also activates expression of whiB, which plays a vital role in developmentally controlled cell division. whiB null mutants fail to arrest aerial tip growth, the normal prelude to sporulation, and are completely blocked in the initiation of sporulation septation, producing abnormally long, undivided aerial hyphae [30]. WhiB is the founding member of a family of proteins confined to the actinomycetes, and several of these WhiB-like (Whi) proteins have been shown to play key roles in the biology of streptomyces and mycobacteria. Whi proteins carry a [4Fe–4S] iron-sulfur cluster coordinated by four invariant cysteines in a C(X29)C(X2)C(X5)C motif [31–35], and although the biochemical role of these unusual proteins has been controversial [36], it seems increasingly certain that they function as transcription factors [33,37–38].

**Group II genes**

Among the Group II targets controlled by the BldM-Whi heterodimer are two loci with well-characterized roles in sporulation: smeA-sffA and whiE. The smeA-sffA operon encodes a DNA translocase (SffA) involved in chromosome segregation into spores that is specifically targeted to sporulation septa by the small membrane protein SmcA [39]. Deletion of smeA-sffA in S. coelicolor results in a defect in spore chromosome segregation and has pleiotropic effects on spore maturation [39]. Like the Group I targets ssgR and whiB, expression of the smeA-sffA operon is also repressed by the master regulator BldM during vegetative growth [25]. whiE is a complex locus that specifies the spore pigment. The structure of the spore pigment has not been determined in any Streptomyces species but its polypeptide nature was first predicted from the sequence of the whiE locus in S. coelicolor, because it encodes proteins that closely resemble the components of type II polyketide synthase involved in the synthesis of aromatic antibiotics [40–42]. Based on their coordinate regulation and proposed functions, we predict the whiE locus of S. venezuelae consists of an operon of seven genes (sven6798–6792) and the divergently transcribed gene sven6799. Two distinct ChIP-Seq peaks were seen in the intergenic region separating sven6799 and the sven6798–6792 operon, and all eight genes fail to be expressed in the bldM and whiI mutants, implying the BldM-Whi heterodimer controls expression of the entire locus.

All Whi-regulated genes belong to Group II

The work presented here suggests that there is no set of genes regulated by a Whi homodimer and that Whi functions as an auxiliary protein to modulate BldM binding specificity through heterodimerization. With no exceptions, all the genes down regulated in a AwhiI mutant were also down regulated in a AblΔM mutant. Although some promoters were exclusively enriched as peaks in the Whi ChIP-Seq experiment, without exception the transcriptional profile of such targets was unaffected in a AwhiI mutant, showing that Whi has no regulatory influence on these genes. Further, in a bldM null mutant, some Whi peaks are seen in ChIP-Seq, but these sites, often internal to ORFs, show no correlation with the wild-type Whi regulon and the targets lacked a consensus binding motif. These results suggest that, in the absence of BldM, any DNA binding by Whi is aberrant and unrelated to its behavior in the wild type. In contrast, in a AwhiI mutant, BldM fails to bind to Group II promoters but binds normally to its Group I promoters.

In a recent study, evidence was presented that the DNA-binding domain of S. coelicolor WhiI (in the absence of the receiver domain) can bind in vitro to the promoter of the sco3900-sco3899 operon encoding a transcriptional regulator (InoR) and an inositol-1-phosphate synthase (InoA), respectively [43]. Our ChIP-Seq data show that Whi does not regulate these genes in S. venezuelae. Further, in wild-type S. venezuelae both genes are actively expressed during vegetative growth but are downregulated during development, and this expression pattern is unaffected in a whiI mutant.

The potential of response regulator heterodimerization as a regulatory mechanism

Transcription factor heterodimerization can coordinate responses to different cues by integrating signals from distinct regulatory pathways. Although heterodimerization is prevalent as a regulatory mechanism in eukaryotes [44], it is rare in bacteria. Prior to the work described here, the only response regulator reported to heterodimerize with an auxiliary regulator was RcsB.

In Escherichia coli, the typical response regulator RcsB plays a central role in the regulation of capsule synthesis. Once phosphorylated by the histidine kinase RcsD, RcsB directly activates target genes including rpa, osmC, osmB and fbsZ, functioning as a homodimer [45,46]. It also activates exopolysaccharide synthesis genes, required for capsule formation, as a heterodimer with RcsA, which is distantly
related to response regulators (like RcsB, RcsA has a typical LuxR-type C-terminal DNA-binding domain, but its N-terminal domain is not related to typical response-regulator receiver domains). Like WhiI, RcsA appears to lack the capacity to activate genes by itself, and therefore functions solely as a modulator of RcsB binding specificity. RcsA is actively degraded by the Lon protease and in *lon* mutants capsule genes are highly upregulated causing a mucoid phenotype, due to enhanced activation by the stabilized RcsB-RcsA heterodimer [45,46].

The activities of numerous bacterial promoters respond to multiple cues, and there are many examples of promoters that depend on two activators for their activity. Several different regulatory mechanisms underpinning such codendependence have been identified [50,51]. The most widely documented is found at promoters where both activators bind independently, and both activators make independent contacts with RNA polymerase. However, there are rare examples of coactivators that exhibit cooperative binding, such as MelR and CRP at the *E. coli* melAB promoter [52]. There are also examples in which DNA binding by a secondary activator leads to the repositioning of the primary activator from a site where it cannot activate transcription to a site where it can, such as the repositioning of MalT by CRP at the *malK* promoter [53]. Response regulator heterodimer formation provides a new model for coactivation of target genes and the integration of regulatory signals at promoters. BldM-WhiI heterodimer formation serves to integrate signals from two independent pathways (*WhiG-WhiI* and *BldN-BldM*) and it may also function as a timing device, since Group II genes are activated later than Group I genes. Thus the BldM-WhiI system exemplifies the potential of response regulator heterodimer formation as a mechanism to expand the signaling capabilities of bacterial cells.

**Materials and Methods**

**Bacterial strains, plasmids, oligonucleotides and growth conditions**

Bacterial strains and plasmids are listed in Table S3 and the oligonucleotide primers with corresponding restriction sites used in
cloning are listed in Table S4. For microarray and ChIP-Seq experiments, S. venezuelae strains were grown at 30°C in MYM liquid sporulation medium [16] made with 50% tap water and supplemented with 200 μl trace element solution [54] per 100 ml. The phenotypes of mutants and complemented strains were scored after 3–4 days growth on MYM-agar at 30°C. bldM and whiI deletion mutants were constructed by ‘Redirect’ PCR targeting [55] and their chromosomal structures were confirmed by PCR analysis and by Southern hybridization using the parental cosmids as probes.

ChIP (Chromatin immunoprecipitation)-Seq

For each strain, two flasks containing 35 ml of MYM were inoculated with spores (or mycelium in case of the Abl Dahl mutant) to give an OD600 ~0.35 after 8 h of growth. The crosslinking reagent formaldehyde was added to a final concentration of 1% (v/v) to the cultures at 16 h of growth and incubated at 30°C with shaking for 30 min before glycine was added to a final concentration of 125 mM to quench the crosslinking reaction. The samples were incubated at room temperature for 5 min and washed twice in PBS buffer pH 7.4 (Sigma). Mycelial pellets were resuspended in 0.5 ml lysis buffer (10 mM Tris-HCl pH 8, 50 mM NaCl, 15 mg/ml lysozyme, 1x protease inhibitor) and incubated at 37°C for 20 min. The lysate was resuspended in 0.5 ml IP buffer (100 mM Tris- HCl pH 8, 250 mM NaCl, 0.1% Triton-X-100, 1x protease inhibitor) and the lysate was kept on ice for 2 min before sonication. The samples were subjected to seven cycles of sonication, 15 s each, at 10 microns, to shear the chromosome into fragments ranging in size from 300–1000 bp. The sample was then centrifuged twice at top speed, 4°C for 15 minutes to clear cell extracts. To pre-clear non-specific binding, 90 μl protein A sepharose (Sigma) was added to cell lysates (about 900 μl) and incubated for 1 h at 4°C with mixing. The beads were cleared by centrifugation at top speed for 15 min. 100 μl BldM or WhiI antibodies were added to the corresponding cell lysates overnight at 4°C with mixing. 100 μl Protein A Sepharose 1:1 suspension was added to immunoprecipitate antibody-BldM or WhiI chromatin complexes and incubated for 4 h at 4°C with mixing. The samples were centrifuged at 3500 rpm for 30 s and the beads were washed four times with IP buffer. The pellets were eluted in 150 μl IP elution buffer (50 mM Tris-HCl pH 7.6, 10 mM EDTA, 1% SDS) overnight at 65°C to reverse crosslink. The samples were centrifuged at top speed for 5 min to remove the beads and the pellets were re-extracted with 50 μl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA). The supernatants were combined and crosslinking was quenched with 3 μl 10 mg/ml proteinase K (Roche) for 2 h at 55°C. The samples were extracted twice with phenol-chloroform to remove protein followed by chloroform extraction to remove traces of phenol and purified with Qiaquick columns (Qiagen). The IP DNA was eluted in 50 μl EB buffer (Qiagen). Sequencing libraries were generated and the IP DNA was sequenced as described previously [20]. The BayesPeak package was used to identify significantly enriched regions and the default parameters were applied [56].

Microarray transcriptional profiling

Microarray transcriptional profiling experiments were carried out as described previously [16,20]. Multi-experiment viewer software (MeV 4.8) was used for viewing and statistical analysis [57]. The non-parametric tool ‘Rank Products’ [58] was used in MeV to assign ‘down regulated’, ‘up regulated’ and ‘not significant’ genes based on expression at 16, 18 and 20 h of growth. Group I genes were defined as direct BldM ChIP-Seq targets that were significantly down regulated in Abl Dahl and not significantly changed in all Awhi mutants (Table S1). Group II genes were defined as direct ChIP-Seq targets of both BldM and WhiI that were significantly down regulated in Abl Dahl, AwhiG and AwhiI (Table S2).

Protein expression and purification

The open reading frame of interest was PCR-amplified using Expand High-Fidelity DNA polymerase (Roche). Plasmids containing the correct inserts were confirmed by sequencing and introduced into electrocompetent E. coli BL21(DE3)/pLysS. The transformed cells were spread on LB-carbenicillin/chloramphenicol and one colony was used for inoculation. Proteins were expressed in two 2.5 litre volumetric flasks each containing 400 ml LB culture and expression was induced with 0.25 mM IPTG. The optimised temperature for expression varied with the protein: His6-BldM was expressed at 25°C for 5 h; GST-WhiI was expressed at 15°C overnight; His6-BldM/WhiI or S8p-WhiI were co-expressed at 30°C for 5 h. The pellets were lysed in a buffer containing 50 mM Tris-HCl pH 8, 250 mM NaCl, 10% glycerol, 0.1% Triton X100, protease inhibitor (complete mini, EDTA-free,
Roche) and incubated at room temperature for 20 min. HisTrap HP Ni and StreptTrap HP affinity columns (GE Healthcare) were used to purify the His- and Strep-tagged proteins in a tandem manner. The Gst-WhiI was purified with 1 ml GSTrap FF column (GE Healthcare).

**DNase I footprinting**

Single 32P end-labelled probes (Table S4) were generated by PCR and purified using Qiagene columns (Qiagen). Transcription factors were incubated with probe DNA (~150,000 cpm) for 30 min at room temperature in 40 µl reaction buffer [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol, 10 mM MgCl2, 2 mM dithiothreitol and 1 µg/reaction poly(dI-dC)], prior to treatment with 1 U DNase I (Promega) for 30–50 s in the case of group-II promoters and 3 U DNase I for 15–20 s in the case of group-I promoters. Reactions were terminated with 140 µl of stop buffer (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, 70 µg/ml yeast tRNA) and samples were extracted with phenol-chloroform prior to ethanol precipitation. Footprinting samples were loaded on 6% polyacrylamide sequencing gels, next to a G+L ladders prepared according to the Sure Track footprinting kit (Amersham Pharmacia Biotech).

**Immunoprecipitation of BldM-FLAG and FLAG-WhiI proteins from S. venezuelae**

C-terminally 3xFLAG-tagged bldM expressed from the native promoter was used to complement the AbldM mutant and N-terminal 3xFLAG-tagged whiI carrying native promoter was used to complement the AwhiI mutant. 50 µl dense spore suspension was used to inoculate 300 ml MYM in 2 litre flasks with spring baffles. After 17 h growth, cultures were harvested by centrifugation at 3000 rpm for 1 min, washed in 5 ml sporulation medium, and resuspended in 0.05 M Tris, 0.9% NaCl, pH 7.6, 0.1% Tween) overnight and sonicated for 5 cycles at 15-micron amplitude for 20 s. FLAG-tagged proteins were immunoprecipitated using M2 beads [anti-FLAG antibodies covalently attached to agarose beads (Sigma)], the beads were washed using TBS buffer containing protease inhibitor (Roche) and 0.05% Triton X100, and the protein was eluted using FLAG peptides as recommended by the manufacturer.

**Preparation of crude cell extracts and immunoblot analysis**

S. venezuelae strains were grown in MYM medium. 10 ml samples were taken at 14, 15, and 16 hours of growth. Strains were harvested by centrifugation at 3000 rpm for 1 min, washed in 5 ml ice-cold washing buffer (20 mM Tris pH 8.0, 5 mM EDTA) and resuspended in 0.4 ml of ice-cold sonication buffer [20 mM Tris pH 8.0, 5 mM EDTA, 1x protease inhibitor (Roche)]. Samples were sonicated immediately for 5 cycles, 20 s at 10 microns with 1 min intervals of ice incubation, then centrifuged at 13000 rpm at 4°C for 15 min to remove cell debris. Protein concentrations of the supernatant crude cell extracts were measured by Bradford assay and samples (10 µg protein) were separated on a 12.5% SDS-PAGE gel and blotted onto nitrocellulose membrane. The membrane was incubated in blocking solution [10% dried milk powder in TBS (0.05 M Tris, 0.9% NaCl, pH 7.6, 0.1% Tween)] overnight and then incubated for 1 h at room temperature with the 1/2500 dilution of anti-BldM antiserum in blocking solution. The membrane was rinsed twice (10 min) in TBS and then incubated for 1 h with 1/5,000 dilutions of horseradish peroxidase-linked goat anti-rabbit immunoglobulin G antibody (GE Healthcare). Blots were developed using the ECL enhanced chemiluminescence system from GE Healthcare and were typically exposed to X-ray film for between 30 s and 5 min.

**Supporting Information**

**Figure S1** Western blot analysis of BldM levels during differentiation of wild-type S. venezuelae grown in MYM liquid sporulation medium. Polyclonal anti-BldM antibodies were used. (TIF)

**Figure S2** A. Alignment of the whiI promoters of S. coelicolor and S. venezuelae showing conservation of the σ70 promoter-10 and -35 sequences. B. Transcriptional profiles of bldM and whiI during differentiation of wild-type S. venezuelae (WT) and its congenic bldM, whiG and whiI mutants, bldM transcript levels are indicated in blue and whiI transcript levels are indicated in red. Note that transcription of bldM and whiI cannot be detected in their respective null mutants because the coding sequences represented on the microarrays are deleted in those strains. Strains were grown in MYM liquid sporulation medium. (TIF)

**Figure S3** A. Complementation of the AbldM mutant with a C-terminal bldM-3xFLAG allele cloned into the integrative vector pMS82. B. Complementation of the AwhiI mutant with an N-terminal 3xFLAG-whiI allele cloned into the integrative vector pMS82. (TIF)

**Figure S4** Co-immunoprecipitation of BldM and WhiI. A. The AwhiI mutant complemented with the N-terminally 3xFLAG-tagged allele of whiI was grown for 16 h in MYM liquid sporulation medium, and FLAG-WhiI was immunoprecipitated using M2 antibody. Polyclonal BldM antibody was used to detect the presence of BldM, and immunoprecipitates from WT and BldM-FLAG strains were used as negative and positive controls, respectively. B. The AbldM mutant complemented with the C-terminally 3xFLAG-tagged allele of bldM was grown for 16 h in MYM liquid sporulation medium and BldM-FLAG was immunoprecipitated as described above. Polyclonal WhiI antibody was used to detect the presence of WhiI, and immunoprecipitates from WT and FLAG-WhiI strains were used as negative and positive controls, respectively. (TIF)

**Table S1** List of Group I genes describing peak positions, annotations, microarray fold change and p values for expression in the AbldM and AwhiI mutant backgrounds. (XLSX)

**Table S2** List of Group II genes describing peak positions, annotations, microarray fold change and p values for expression in the AbldM and AwhiI mutant backgrounds. The presence or absence of peaks in the corresponding FLAG-WhiI ChIP-Seq experiment is also shown. (XLSX)

**Table S3** Strains and plasmids used in this study. (DOCX)

**Table S4** Oligonucleotides used for cloning and DNase I footprinting. Restriction sites in the primers are shown. (XLSX)

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Author Contributions
Conceived and designed the experiments: MMA MJBu MJBut. Performed the experiments: MMA MJBu. Analyzed the data: MMA MJBu.

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