

σ^{BldN} , an Extracytoplasmic Function RNA Polymerase Sigma Factor Required for Aerial Mycelium Formation in *Streptomyces coelicolor* A3(2)

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Received 3 April 2000/Accepted 15 May 2000

Sporulation mutants of *Streptomyces coelicolor* appear white because they are defective in the synthesis of the gray polyketide spore pigment, and such white (*whi*) mutants have been used to define 13 sporulation loci. *whiN*, one of five new *whi* loci identified in a recent screen of NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine)-induced *whi* strains (N. J. Ryding et al., *J. Bacteriol.* 181:5419–5425, 1999), was defined by two mutants, R112 and R650. R650 produced frequent spores that were longer than those of the wild type. In contrast, R112 produced long, straight, undifferentiated hyphae, although rare spore chains were observed, sometimes showing highly irregular septum placement. Subcloning and sequencing showed that *whiN* encodes a member of the extracytoplasmic function subfamily of RNA polymerase sigma factors and that the sigma factor has an unusual N-terminal extension of approximately 86 residues that is not present in other sigma factors. A constructed *whiN* null mutant failed to form aerial mycelium (the “bald” phenotype) and, as a consequence, *whiN* was renamed *bldN*. This observation was not totally unexpected because, on some media, the R112 point mutant produced substantially less aerial mycelium than its parent, M145. The *bldN* null mutant did not fit simply into the extracellular signaling cascade proposed for *S. coelicolor* *bld* mutants. Expression of *bldN* was analyzed during colony development in wild-type and aerial mycelium-deficient *bld* strains. *bldN* was transcribed from a single promoter, *bldNp*. *bldN* transcription was developmentally regulated, commencing approximately at the time of aerial mycelium formation, and depended on *bldG* and *bldH*, but not on *bldA*, *bldB*, *bldC*, *bldF*, *bldK*, or *bldJ* or on *bldN* itself. Transcription from the *p1* promoter of the response-regulator gene *bldM* depended on *bldN* in vivo, and the *bldMp1* promoter was shown to be a direct biochemical target for σ^{BldN} holoenzyme in vitro.

Filamentous bacteria of the genus *Streptomyces* have a complex developmental cycle. At the start of differentiation, an aerial mycelium is formed, consisting of hyphae that grow out of the aqueous environment of the substrate mycelium into the air. These multigenomic aerial hyphae, which give the developing colonies a characteristic fuzzy appearance, subsequently differentiate to form chains of exospores (8, 20).

The isolation of *bld* mutants that lack aerial mycelium and, therefore, have a shiny, “bald” appearance has facilitated study of the development of aerial hyphae in *Streptomyces coelicolor*. Unlike a second class of developmental mutations (*whi*), which appear only to affect the differentiation of aerial hyphae into spores, *bld* mutations have pleiotropic effects which often cause defects in carbon catabolite repression and in cell-cell signaling and sometimes cause loss of antibiotic production, in addition to blocking differentiation (6, 20, 31, 38, 43, 53).

The behavior of *bld* mutants on different media suggests that aerial mycelium formation can occur by at least two different pathways. The aerial mycelium of *S. coelicolor*, when grown on rich media such as R2YE, is associated with the production of a small, hydrophobic peptide called SapB, a putative morphogen that coats the surface of aerial hyphae (52). SapB is a surfactant that allows aerial hyphae to break the surface tension of the aqueous environment of the substrate mycelium and grow into the air (51). On R2YE, SapB production and aerial mycelium formation depend on *bldA*, *bldB*, *bldC*, *bldD*,

bldF, *bldG*, *bldH*, *bldI*, *bldJ* (formerly *bld261* [38]), and *bldK*. However, aerial mycelium formation in these mutants can be restored by the exogenous addition of purified SapB protein or by growing them close to SapB-producing colonies (52). But, via a second pathway, aerial mycelium formation and sporulation can be restored to most *bld* mutants (an exception being *bldB*) simply by growing them on a minimal medium containing mannitol as the sole carbon source: conditions where no SapB is detectable (52).

A complex extracellular signaling cascade has been proposed to initiate the formation of aerial hyphae in *S. coelicolor* grown on rich media (36, 37, 38, 53). When some pairs of *bld* mutants are grown on R2YE in close proximity to each other, one mutant induces the other both to synthesize SapB and to erect aerial hyphae and sporulate. This “extracellular complementation” is always unidirectional, with one *bld* mutant acting as a “donor” and the other as a “recipient.” Experiments with the whole range of *bld* mutants showed that most could be arranged into the following hierarchy, in which each mutant can rescue the developmental defect in all the mutants to the left, but not to the right:

bldJ < *bldK*, *bldL* < *bldA*, *bldH* < *bldG* < *bldC* < *bldD*, *bldM*

These data have led to a model in which aerial mycelium formation on rich media is initiated by a signaling cascade involving at least five different extracellular signals. Each signal causes the synthesis and/or release of the next signal, eventually causing the *bldD*-dependent production of SapB, and perhaps other morphogens, which allow aerial hyphae to overcome surface tension and grow into the air (36, 37, 38, 53). To date, there is biochemical evidence only for one of these pu-

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TABLE 1. Derivatives of *S. coelicolor* A3(2) used in this study

Strain	Genotype	Source or reference
J1915	$\Delta glkA119$ SCP1 ⁻ SCP2 ⁻	21
J2177	$\Delta glkA119$ <i>bldN::hyg</i> SCP1 ⁻ SCP2 ⁻	This work
R112	<i>bldN112</i> SCP1 ⁻ SCP2 ⁻	46
R650	<i>bldN650</i> SCP1 ⁻ SCP2 ⁻	46
J1700	<i>bldA39 hisA1 uraA1 strA1</i> SCP1 ⁻ SCP2 ⁻	24
J669	<i>bldB43 mthB2 cysD18 agaA7</i> SCP1 ^{NF} SCP2 [*]	31
J660	<i>bldC18 mthB2 cysD18 agaA7</i> SCP1 ^{NF} SCP2 [*]	31
J774	<i>bldD53 cysA15 pheA1 mthB2 strA1</i> SCP1 ^{NF} SCP2 [*]	31
161	<i>bldF hisD3 pheA1 strA1</i> SCP1 ⁺ SCP2 ⁺	44
J1501	<i>hisA1 uraA1 strA1 Pgl⁻</i> SCP1 ⁻ SCP2 ⁻	9
WC103	<i>bldG103 hisA1 uraA1 strA1 Pgl⁻</i> SCP1 ⁻ SCP2 ⁻	6
WC109	<i>bldH109 hisA1 uraA1 strA1 Pgl⁻</i> SCP1 ⁻ SCP2 ⁻	6
HU261	<i>bldJ261 hisA1 uraA1 strA1 Pgl⁻</i> SCP1 ^{NF} SCP2 [*]	53
NS17	<i>bldK::aadA</i> SCP1 ⁻ SCP2 ⁻	37

tative extracellular signaling molecules: a covalently modified oligopeptide has been identified that rescues the mutant phenotype of the *bldJ* mutant in a *bldK*-dependent manner (36).

whiN is one of five new *whi* loci identified after NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) mutagenesis and was defined by two mutants, R112 and R650, which had strikingly different phenotypes (46). On minimal medium, colonies of R650 were medium gray and produced frequent spores that were longer than those of the wild type. In contrast, colonies of R112 were white and produced long, straight, undifferentiated hyphae, although rare spore chains were observed, sometimes showing highly irregular septum placement. The R112 mutation also showed clear signs of pleiotropic effects; in addition to the defects in sporulation, on some media R112 produced substantially less aerial mycelium than did the parental strain M145.

Here we show that *WhiN* is a member of the extracytoplasmic function (ECF) subfamily of RNA polymerase sigma factors and that it has an unusual *N*-terminal extension of approximately 86 residues that is not found in other sigma factors. We show that a constructed *whiN* null mutant is bald and, as a consequence, *whiN* was renamed *bldN*. *bldN* did not fit simply into the extracellular signaling cascade proposed for *S. coelicolor* (53). We show that *bldN* transcription is developmentally regulated, commencing approximately at the time of aerial mycelium formation, and that *bldN* transcription depends on *bldG* and *bldH*. Finally, we identify the *p1* promoter of the response-regulator gene *bldM* as a direct biochemical target for σ^{BldN} holoenzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and conjugal plasmid transfer from *Escherichia coli* to *Streptomyces* spp. The *S. coelicolor* strains used here are summarized in Table 1 and were cultured on R2YE, minimal medium (MM) containing 0.5% (wt/vol) mannitol as a carbon source, or MS (mannitol plus soya flour) agar (23). To bypass the methyl-specific restriction system of *S. coelicolor*, unmethylated plasmids were transferred by conjugation from the *dam dem hsdS* *E. coli* strain ET12567 (30) as described by Ryding et al. (46). The plasmids used were pSET152 (3) and pIJ6650, a derivative of pKC1132 that has a 1.3-kb *glkA* fragment inserted at the *Bgl*II site (23).

Sequencing. The nucleotide sequence of the 2.07-kb *BspEI-XbaI* fragment carrying *whiN/bldN* has been deposited in the DDBJ, EMBL, and GenBank databases under accession number AJ010584. The mutant alleles that originally defined the *whiN* locus were amplified from the chromosomes of R650 and R112 by PCR and sequenced directly without cloning. The oligonucleotides used were 5'-AGCGCGTCTCTCTCGTCCGG-3' and 5'-GTCAACGGACTTTCACCA GTG-3' for the coding region and 5'-CTGACCGCCGCCGCTTGAC-3' and 5'-CGGACGAGGACGACCGCTCG-3' for the upstream sequence. PCR reaction conditions were 20 cycles of 96°C for 30 s, 62°C for 50 s, and 72°C for 50 s, followed by an extension reaction at 72°C for 10 min. The reaction was performed in 100 μ l 1 \times PCR buffer (Boehringer Mannheim) containing 200 μ M

concentrations of each of the four deoxynucleoside triphosphates, 5% (vol/vol) dimethyl sulfoxide, 50 pmol of each primer, and 50 ng of chromosomal DNA.

Construction of a *whiN* null mutant. A *whiN* null mutant derivative of J1915, a plasmid-free, $\Delta glkA$ derivative of the wild-type strain, was constructed using the method of Buttner et al. (5). This method makes use of the counterselectable glucose kinase gene (*glkA*), which allows a positive selection to be made for gene replacement, provided that the mutations are constructed in a strain carrying a deletion of *glkA*.

A 4.05-kb *Aat*II fragment, isolated from pIJ6706, was cloned into pUC19 digested with *Aat*II to give pIJ6718, and a 1.8-kb *hyg* cassette (56) was blunt-end cloned into pIJ6718 as a replacement for a 66-bp *Xho*I fragment internal to *whiN*, yielding pIJ6719. The constructed *whiN::hyg* null mutant allele was removed from pIJ6719 as a 6.1-kb *SspI-NdeI* fragment and blunt-end cloned into the counterselectable delivery vector pIJ6650 digested with *EcoRV* to give pIJ6720.

pIJ6720 was introduced into *S. coelicolor* J1915 ($\Delta glkA119$) by mating from *E. coli* and exconjugants in which the plasmid had presumptively integrated at the *whiN* locus by single crossover homologous recombination were selected with apramycin. Because the two intervals flanking the *hyg* cassette in pIJ6720 were of unequal size (2.75 and 1.3 kb), 12 apramycin-resistant colonies were screened using Southern hybridization to look for isolates in which the first crossover had occurred in the smaller, 1.3-kb interval; one such strain was identified (J2176). After two rounds of nonselective growth of J2176, isolates from which the delivery plasmid had been lost were selected on MM containing 100 mM 2-deoxyglucose. These 2-deoxyglucose-resistant isolates were a mixture of bald and sporulating colonies. Southern hybridization showed that in the bald colonies the plasmid had excised leaving the *whiN::hyg* mutant allele in the chromosome, and that in the sporulating colonies the plasmid had excised leaving the wild-type *whiN* gene in the chromosome; a representative *whiN* null mutant was designated J2177. (Note that the level of hygromycin resistance conferred by *whiN::hyg* was too low to permit effective selection of the null mutant allele in these experiments.)

Isolation of RNA and S1 nuclease mapping. For RNA preparation, *S. coelicolor* strains were grown on cellophane disks on R2YE medium, and RNA was extracted as described by Kelemen et al. (22). For each S1 nuclease reaction, 30 μ g of RNA was hybridized to a ³²P-end-labeled probe at 45°C for 3 to 15 h, following denaturation at 65°C for 15 min. S1 nuclease (Boehringer) digestions and analysis of RNA-protected fragments were performed as described by Kieser et al. (23). Uniquely end-labeled probes were generated by PCR as follows. For low-resolution S1 nuclease mapping of *bldN*, the oligonucleotide 5'-GACGAG GACGACCGCGCTCGTC-3' was 5' end labeled using [γ -³²P]ATP and T4 polynucleotide kinase (Pharmacia) and used in a PCR reaction with unlabeled universal primer and pIJ6714 as the template, generating a 1,070-bp product. The 904-bp probe used for high-resolution S1 nuclease mapping of *bldN* was generated in the same way but using the radiolabeled oligonucleotide 5'-AGC CAGGCCCGAGGCGTCAAC-3'. The 1.168-kb *bldM* probe was generated using the radiolabeled oligonucleotide 5'-GGCTGGTACGAAATCGTCAC-3', unlabeled universal primer, and pIJ6626 (34) as a template. The 343-bp *hrdB* probe was generated using the radiolabeled oligonucleotide 5'-GCCATGACA GAGACGGACTCGGCG-3', the unlabeled oligonucleotide 5'-CGGCCGCAA GGTACGCGTTGATGA-3', and pIJ2034 (5) as the template. Sequencing ladders for high-resolution mapping were generated with the *Taq* Track kit (Promega) and the same oligonucleotide that was used to generate the S1 nuclease mapping probe.

Overproduction and purification of σ^{BldN} . A 1-kb *ApaI-Bam*HI fragment carrying most of the *bldN* gene but lacking the 5' end was isolated from pIJ6715 and cloned into pRSET (Invitrogen) digested with *NdeI* and *Bam*HI using two complementary adapter oligonucleotides 5'-TATGATGGAAGTGGTTGAAC GGGCC-3' and 5'-CGTTCAACCAGTTCATCA-3'. These oligonucleotides introduced an *NdeI* restriction site at the 5' end of the *bldN* gene and replaced

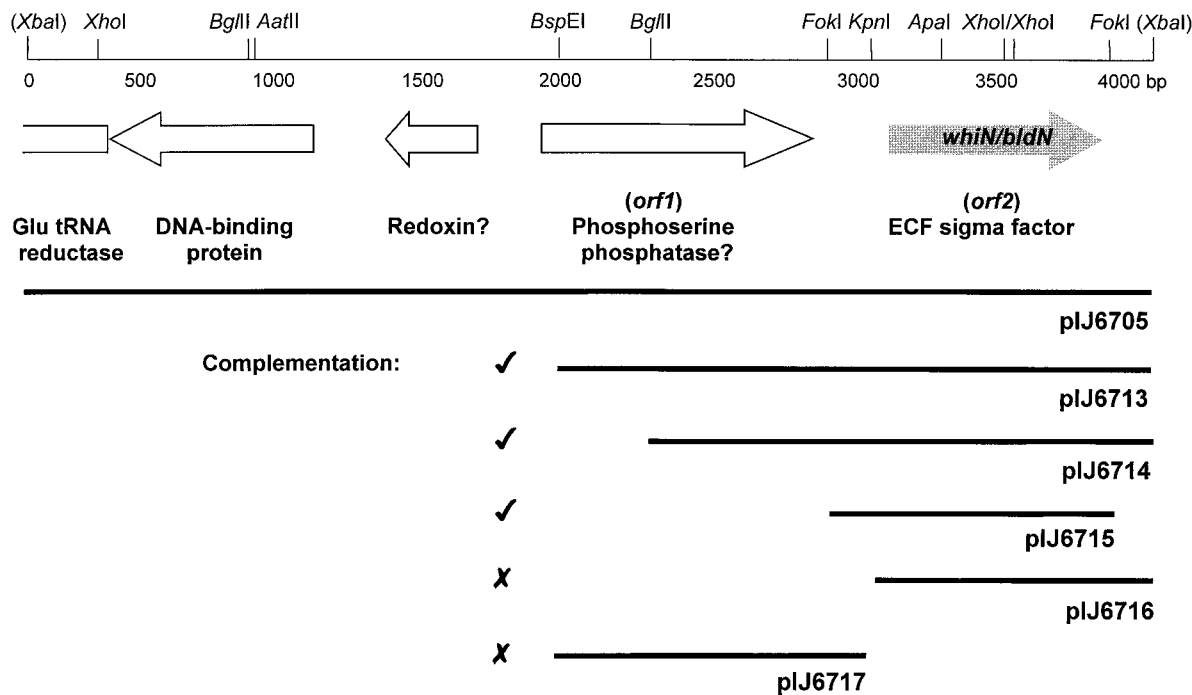


FIG. 1. Genetic organization of the 4.1-kb DNA fragment carrying *whiN/bldN*. The positions of the protein coding regions are indicated by arrows, and restriction sites referred to in the text are marked. The extent of the subclones described in the text and their ability to complement the *whiN* mutants R112 and R650 are shown below. *XbaI* sites are derived from the cloning vector, pIJ6798. The DNA sequence of the lefthand 2-kb *XbaI*-*BspEI* fragment is taken from the ongoing *S. coelicolor* genome sequence (accession number AL079345).

the third, fifth, and sixth codons with synonymous codons commonly associated with genes expressed at high levels in *E. coli*. The cassette replacement was verified by sequencing the resulting plasmid, pIJ6721. Because of low expression levels, pIJ6721 was subsequently modified using PCR-based site-directed mutagenesis to replace the seventh codon, CGG, which is also rare in *E. coli*, with CGT. In this method two abutting oligonucleotides were used to amplify the entire pIJ6721 plasmid, simultaneously introducing the single-base-pair change. The two oligonucleotides were 5'-GCCAGGCCGCGAGGCCGAC-3' and 5'-ACGTTCAACCAGTTCATCAT-3', and the PCR program was 10 cycles of 1 min at 96°C, 45 s at 60°C, and 7 min at 72°C, followed by 10 cycles of 1 min at 96°C, 45 s at 60°C, and 10.5 min at 72°C, with a final incubation at 72°C for 15 min. The reaction was performed in 50 μ l of 1 \times PCR buffer (Promega) containing 200 μ M concentrations each of the four deoxynucleoside triphosphates, 10% (vol/vol) glycerol, 50 pmol of each primer, 10 ng of template DNA, and 2.5 U of high-fidelity *Pfu* DNA polymerase (Promega). The PCR product was self-ligated to create pIJ6722, and the resulting *bldN* allele was sequenced over its entire length to ensure that only the desired mutation had been introduced.

pIJ6722 was introduced into *E. coli* BL21 Δ DE3(pLysS) (50), and *bldN* expression was induced in exponentially growing cells (optical density 0.5 at 600 nm) by the addition of 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG), followed by a further 3 h of growth.

σ^{bldN} was recovered from inclusion bodies by a minor modification of the method of Nguyen et al. (35). The cell pellet was resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, 1 mM dithiothreitol [DTT], 50 mM NaCl, 0.2% [wt/vol] sodium deoxycholate [NaDOC], 5% [vol/vol] glycerol, 200 μ g of lysozyme per ml) and incubated on ice for 30 min before lysis was completed by three 30-s cycles of sonication. The cell lysate was centrifuged for 20 min at 15,000 rpm in an SS34 rotor, and the supernatant was discarded. The inclusion bodies were purified by resuspension in 20 ml of wash buffer, which was TGED (50 mM Tris-HCl, pH 8.0; 5% [vol/vol] glycerol; 0.1 mM EDTA, 0.1 mM DTT) containing 50 mM NaCl and 2% (wt/vol) NaDOC, followed by stirring at 4°C for 1 h and repeated sonication as before. The inclusion bodies were recovered by centrifugation, and the washing procedure was repeated once again. The purified inclusion bodies were solubilized by resuspension in 20 ml of solubilisation buffer (TGED containing 50 mM NaCl and 0.25 [wt/vol] Sarkosyl [*N*-lauroylsarcosine]) and stirred for 1 h at 4°C. The solubilized material was dialyzed for at least 24 h against 2 liters of TGED containing 50 mM NaCl, with several changes of buffer to attempt the complete removal of Sarkosyl, followed by centrifugation and filtration through a 0.2- μ m (pore size) cellulose acetate filter (Sartorius GmbH). Finally, the purified protein was dialyzed against TGED containing 50 mM NaCl and 50% (vol/vol) glycerol before storage at -20°C.

In vitro transcription. Runoff transcription assays were performed using [α -³²P]CTP (New England Nuclear) at 600 Ci/mmol as described by Buttner et al. (4). Transcription from the *bldMpl* promoter region was assayed using two PCR-derived templates of 1.194 kb (template 1) and 1.168 kb (template 2), generated using the template pIJ6626 (31), the universal primer, and the *bldM*-specific oligonucleotide primers 5'-CTCTTGC GCGTCACGTTGAGC-3' (template 1) and 5'-GGCTGGTACGAAATCGTCAC-3' (template 2). Transcripts were analyzed on 6% polyacrylamide-7 M urea gels using a heat-denatured, ³²P-labeled *HinFI* digest of Φ X174 as the size standards. *E. coli* core RNA polymerase was purchased from Epicentre Technologies (Madison, Wis.).

Scanning electron microscopy. Scanning electron microscopy was performed as described by Ryding et al. (46).

RESULTS

***whiN* encodes an extracytoplasmic function RNA polymerase sigma factor.** We previously isolated two SCP2*-based clones, pIJ6705 and pIJ6706, that complemented the two NTG-generated *whiN* mutants R112 and R650 (46). Further analysis showed that pIJ6705 and pIJ6706 carried 4- and 9-kb inserts, respectively, and that the two inserts overlapped. Subcloning from the 4-kb insert of pIJ6705 into the conjugative vector pSET152, which integrates site specifically into the *S. coelicolor* chromosome at the phage Φ C31 *attB* site, identified a 2.07-kb *BspEI*-*XbaI* fragment that restored wild-type levels of sporulation to both mutants (pIJ6713; Fig. 1). The nucleotide sequence of this 2.07-kb fragment was determined and one partial (*orf1*; Fig. 1) and one complete (*orf2*; Fig. 1) protein-coding sequence were identified with the aid of the FRAME program (2). The incomplete coding sequence, *orf1*, encoded the 266 C-terminal residues of a protein showing 25% identity to *E. coli* phosphoserine phosphatase. When the unique *KpnI* site lying in the noncoding region between *orf1* and *orf2* was used to generate two 1-kb subclones, pIJ6716 and pIJ6717 (Fig. 1), neither subclone complemented the *whiN* mutants

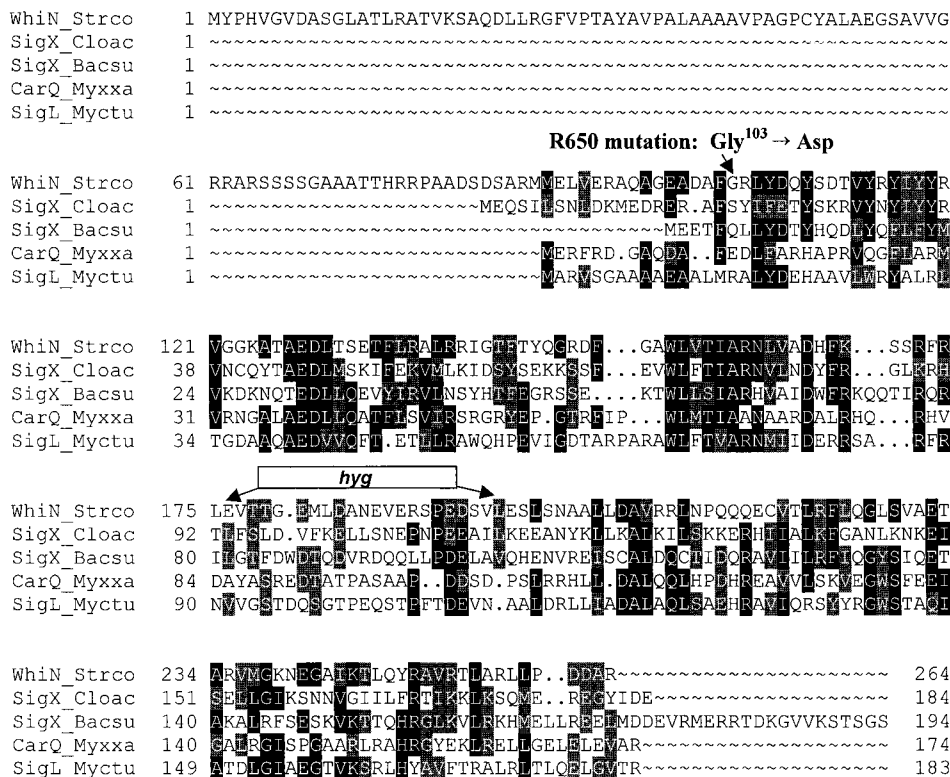


FIG. 2. Amino acid alignment of WhiN/BldN with other members of the ECF subfamily of sigma factors. The proteins and their corresponding amino acid sequence accession numbers are WhiN_Strco, *S. coelicolor* WhiN (CAB55345); SigX_Cloac, *Clostridium acetobutylicum* SigX (AAC12856); SigX_Bacsu, *B. subtilis* SigX (P35165); CarQ_Myxxa, *M. xanthus* CarQ (S39877) and SigL_Myctu, *Mycobacterium tuberculosis* SigL (CAA17502). The amino acid substitution carried by the *whiN* point mutant, R650, and the location of the *hyg* cassette (resulting from the replacement of a 66-bp *XhoI* fragment) in the *whiN::hyg* null mutant are shown.

(for an explanation of this result, see the section below on promoter mapping). However, a slightly larger *FokI* fragment (pIJ6715; Fig. 1) carrying all of *orf2* and more sequence upstream of the *KpnI* site fully complemented both *whiN* mutants. As a consequence, *orf2* was designated *whiN*.

Global similarity searches of the EMBL databases showed that WhiN is a member of the ECF subfamily of RNA polymerase sigma factors (27, 32), showing, for example, 27% identity to *Bacillus subtilis* σ^X (14, 15), and 30% identity to *Myxococcus xanthus* σ^{CarQ} (11, 29) (Fig. 2). The *whiN* start codon cannot be unambiguously assigned from FRAME analysis and sequence inspection. There are potential GTG start codons at positions 133 and 262 and two adjacent potential ATG start codons at positions 391 and 394 (Fig. 3B and 4A). Of these, only the upstream GTG codon at position 133 is preceded by an appropriately positioned potential ribosome binding site (GGAG). The discovery that the NTG-induced mutation in the more severe *whiN* mutant, R112, is a change in this putative ribosome binding site (Fig. 3B; see below) strongly implicates GTG-133 as an in vivo translation start codon. The sigma factor arising from translation initiation at GTG-133 would carry an unusual N-terminal extension of approximately 86 amino acids that is not present in other sigma factors (Fig. 2). The DNA encoding this extension has somewhat unusual codon usage, as can be seen from the *whiN* FRAME plot (Fig. 4A). Analysis of this extension using the TMPRED program (European Molecular Biology Network-Swiss node, http://www.ch.embnet.org/software/TMPRED_form.html) (Fig. 4B) revealed a stretch of 20 hydrophobic amino acids (YAVPALAAAAPAGPCYALA) from positions 34 to 53 (Fig. 3B) that

might cause WhiN to interact with the membrane. If translation initiation were also to occur from either of the adjacent ATG start codons at positions 391 and 394, it would give rise to a protein lacking the unusual extension that would be approximately co-N-terminal with most other members of the ECF subfamily of sigma factors (Fig. 2 and 3B).

The NTG-induced *whiN* mutations affect region 2.1 and the putative ribosome binding site. The two *whiN* alleles that originally defined the locus were amplified from the chromosomes of R650 and R112 and sequenced. The “weak” mutant, R650 (Fig. 3A), carries a GGC to GAC change, giving rise to a glycine to aspartate (G103D) substitution in region 2.1 of WhiN (Fig. 2 and 3B). In other sigma factors, region 2.1 has been implicated in the interaction with core RNA polymerase. Deletion of region 2.1 in σ^{70} and σ^{32} of *E. coli* (25, 26) and point mutations in region 2.1 of *E. coli* σ^{70} (47) and *B. subtilis* σ^E (47) all reduce binding of the sigma factor to core RNA polymerase. In addition, the RAP30 subunit of the human heteromeric general transcription initiation factor RAP30/74 contains a region that is similar in amino acid sequence to region 2.1 of bacterial sigma factors, and RAP30/74 binds *E. coli* core RNA polymerase and is displaced by σ^{70} (28).

The more “severe” mutant, R112 (Fig. 3A), has a wild-type coding sequence but carries a GGAG to GGAA change in the putative ribosome binding site upstream of GTG-133, reducing complementarity to the 3' end of the 16S rRNA to 3 bp (Fig. 3B). Presumably, as a consequence, *whiN* mRNA is poorly translated in R112. Unlike the other *whi* mutants described by Chater (7) and Ryding et al. (46), R112 frequently throws off colonies that sporulate much more efficiently than R112 itself.

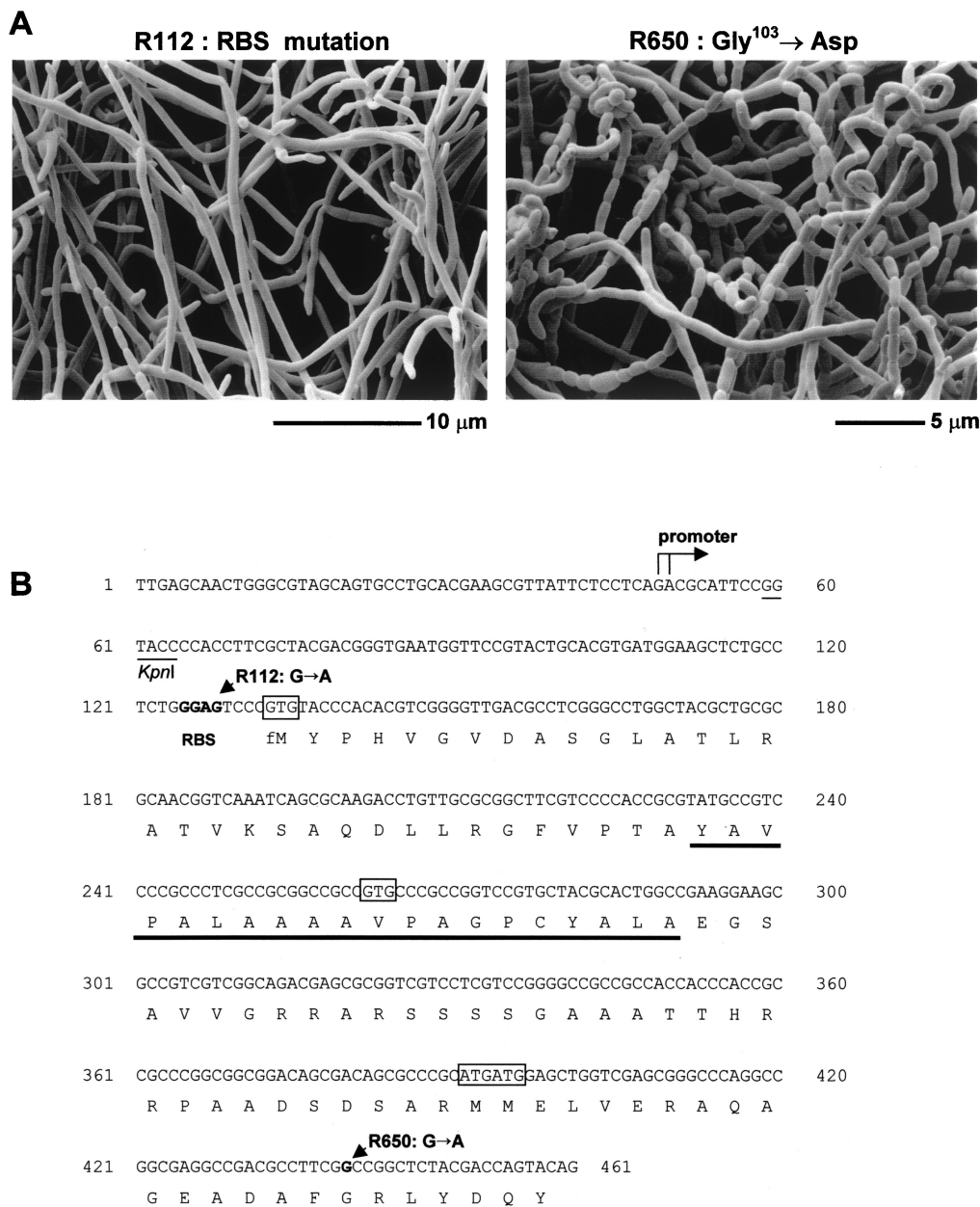


FIG. 3. (A) Scanning electron micrographs showing the phenotypes of the *whiN/bldN* point mutants, R112 and R650. (B) Nucleotide sequence of the promoter region and the 5' end of the *whiN/bldN* gene showing the transcription start point, the putative ribosome binding site (RBS), the potential start codons (boxed), the nucleotide substitutions carried by the *whiN* point mutants, R112 and R650, and the *KpnI* site and the stretch of hydrophobic residues (underlined) discussed in the text.

Perhaps these isolates contain suppressor mutations that affect the ribosome and allow increased translation of the mutant *whiN* mRNA in the absence of the wild-type ribosome binding site.

A constructed *whiN* null mutant cannot erect aerial hyphae. A *whiN* null mutant allele was constructed in vitro by replacing a 66-bp *XhoI* fragment internal to *whiN* with a hygromycin-resistance gene (*hyg*) (Fig. 1 and 2). This mutant allele was used to replace the wild-type allele in J1915 as described in Materials and Methods. The chromosomal structure of a representative *whiN* mutant was confirmed by Southern blot analysis (data not shown), and the strain was designated J2177.

J2177 was found to have a bald phenotype, being unable to erect aerial hyphae on MS agar or R2YE (Fig. 5). This observation was not totally unexpected. As previously noted (46), the more severe NTG-induced *whiN* mutant, R112, showed clear signs of pleiotropic effects; in addition to the defects in sporulation, on some media such as MM, R112 produced significantly less aerial mycelium than its parental strain, M145. In this respect, R112 did not fit the classical definition of a *whi* mutant, which should be solely defective in the differentiation of aerial hyphae into spores. Unlike some *S. coelicolor bld* mutants (31, 38), J2177 was not blocked in the production of actinorhodin or undecylprodigiosin, the two pigmented antibi-

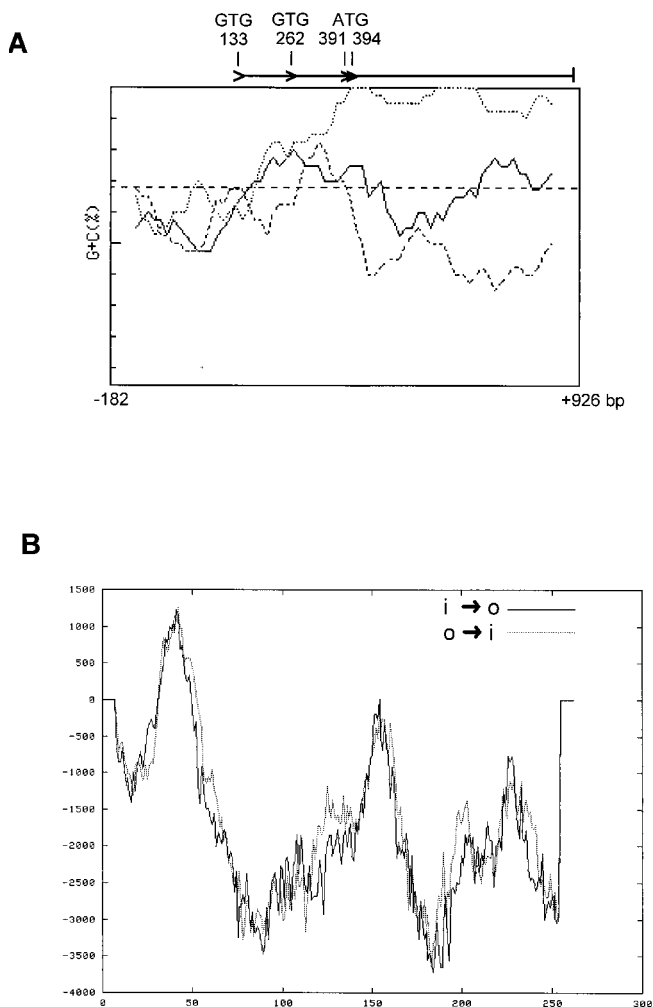


FIG. 4. (A) FRAME plot (2) of the *whiN/bldN* gene showing the unusual codon usage at the 5' end. The GTG and ATG potential translation start codons are marked. The window size was 40 codons, and the step size was five codons. The sequence analyzed begins immediately after the stop codon of the upstream gene, and the numbering is that used in Fig. 3B. (B) TMPRED hydrophobicity plot of WhiN/BldN showing possible transmembrane helices. Predictions for both inside-to-outside helices (i \rightarrow o) and outside-to-inside helices (o \rightarrow i) are shown. Scores above 500 are considered significant.

otics made by this strain. For most of the *bld* mutants (an exception being *bldB*), mycelium formation and sporulation can be restored simply by growing them on MM containing mannitol as the sole carbon source. However, J2177 showed no signs of aerial mycelium formation on MM containing mannitol. As a consequence of the phenotype of the null mutant, *whiN* was renamed *bldN*. The *bldN* null mutant was fully complemented by pIJ6715, the pSET152 derivative carrying *bldN* (Fig. 5).

***bldN* does not fit simply into the extracellular signaling hierarchy proposed for *S. coelicolor*.** To see if *bldN* could be positioned in the extracellular complementation hierarchy proposed by Willey et al. (53), the *bldN* null mutant J2177 was grown on R2YE close to each *bld* mutant in turn. *bldN* restored a fringe of aerial mycelium formation to *bldJ* (formerly referred to as *bld261* [38]), *bldK* and, to a very limited degree, *bldH*. It was not, however, able to restore aerial mycelium

formation to any of the remaining *bld* mutants tested (*bldA*, *bldC*, *bldD*, and *bldG*), and none of the *bld* mutants restored aerial mycelium formation to *bldN*.

***bldN* is transcribed from a single promoter.** High-resolution S1 nuclease mapping of the *bldN* promoter region was performed using a PCR-generated probe and RNA isolated from J1915 grown on R2YE solid medium. A single promoter (*bldNp*) was identified, initiating transcription 83 to 84 bp upstream of the *bldN* GTG-133 start codon (Fig. 3B and 6A). The position of the promoter explains the results of the subcloning complementation experiments. Although the 1-kb *KpnI-XbaI* fragment contains the complete *bldN* coding sequence (pIJ6716; Fig. 1), it failed to complement R112 and R650 because the *KpnI* site used in the subcloning lies downstream of the *bldN* promoter (Fig. 3B), whereas the complementing *FokI* fragment encompasses both the *bldN* coding sequence and the promoter (pIJ6715; Fig. 1).

***bldN* transcription is temporally regulated.** *bldN* transcription was monitored by S1 nuclease protection analysis during development of *S. coelicolor* J1915 on R2YE solid medium. The *bldN* promoter was found to be temporally regulated; the *bldN* transcript was almost undetectable during vegetative growth, but its abundance increased dramatically during aerial mycelium formation and remained at a high level during sporulation (Fig. 6B). Because no attempt was made to fractionate the harvested cell material used for RNA preparation (thus, for example, the late sample contained vegetative and aerial mycelium as well as spores), no conclusions about the spatial location of *bldN* transcription within the colony could be drawn.

***bldN* transcription depends on *bldG* and *bldH*.** To see if *bldN* transcription depended on any of the other *bld* genes required for aerial mycelium formation, RNA was isolated from representative *bldA*, *bldB*, *bldC*, *bldF*, *bldG*, *bldH*, *bldJ*, and *bldK* mutants, as well as from the *bldN* null mutant itself. Each strain was grown on R2YE, a solid medium on which all of the *bld* mutants fail to produce aerial mycelium. These *bld* mutations exist in a complicated variety of genetic backgrounds (Table 1). Therefore, a preliminary experiment was conducted looking for any striking effects, keeping in mind the possible influence of the variable genetic background. *bldN* transcripts were readily detected in the *bldA*, *bldB*, *bldC*, *bldF*, *bldK*, and *bldJ* mutants, as well as in the *bldN* null mutant (data not shown), showing that aerial mycelium formation per se is not required for *bldN* transcription. Strikingly, however, *bldN* transcripts were undetectable in the *bldG* mutant, WC103, and the *bldH* mutant, WC109 (data not shown). Therefore, we repeated these experiments after the isolation of time courses of RNA samples from WC103, WC109, and their congenic parent, J1501 (*bldG*⁺ *bldH*⁺), again grown on R2YE solid medium. As in J1915 (Fig. 6B), *bldN* transcripts were almost undetectable in J1501 during vegetative growth but were readily detected during aerial mycelium formation and sporulation (Fig. 6C). However, *bldN* transcripts were again undetectable in the *bldG* and *bldH* mutants (Fig. 6C). In the absence of a *bldN* signal in the *bldG* and *bldH* mutants, as a positive internal control we examined the level of the transcript for *hrdB*, which encodes the principal, essential sigma factor of *S. coelicolor* (5). *hrdB* transcripts were readily detected at all time points in the *bldG* and *bldH* mutants and in J1501 (Fig. 6C). Therefore, *bldN* transcription depends, directly or indirectly, on *bldG* and *bldH*.

The *bldMp1* promoter depends on *bldN* in vivo. Promoters regulated by ECF sigma factors are similar in their -10 and -35 sequences (14, 16, 17, 27, 32, 40, 41). Visual inspection of the promoter regions of published *bld* gene sequences revealed a possible ECF consensus-like promoter (Fig. 7B) upstream of

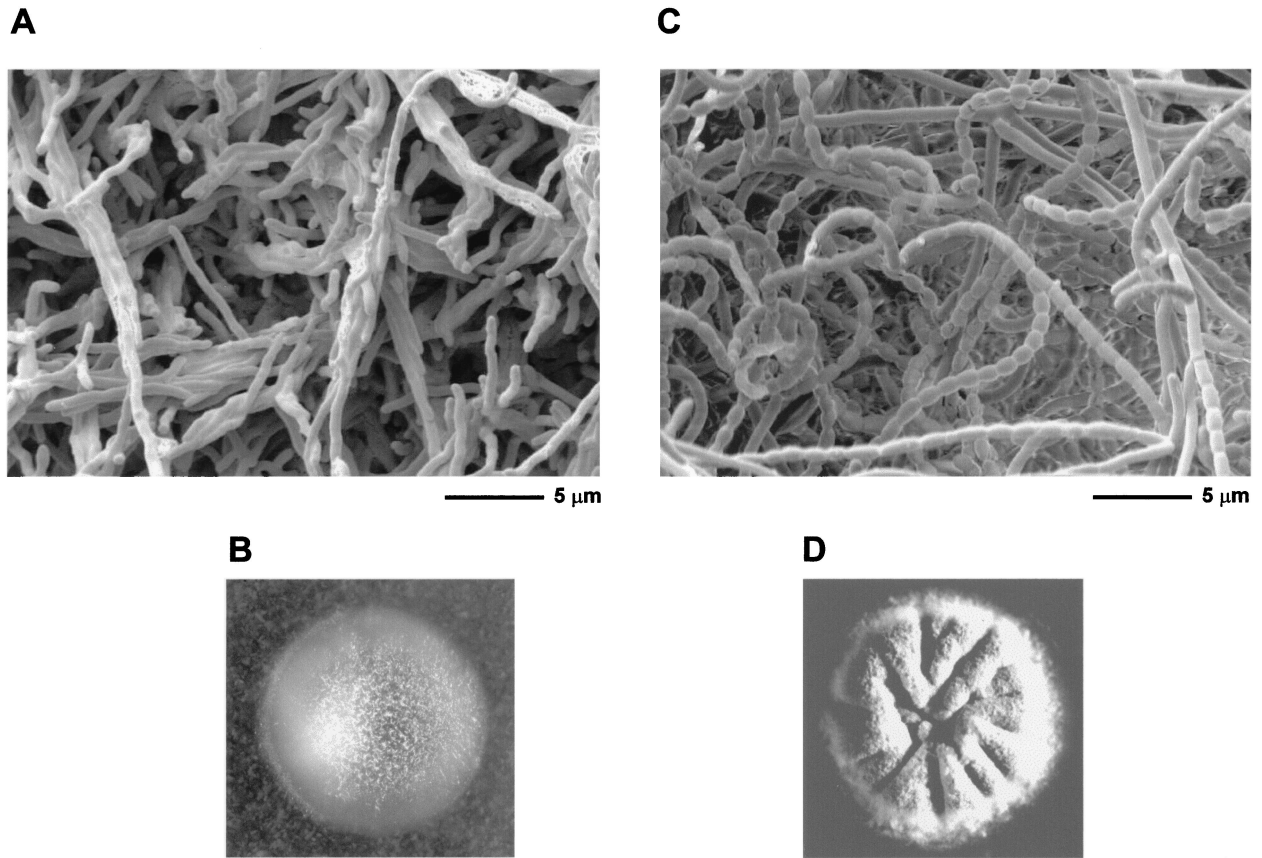


FIG. 5. Scanning electron micrographs and photographs showing the bald phenotype of the constructed *bldN* null mutant, J2177 (A and B), and its complementation by pIJ6715, the pSET152 derivative carrying *bldN* (see Fig. 1) (C and D).

bldM. *bldM* encodes an apparently typical member of the FixJ subfamily of response regulators; however, aspartate-54, the putative site of phosphorylation, is not required for BldM function (33). The *bldM* null mutant fits into the extracellular

signaling cascade proposed for *S. coelicolor* and is a member of the *bldD* extracellular complementation group (33). To determine if the sequence identified upstream of *bldM* functioned as a promoter, high-resolution S1 nuclease mapping was per-

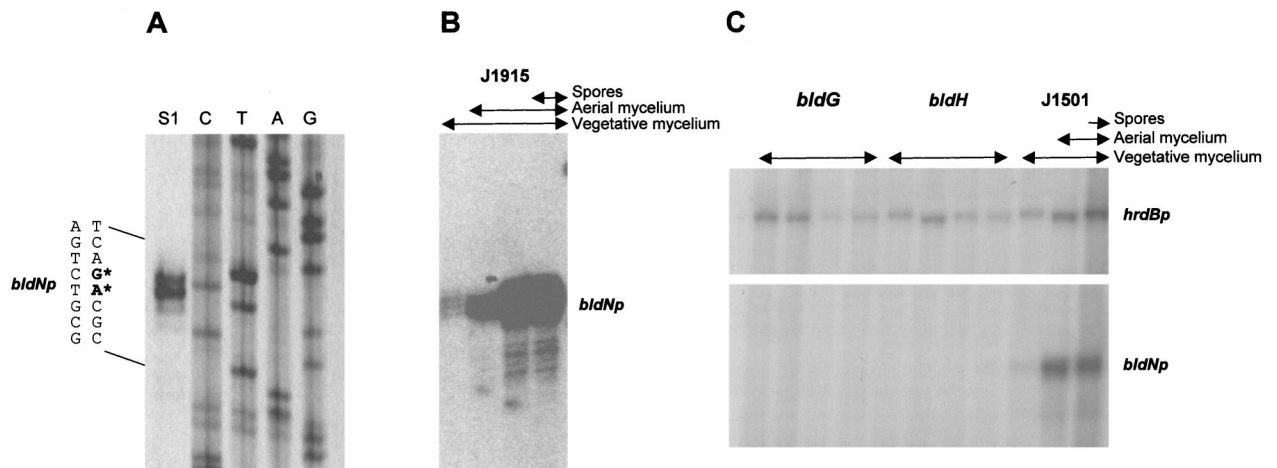


FIG. 6. Transcriptional analysis of *bldN*. (A) High-resolution S1 nuclease mapping of the 5' end of the *bldN* transcript in *S. coelicolor* J1915. The most likely transcription start points are indicated by the asterisks. Lanes C, T, A, and G represent a dideoxy sequencing ladder generated using the same oligonucleotide that was used to make the S1 mapping probe. (B) S1 nuclease protection analysis of *bldN* transcription during development of *S. coelicolor* J1915 on R2YE solid medium. For each time point, the presence of vegetative mycelium, aerial mycelium, and spores, as judged by microscopic examination, is shown. (C) S1 nuclease protection analysis of *bldN* and *hrdB* transcription during development of *S. coelicolor* WC103 (*bldG*) and WC109 (*bldH*) and their congenic parent J1501 (*bldG*⁺ *bldH*⁺) on R2YE solid medium. For each time point, the presence of vegetative mycelium, aerial mycelium, and spores, as judged by microscopic examination, is shown.

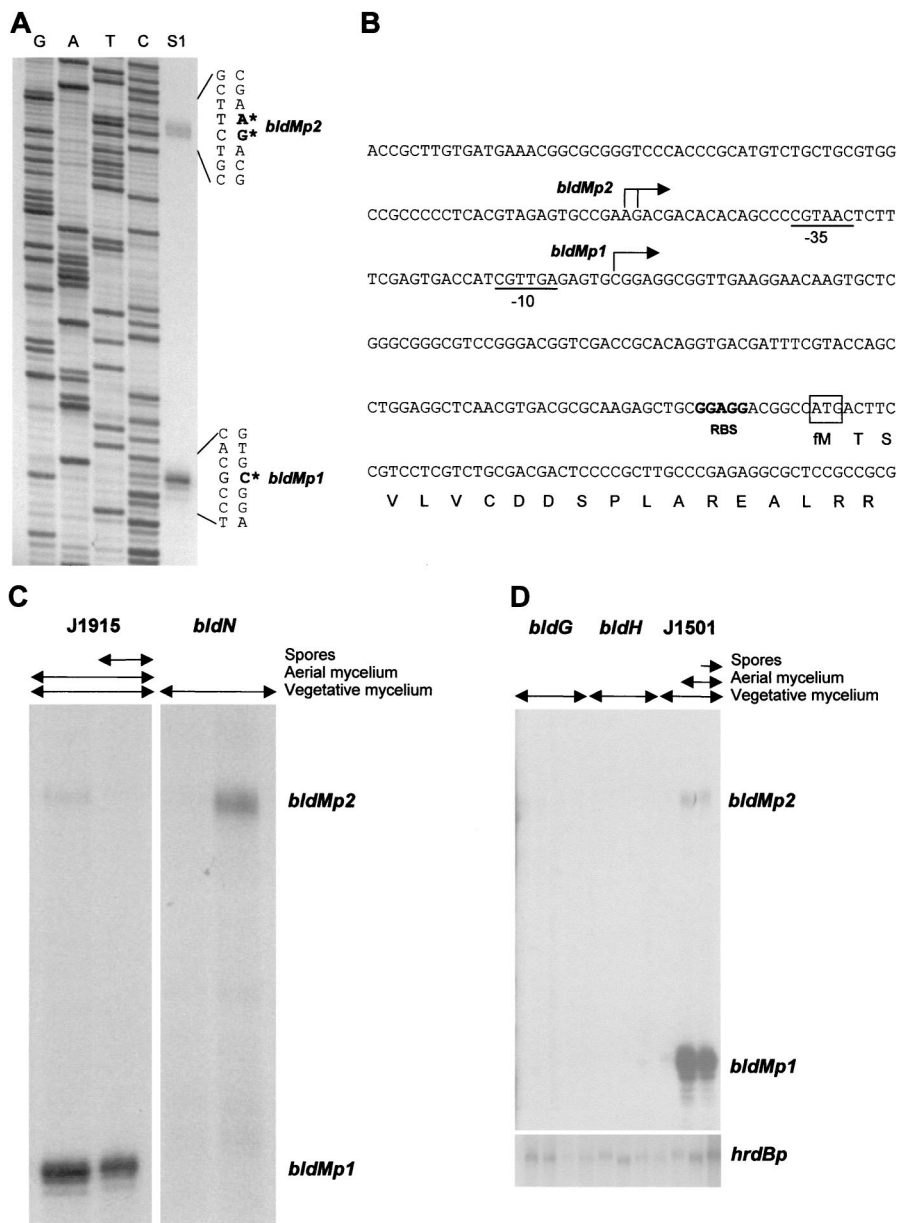


FIG. 7. Transcriptional analysis of *bldM*. (A) High-resolution S1 nuclease mapping of the 5' ends of the *bldMp1* and *bldMp2* transcripts in *S. coelicolor* J1915. The most likely transcription start points are indicated by the asterisks. Lanes G, A, T, and C represent a dideoxy sequencing ladder generated using the same oligonucleotide that was used to make the S1 mapping probe. (B) Nucleotide sequence of the *bldM* promoter region showing the *bldMp1* and *bldMp2* transcription start points, the putative -10 and -35 sequences of the *bldMp1* promoter, the putative ribosome binding site (RBS), and the start of the *bldM* coding sequence. (C) S1 nuclease protection analysis of *bldM* transcription during development of *S. coelicolor* J2177 (*bldN*) and its congenic parent J1915 (*bldN*⁺) on R2YE solid medium. For each time point, the presence of vegetative mycelium, aerial mycelium, and spores, as judged by microscopic examination, is shown. (D) S1 nuclease protection analysis of *bldM* and *hrdB* transcription during development of *S. coelicolor* WC103 (*bldG*) and WC109 (*bldH*) and their congenic parent J1501 (*bldG*⁺ *bldH*⁺) on R2YE solid medium. The RNA samples used were those described in Fig. 6C. For each time point, the presence of vegetative mycelium, aerial mycelium, and spores, as judged by microscopic examination, is shown.

formed using a PCR-generated probe and RNA isolated from J1915. Two promoters were identified, initiating transcription 119 bp (*bldMp1*) and 167 to 168 bp (*bldMp2*) upstream of the *bldM* ATG start codon (Fig. 7A and B). The *bldMp1* promoter corresponded to the putative ECF sigma factor consensus-like sequence (Fig. 7B).

To determine if *bldMp1* depended on *bldN*, RNA was isolated from the constructed *bldN* null mutant, J2177, and its congenic parent, J1915 (*bldN*⁺). *bldMp2* transcripts were de-

tectable in J1915 and in the *bldN* null mutant (Fig. 7C). In contrast, *bldMp1* transcripts were abundant in J1915 but were absent from the *bldN* null mutant, showing that *bldMp1* transcription depended, directly or indirectly, on *bldN* (Fig. 7C).

Given that *bldMp1* transcription depended on *bldN* and that *bldN* transcription in turn depended on *bldG* and *bldH*, it followed that *bldMp1* transcription should be undetectable in *bldG* and *bldH* mutants. To determine if this were true, we examined the transcription of *bldM* in WC103 (*bldG*), WC109

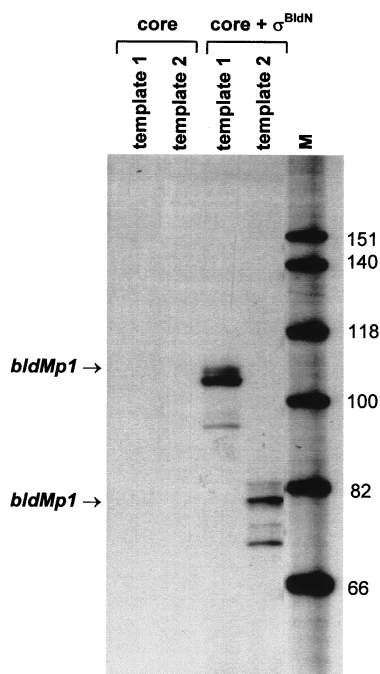


FIG. 8. In vitro transcription of the *bldMp1* promoter by reconstituted $E\sigma^{BldN}$ holoenzyme. Transcripts were generated from templates 1 and 2 (see Materials and Methods) using core RNA polymerase alone or core enzyme plus σ^{BldN} . The expected sizes of the runoff transcripts from the *bldMp1* promoter were 104 nucleotides (template 1) and 78 nucleotides (template 2). The size markers (M) were a ^{32}P -end-labeled *Hin*II digest of Φ X174.

(*bldH*), and J1501 (*bldG*⁺ *bldH*⁺), using the same time courses of RNA samples that were used to examine the transcription of *bldN*. Like *bldN* transcription (Fig. 6B and C), *bldMp1* transcription was temporally regulated, being undetectable during vegetative growth but readily detectable during aerial mycelium formation and sporulation in J1501 (Fig. 7D). In contrast, *bldMp1* transcripts were undetectable in the *bldG* and *bldH* mutants, confirming that *bldMp1* transcription does indeed depend on *bldG* and *bldH* in vivo (Fig. 7D).

The *bldMp1* promoter is a direct biochemical target for σ^{BldN} . To determine if the *bldMp1* promoter is a direct biochemical target for σ^{BldN} -containing RNA polymerase holoenzyme ($E\sigma^{BldN}$), we performed in vitro transcription assays using reconstituted $E\sigma^{BldN}$. A truncated form of σ^{BldN} lacking the N-terminal extension (starting at Met-87) was overexpressed in *E. coli* and purified to near homogeneity, as described in Materials and Methods. In addition, two different *bldMp1* promoter templates, designed to give runoff transcripts of 104 and 78 nucleotides, were amplified by PCR. σ^{BldN} (1 pmol) was added to core RNA polymerase (1 pmol) to reconstitute $E\sigma^{BldN}$ holoenzyme and then incubated separately in the presence of each *bldMp1* promoter template. A runoff product of the predicted size was produced on each template (Fig. 8) in the presence of $E\sigma^{BldN}$ but not in the presence of core enzyme alone, showing that *bldMp1* is recognized by $E\sigma^{BldN}$ in the absence of other transcriptional activators. The *bldMp1* promoter is therefore a direct biochemical target for $E\sigma^{BldN}$ holoenzyme.

DISCUSSION

Different *bldN* alleles arrest *S. coelicolor* development at distinct stages. In the first genetic screen for *whi* loci, con-

ducted by Chater (7), most of the mutants assigned to a given locus were very similar in phenotype, as would be expected if most of the alleles were null, or close to null. In contrast, in the second screen there was wide phenotypic variation among the alleles of both *whiK* and *whiN* (46). Like *whiN/bldN*, disruption of *whiK* also gives rise to a *bld* phenotype (causing *whiK* to be renamed *bldM* [33]). Therefore, the phenotypic variation observed among the *whiN* and *whiK* mutants arises because the NTG-induced alleles retain various degrees of activity (the original screen specifically looked for white mutants and so would not have identified null mutants at either of these loci). In the case of *whiN/bldN*, R112 carries a mutation in the ribosome binding site and presumably produces reduced amounts of wild-type σ^{BldN} , while in R650 the σ^{BldN} produced carries an amino acid substitution in region 2.1 and is therefore likely to interact less efficiently with core RNA polymerase than the wild-type protein.

Are there further *whi* loci to be identified in *S. coelicolor*? It is now clear that, in the cases of *whiN/bldN* and *whiK/bldM* at least, the *whi* mutant screen of Ryding et al. (46) identified special alleles of *bld* loci rather than null mutations at *whi* loci. However, there is reason to believe that there are further *whi* loci to identify in *S. coelicolor*. First, we have recently shown that *whiL* null mutants are white (M. J. Bibb and M. J. Buttner, unpublished data). Second, it is probable that a variety of potentially novel mutants were discarded by Ryding et al. (46) because they were partially suppressed by previously cloned *whi* genes. Of the 115 *whi* mutants chosen for study, 25 were excluded from further analysis because the introduction of either *whiA*, *whiB*, *whiG*, *whiH*, or *whiJ* (and sometimes more than one of these genes) increased gray pigmentation of the colonies, even though the morphological defects of the strains were not complemented (46). It seems very likely that at least some of these 25 strains will define novel *whi* loci. Until these experiments, suppression effects had not been reported in developmental work in *Streptomyces* spp., although the ability of an additional copy of *whiG* partially to suppress the spore pigment defect of *whiH* mutants without affecting their morphological phenotype has recently been noted (K. Flårdh, personal communication).

***bldN* and the *S. coelicolor* extracellular signaling hierarchy.** *bldN* does not fit simply into the extracellular signaling cascade proposed by Willey et al. (53):

$$bldJ < bldK, bldL < bldA, bldH < bldG < bldC < bldD, bldM$$

The *bldN* null mutant restored a fringe of aerial mycelium formation to *bldJ* (formerly referred to as *bld261*) and *bldK*, mutants that define the first two extracellular complementation groups in the cascade, and seemed to have some limited effect on *bldH*, which belongs to the third extracellular complementation group, but not on *bldA*, which also belongs to the third group. The *bldN* mutant was not, however, able to restore aerial mycelium formation to *bld* mutants that define the remaining three extracellular complementation groups in the cascade (*bldG*, *bldC*, and *bldD*), and none of the *bld* mutants restored aerial mycelium formation to *bldN*. Two other *bld* mutants have been described that do not fit cleanly into the hierarchy. *bldI* appears to be a member of the *bldAH* complementation group except that it is not complemented by *bldC*, and *bldB* appears to be in the same extracellular complementation group as *bldC* except that it fails to complement *bldA* and *bldH* (53). The extracellular signaling cascade was proposed in the light of experiments performed using *bld* mutants isolated by Merrick (31) and Champness (6). Recently, a large number of new *bld* mutants have been isolated, and the vast

majority of these mutants fit into the cascade hierarchy (33, 38). Nevertheless, the behavior of *bldB*, *bldI*, and *bldN* emphasizes the need for further elaboration of the model.

Transcriptional dependence between *bld* genes. Transcription of *bldN* depends, directly or indirectly, on *bldG* and *bldH*. *bldH* has not been characterized, but *bldG* (database accession number AF134889) encodes a homologue of SpoIIAA and RsbV, proteins that function as anti-anti-sigma factors in the regulation of σ^F and σ^B , respectively, in *B. subtilis* (1, 10, 49, 55). The genes encoding SpoIIAA, the anti-sigma factor SpoIIAB, and σ^F lie in the same operon, as do the genes encoding RsbV, the anti-sigma factor RsbW, and σ^B . In contrast, there is an anti-sigma factor gene adjacent to *bldG*, but no sigma factor gene. Since σ^{BldN} does not direct transcription of its own gene, the effects of *bldG* on *bldN* transcription must be indirect, implying the involvement of a further sigma factor in the control of aerial mycelium formation in *S. coelicolor*.

In turn, transcription of the *bldMp1* promoter depends on *bldN*, and this dependence arises because *bldMp1* is a direct biochemical target for $E\sigma^{BldN}$ holoenzyme. The second *bldM* promoter, *bldMp2*, however, does not depend on σ^{BldN} .

What is the functional significance of the N-terminal extension of σ^{BldN} ? Although the *bldN* start codon cannot be unambiguously assigned from FRAME analysis and sequence inspection, the discovery that the NTG-induced mutation in R112 is a change in the putative ribosome binding site upstream of GTG-133 strongly implies that σ^{BldN} is synthesized with an N-terminal extension of approximately 86 residues that is absent from other sigma factors. During *B. subtilis* development, the mother cell-specific sigma factors σ^E and σ^K are synthesized as inactive pro- σ factors that are subsequently activated by proteolysis of the N-terminal 29 and 20 amino acids, respectively (10, 49), by membrane-localized proteases (42, 45). In both cases, the activation of this processing event is triggered by signals derived from the forespore, and this "cross talk" serves to coordinate the divergent programs of gene expression between the two cellular compartments within the sporangium (10, 49). By analogy, it is possible that σ^{BldN} is synthesized as an inactive pro- σ factor, and it was for this reason that we overexpressed a truncated form of σ^{BldN} that was approximately co-N-terminal with other ECF sigma factors for the in vitro transcription studies on the *bldMp1* promoter. Given the stretch of 20 hydrophobic residues within the N-terminal extension of σ^{BldN} , it is interesting to note that in *B. subtilis* the pro-sequences of both pro- σ^E and pro- σ^K promote membrane association (12, 18, 19, 57). Upon subcellular fractionation, the majority of pro- σ^E and pro- σ^K are present in the membrane fraction, whereas the majority of σ^E and σ^K are associated with core RNA polymerase in the cytoplasm (12, 57). In agreement with these observations, immunofluorescence microscopy showed that pro- σ^E is associated with the cytoplasmic membrane in the predivisional sporangium and with the sporulation septum at the stage of asymmetric division (12, 18, 19). Similarly, immunofluorescence microscopy showed that pro- σ^K is localized to the mother cell membranes that surround both the mother cell and the forespore (57).

The orthologue of σ^{BldN} in *Streptomyces griseus* is also required for aerial mycelium formation and is under the control of the A-factor cascade. In an accompanying study, Yamazaki et al. (54) show that the orthologue of σ^{BldN} in *S. griseus* also plays an important role in differentiation. In *S. griseus*, the γ -butyrolactone signaling molecule A-factor (2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone) triggers a regulatory cascade required for both aerial mycelium formation and production of the antibiotic streptomycin (13). A-factor causes expression of a transcriptional activator called AdpA, which

induces streptomycin biosynthesis by activating transcription of *strR*, the gene encoding the pathway-specific activator of the streptomycin cluster (39). Until now, no targets for AdpA have been identified to explain the morphological defects of an *adpA* mutant. However, Yamazaki et al. (54) report the isolation of new AdpA binding sites from *S. griseus* chromosomal DNA, one of which is the promoter of an ECF sigma factor gene they have named *adsA* (AdpA-dependent sigma factor), the *S. griseus* orthologue of *bldN*. As is true for *S. coelicolor bldN*, transcription of *S. griseus adsA* begins approximately at the time of aerial mycelium formation, and disruption of *adsA* also results in loss of aerial mycelium formation. Neither *S. coelicolor bldN* nor *S. griseus adsA* is required for antibiotic production.

The predicted AdpA binding site is not clearly conserved in the promoter region of *S. coelicolor bldN*. However, there is a likely orthologue of *adpA* in the emerging *S. coelicolor* genome sequence (*Streptomyces coelicolor* Genome Project, http://www.sanger.ac.uk/Projects/S_coelicolor/ [Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom]). It will be interesting to see the extent to which the regulatory pathways governing aerial mycelium formation are conserved between these two distantly related *Streptomyces* species.

ACKNOWLEDGMENTS

We thank Mark Paget and Gabriella Kelemen for helpful discussion, Kim Findlay for taking the scanning electron micrographs of R112 and R650, and Mervyn Bibb, Keith Chater, and David Hopwood for their comments on the manuscript.

This work was supported by BBSRC grant 83/P07658 (to M. J. Buttner), by a John Innes Foundation studentship (to V. Molle), by a Lister Institute Research Fellowship (to M. J. Buttner), and by a grant-in-aid to the John Innes Centre from the BBSRC.

REFERENCES

- Alper, S., A. Dufour, D. A. Garsin, L. Duncan, and R. Losick. 1996. Role of adenosine nucleotides in the regulation of a stress-response transcription factor in *Bacillus subtilis*. *J. Mol. Biol.* **260**:165–177.
- Bibb, M. J., P. R. Findlay, and M. W. Johnson. 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene* **30**:157–166.
- Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**:43–49.
- Buttner, M. J., I. M. Fearnley, and M. J. Bibb. 1987. The agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. *Mol. Gen. Genet.* **209**:101–109.
- Buttner, M. J., K. F. Chater, and M. J. Bibb. 1990. Cloning, disruption and transcriptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **172**:3367–3378.
- Champness, W. C. 1988. New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J. Bacteriol.* **170**:1168–1174.
- Chater, K. F. 1972. A morphological and genetic mapping study of white colony mutants of *Streptomyces coelicolor*. *J. Gen. Microbiol.* **72**:9–28.
- Chater, K. F. 1998. Taking a genetic scalpel to the *Streptomyces* colony. *Microbiology* **144**:1465–1478.
- Chater, K. F., C. J. Bruton, A. A. King, and J. E. Suarez. 1982. The expression of *Streptomyces* and *Escherichia coli* drug resistance determinants cloned into the *Streptomyces* phage Φ C31. *Gene* **19**:21–32.
- Errington, J. 1996. Determination of cell fate in *Bacillus subtilis*. *Trends Genet.* **12**:31–34.
- Gorham, H. C., S. J. McGowan, P. R. H. Robson, and D. A. Hodgson. 1996. Light-induced carotogenesis in *Mycococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. *Mol. Microbiol.* **19**:171–186.
- Hofmeister, A. 1998. Activation of the proprotein transcription factor pro- σ^E is associated with its progression through three patterns of subcellular localization during sporulation in *Bacillus subtilis*. *J. Bacteriol.* **180**:2426–2433.
- Horinouchi, S., and T. Beppu. 1994. A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. *Mol. Microbiol.* **12**:859–864.
- Huang, X., and J. D. Hermann. 1998. Identification of target promoters for

- the *Bacillus subtilis* σ^X factor using a consensus-directed search. *J. Mol. Biol.* **279**:165–173.
15. Huang, X., A. Decatur, A. Sorokin, and J. D. Helmann. 1997. The *Bacillus subtilis* σ^X protein is an extracytoplasmic function σ factor contributing to survival at high temperature. *J. Bacteriol.* **179**:2915–2921.
 16. Huang, X., K. L. Fredrick, and J. D. Helmann. 1998. Promoter recognition by *Bacillus subtilis* σ^W : autoregulation and partial overlap with the σ^X regulon. *J. Bacteriol.* **180**:3765–3770.
 17. Huang, X., A. Gaballa, M. Cao, and J. D. Helmann. 1999. Identification of target promoters for the *Bacillus subtilis* extracytoplasmic function σ factor σ^W . *Mol. Microbiol.* **31**:361–371.
 18. Ju, J., and W. G. Haldenwang. 1999. The “pro” sequence of the sporulation-specific σ transcription factor σ^E directs it to the mother cell side of the sporulation septum. *J. Bacteriol.* **181**:6171–6175.
 19. Ju, J., T. Luo, and W. G. Haldenwang. 1997. *Bacillus subtilis* pro- σ^E fusion protein localises to the forespore septum and fails to be processed when synthesized in the forespore. *J. Bacteriol.* **179**:4888–4893.
 20. Kelemen, G. H., and M. J. Buttner. 1998. Initiation of aerial mycelium formation in *Streptomyces*. *Curr. Opin. Microbiol.* **1**:656–662.
 21. Kelemen, G. H., K. A. Plaskitt, C. G. Lewis, K. Findlay, and M. J. Buttner. 1995. Deletion of DNA lying close to the *glkA* locus induces ectopic sporulation in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **17**:221–230.
 22. Kelemen, G. H., G. L. Brown, J. Kormanec, L. Potůčková, K. F. Chater, and M. J. Buttner. 1996. The positions of the sigma factor genes, *whiG* and *sigF*, in the hierarchy controlling the development of spore chains in the aerial hyphae of *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **21**:593–603.
 23. Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
 24. Lawlor, E. J., H. A. Baylis, and K. F. Chater. 1987. Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Genes Dev.* **1**:1305–1310.
 25. Lesley, S. A., and R. R. Burgess. 1989. Characterization of the *Escherichia coli* transcription factor σ^{70} : localization of a region involved in the interaction with core RNA polymerase. *Biochemistry* **28**:7728–7734.
 26. Lesley, S. A., M. A. Brow, and R. R. Burgess. 1991. Use of *in vitro* protein synthesis from polymerase chain reaction-generated templates to study interaction of *Escherichia coli* transcription factors with core RNA polymerase and for epitope mapping of monoclonal antibodies. *J. Biol. Chem.* **266**:2632–2638.
 27. Lonetto, M. A., K. L. Brown, K. E. Rudd, and M. J. Buttner. 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions. *Proc. Natl. Acad. Sci. USA* **91**:7573–7577.
 28. McCracken, S., and J. Greenblatt. 1991. Related RNA polymerase-binding regions in human RAP30/74 and *Escherichia coli* σ^{70} . *Science* **253**:900–902.
 29. McGowan, S. J., H. C. Gorham, and D. A. Hodgson. 1993. Light-induced carotogenesis in *Mycococcus xanthus*: DNA sequence of the *carR* region. *Mol. Microbiol.* **10**:713–735.
 30. MacNeil, D. J., J. L. Occi, K. M. Gewain, T. MacNeil, P. H. Gibbons, C. L. Ruby, and S. L. Danis. 1992. Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. *Gene* **115**:119–125.
 31. Merrick, M. J. 1976. A morphological and genetic mapping study of bald mutants of *Streptomyces coelicolor*. *J. Gen. Microbiol.* **96**:299–315.
 32. Missiakas, D., and S. Raina. 1998. The extracytoplasmic function sigma factors: role and regulation. *Mol. Microbiol.* **28**:1059–1066.
 33. Molle, V., and M. J. Buttner. 2000. Different alleles of the response regulator gene *blmD* arrest *Streptomyces coelicolor* development at distinct stages. *Mol. Microbiol.* **36**:1265–1278.
 34. Molle, V., W. J. Palframan, K. C. Findlay, and M. J. Buttner. 2000. WhiD and WhiB, homologous proteins required for different stages of sporulation in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **182**:1286–1295.
 35. Nguyen, L. H., D. B. Jensen, and R. R. Burgess. 1993. Overexpression and purification of σ^{32} , the *Escherichia coli* heat shock transcription factor. *Protein Expr. Purif.* **4**:425–433.
 36. Nodwell, J. R., and R. Losick. 1998. Purification of an extracellular signalling molecule involved in production of aerial mycelium by *Streptomyces coelicolor*. *J. Bacteriol.* **180**:1334–1337.
 37. Nodwell, J. R., K. McGovern, and R. Losick. 1996. An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. *Mol. Microbiol.* **22**:881–893.
 38. Nodwell, J. R., M. Yang, D. Kuo, and R. Losick. 1999. Extracellular complementation and the identification of genes involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genetics* **151**:569–584.
 39. Ohnishi, Y., S. Kameyama, H. Onaka, and S. Horinouchi. 1999. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene for the A-factor receptor. *Mol. Microbiol.* **34**:102–111.
 40. Paget, M. S. B., J.-G. Kang, J.-H. Roe, and M. J. Buttner. 1998. σ^R , an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in *Streptomyces coelicolor* A3(2). *EMBO J.* **17**:5776–5782.
 41. Paget, M. S. B., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner. 1999. Evidence that the extracytoplasmic function sigma factor, σ^E , is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **181**:204–211.
 42. Peters, H. K., and W. G. Haldenwang. 1991. Synthesis and fractionation properties of SpoIIIGA, a protein essential for pro- σ^E processing in *Bacillus subtilis*. *J. Bacteriol.* **173**:7821–7827.
 43. Pope, M. K., B. D. Green, and J. Westpheling. 1996. The *blD* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilisation, morphogenesis and cell-cell signalling. *Mol. Microbiol.* **19**:747–756.
 44. Puglia, A.-M., and E. Capelletti. 1984. A bald, superfertile, UV-resistant strain in *Streptomyces coelicolor* A3(2). *Microbiologica* **7**:263–266.
 45. Rudner, D. Z., P. Fawcett, and R. Losick. 1999. A family of membrane-embedded metalloproteases involved in regulated proteolysis of membrane-associated transcription factors. *Proc. Natl. Acad. Sci. USA* **96**:14765–14770.
 46. Ryding, N. J., M. J. Bibb, V. Molle, K. C. Findlay, K. F. Chater, and M. J. Buttner. 1999. New sporulation loci in *Streptomyces coelicolor* A3(2). *J. Bacteriol.* **181**:5419–5425.
 47. Schuler, M. F., K. M. Tatti, K. H. Wade, and C. P. Moran. 1995. A single amino acid substitution in σ^E affects its ability to bind core RNA polymerase. *J. Bacteriol.* **177**:3687–3694.
 48. Sharp, M. M., C. L. Chan, C. Z. Lu, M. T. Marr, S. Nechaev, E. W. Merritt, K. Severinov, J. W. Roberts, and C. A. Gross. 1999. The interface of σ with core RNA polymerase is extensive, conserved, and functionally specialised. *Genes Dev.* **13**:3015–3026.
 49. Stragier, P., and R. Losick. 1996. Molecular genetic analysis of sporulation in *Bacillus subtilis*. *Annu. Rev. Genet.* **30**:297–341.
 50. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
 51. Tillotson, R. D., H. A. B. Wösten, M. Richter, and J. M. Willey. 1998. A surface active protein involved in aerial mycelium formation in the filamentous fungus *Schizophyllum commune* restores the capacity of a bald mutant of the filamentous bacterium *Streptomyces coelicolor* to erect aerial structures. *Mol. Microbiol.* **30**:595–602.
 52. Willey, J., R. Santamaria, J. Guizarro, M. Geistlich, and R. Losick. 1991. Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. *Cell* **65**:641–650.
 53. Willey, J., J. Schwedock, and R. Losick. 1993. Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Dev.* **7**:895–903.
 54. Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2000. An A-factor-dependent extracytoplasmic function sigma factor (σ^{AfsA}) that is essential for morphological development in *Streptomyces griseus*. *J. Bacteriol.* **182**:4596–4605.
 55. Yang, X., C. M. Kang, M. S. Brody, and C. W. Price. 1996. Opposing pairs of serine protein kinases and phosphatases transmit signals of environmental stress to activate a bacterial transcription factor. *Genes Dev.* **10**:2265–2275.
 56. Zalacain, M., A. González, M. C. Guerrero, R. J. Mattaliano, F. Malpartida, and A. Jiménez. 1986. Nucleotide sequence of the hygromycin B phosphotransferase gene from *Streptomyces hygroscopicus*. *Nucleic Acids Res.* **14**:1565–1581.
 57. Zhang, B., A. Hofmeister, and L. Kroos. 1998. The prosequence of pro- σ^K promotes membrane association and inhibits RNA polymerase core binding. *J. Bacteriol.* **180**:2434–2441.