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The *Streptomyces coelicolor* Developmental Transcription Factor σ^{BldN} Is Synthesized as a Proprotein

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***bldN* is one of a set of genes required for the formation of specialized, spore-bearing aerial hyphae during differentiation in the mycelial bacterium *Streptomyces coelicolor*. Previous analysis (M. J. Bibb et al., J. Bacteriol. 182:4606-4616, 2000) showed that *bldN* encodes a member of the extracytoplasmic function subfamily of RNA polymerase σ factors and that translation from the most strongly predicted start codon (GTG^1) would give rise to a σ factor having an unusual N-terminal extension of ca. 86 residues. Here, by using a combination of site-directed mutagenesis and immunoblot analysis, we provide evidence that all *bldN* translation arises from initiation at GTG^1 and that the primary translation product is a proprotein (pro-σ^{BldN}) that is proteolytically processed to a mature species (σ^{BldN}) by removal of most of the unusual N-terminal extension. A time course taken during differentiation of the wild type on solid medium showed early production of pro-σ^{BldN} and the subsequent appearance of mature σ^{BldN}, which was concomitant with aerial mycelium formation and the disappearance of pro-σ^{BldN}. Two genes encoding members of a family of metalloproteases that are involved in the regulated proteolytic processing of transcription factors in other organisms were identified in the *S. coelicolor* genome, but their disruption did not affect differentiation or pro-σ^{BldN} processing.**

The regulated proteolysis of transcription factors is an important theme in the control of gene expression in both eukaryotes and bacteria. Well-characterized examples include the sterol regulatory element binding protein (SREBP) in mammals (3) and σ^E and σ^K in the bacterium *Bacillus subtilis* (30). SREBP, which activates genes involved in cholesterol biosynthesis and uptake, has an N-terminal transcription activation domain and a C-terminal regulatory domain separated by two transmembrane helices. The protein is inserted into the membrane of the endoplasmic reticulum and the nuclear envelope in a hairpin fashion such that the N-terminal and C-terminal domains are in the cytoplasm and the short "luminal loop" between the two transmembrane helices projects into the lumen of the organelle (18, 36). In order to activate its target genes, the N-terminal domain has to be released from the membrane in a two-step proteolytic process that is activated by a drop in sterol levels (35, 38). In the first step, SREBP is cleaved at site 1 within the luminal loop in a sterol-dependent manner, separating the N-terminal and C-terminal domains but leaving both still anchored in the membrane (10). In the second step, which is not regulated by sterols but requires prior cleavage at site 1, a second protease cleaves at site 2 within the first transmembrane helix (11). This releases the mature N-terminal domain into the cytosol, from which it rapidly enters the nucleus and activates gene expression to bring about an increase in cellular sterol levels (3).

In the gram-positive bacterium *B. subtilis*, the mother cell-specific σ factors, σ^E and σ^K, play central roles in the control of gene expression during endospore formation. σ^E and σ^K are synthesized as inactive, membrane-associated pro-σ factors that are subsequently activated and released into the mother cell cytoplasm through proteolysis of the N-terminal 27 to 29 and 20 amino acids, respectively, in reactions catalyzed by membrane-localized proteases. The prosequences of both pro-σ^E and pro-σ^K are responsible for promoting membrane association, and the mature forms of these σ factors are found in the cytoplasm associated with core RNA polymerase (13, 16, 19, 20, 43). In both cases, the activation of pro-σ processing in the mother cell is triggered by signals derived from the forespore; in the case of σ^K, pro-σ processing is triggered by a putative signaling protein (SpoIVB), which is produced in the forespore and is secreted either into the mother cell or the space between the inner and outer forespore membranes (14, 15). These intercellular signaling pathways are critical for coordination of the divergent programs of gene expression between the two cells. Thus, for example, the engineered removal of the DNA encoding the prosequence from the gene encoding σ^K results in the premature appearance of σ^K activity in the mother cell and the consequent production of highly defective spores (8).

σ^{BldN} is an extracytoplasmic function (ECF) σ factor that carries an unusual N-terminal extension of ca. 86 amino acids that is absent from other σ factors. σ^{BldN} was discovered following the isolation of two NTG-induced point mutants in the *bldN* gene that affected morphological differentiation in the mycelial bacterium *Streptomyces coelicolor* (1, 34). *bldN*-null mutants cannot develop the specialized aerial hyphae that give *Streptomyces* colonies their characteristic fuzzy appearance and which ultimately differentiate to form chains of exospores (1, 6, 21).

bldN expression is developmentally regulated. The *bldN* transcript is almost undetectable during vegetative growth but its abundance increases dramatically during aerial mycelium formation (1). Three other *bld* loci have been implicated in this transcriptional regulation. Activation of *bldN* transcription de-

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

Strain	Genotype	Source or reference
M145	SCP1$^-$ SCP2$^-$	24
J1501	*hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	7
J1915	Δ*glkA119* SCP1$^-$ SCP2$^-$	22
J2177	Δ*glkA119 bldN*::*hyg* SCP1$^-$ SCP2$^-$	1
J2178	Δ*glkA119 bldN*::*his* SCP1$^-$ SCP2$^-$	This work
J2180	Δ*glkA119* SCO2260::*hyg* SCP1$^-$ SCP2$^-$	This work
J2182	SCO5695::*apr* SCP1$^-$ SCP2$^-$	This work
J2183	Δ*bldN*::*aadA* SCP1$^-$ SCP2$^-$	This work
J2184	*hisA1 uraA1 strA1* Δ*bldN*::*aadA* Pgl$^-$ SCP1$^-$ SCP2$^-$	This work
J2188	Δ*glkA119* SCO2260::*hyg* SCO5695::*apr* SCP1$^-$ SCP2$^-$	This work
R112	*bldN112* SCP1$^-$ SCP2$^-$	34
R650	*bldN650* SCP1$^-$ SCP2$^-$	34
WC103	*bldG103 hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	5
WC109	*bldH109 hisA1 uraA1 strA1* Pgl$^-$ SCP1$^-$ SCP2$^-$	5

pends, directly or indirectly, on both *bldG* and *bldH* (1), and BldD directly represses *bldN* transcription by binding to the *bldN* promoter at two sites, one at either side of the transcription start site (12). In addition to *bldN*, BldD also negatively regulates two other σ factor genes, *whiG* and *sigH*, and is thought specifically to repress transcription of these genes prior to aerial mycelium formation (12, 23).

σ^{BldN}, in turn, directly activates transcription of another gene required for aerial mycelium formation, *bldM* (1, 27). *bldM* has two promoters, a strong σ^{BldN}-dependent promoter (*bldMp1*) and a weak σ^{BldN}-independent promoter (*bldMp2*). As a consequence, *bldM* mRNA and protein levels are very low in a *bldN* mutant (1; M. Elliot, unpublished data).

The *bldN* orthologue of *Streptomyces griseus*, *adsA*, has also been characterized, and the unusual N-terminal extension that is present in σ^{BldN} is conserved in σ^{AdsA} (40). Like *bldN*, *adsA* is required for aerial mycelium formation and is developmentally regulated at the level of transcription. In *S. griseus*, the γ-butyrolactone signaling molecule A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) triggers a regulatory cascade required for both aerial mycelium formation and production of the antibiotic streptomycin (17). *adsA* is under the immediate control of the A-factor cascade, being directly activated by AdpA (A-factor-dependent protein A), a transcriptional activator required for both differentiation and streptomycin production (28).

Here we provide evidence that σ^{BldN}, in addition to being developmentally regulated at the transcriptional level, is synthesized as a proprotein (pro-σ^{BldN}) that is processed to a mature species (σ^{BldN}) through the proteolytic removal of the unusual N-terminal extension.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, transformation, and conjugal plasmid transfer from *Escherichia coli* to *Streptomyces*. *S. coelicolor* strains (Table 1) were cultured on R2YE, on minimal medium containing 0.5% (wt/vol) mannitol as a carbon source, on MS agar (mannitol plus soya flour), or in liquid YEME medium (24). To bypass the methyl-specific restriction system of *S. coelicolor* during conjugation from *E. coli*, unmethylated plasmids were transferred by conjugation from the *dam dcm hsdS E. coli* strain ET12567 (26) as described by Ryding et al. (34). *Streptomyces* protoplast transformation was as described by Kieser et al. (24). *E. coli* BL21λ(DE3)(pLysS) (37) was used to express σ^{BldN}, and DH5α was the host strain for standard manipulations. The plasmids used were pET11a (Novagen), pSK+ (Stratagene), pSET152 (2), pRSET (Invitrogen), pIJ487 (39), and pIJ6650 (24).

PCR-based site-directed mutagenesis. A 1.2-kb *Bam*HI-*Eco*RI fragment containing *bldN* was isolated from pIJ6715 (1) and cloned into pSK+ digested with *Bam*HI and *Eco*RI to give pIJ6723. Pairs of abutting oligonucleotides were used to amplify the entire pIJ6723 plasmid, simultaneously introducing specific mutations. The oligonucleotides used were pIJ6724 (GTG[1] to GTC; 5′-CTACCC ACACGTCGGGGTTGA-3′ and 5′-ACGGGACTCCCAGAGGCAGAG-3′), pIJ6725 (GTG[2] to GTC; 5′-CCCCGCCGGTCCGTGCTACGCA-3′ and 5′-AC GGCGGCCGCGGCGAGGGCG-3′), pIJ6726 (TTG to TGA[1]; 5′-TGACGCG GCTTCGTCCCCACCGCG and 5′-CAGGTCTTGCGCTGATTTGAC-3′), pIJ6727 (GGA to TGA[2]; 5′-TGAAGCGCCGTCGTCGGCAGAC-3′ and 5′-T TCGGCCAGTGCGTAGCACGG-3′), and pIJ6728 (AGTATG to GCGGCG; 5′-GCGGAGCTGGTCGAGCGGGCCCAG-3′ and 5′-CGCGCGGGCGCTG TCGCTGTCCGC-3′). After phosphorylation of the oligonucleotides, the PCR conditions used were 10 cycles of 96°C for 1 min, 60°C for 45 s, and 72°C for 8 min 30 s; followed by 10 cycles of 96°C for 1 min, 60°C for 45 s, and 72°C for 12 min 30 s; followed by an extension reaction at 72°C for 15 min. In each case the PCR product was self-ligated to recreate a circular plasmid, and the resulting *bldN* allele was sequenced over its entire length to ensure that only the desired mutation had been introduced. Lastly, each *bldN* allele was isolated as a *Bam*HI-*Eco*RI fragment and ligated into pSET152 cut with the same enzymes. The plasmid numbers given above refer to the final pSET152 clones.

Immunoblot analysis. *Streptomyces* liquid cultures were harvested by centrifugation, whereas surface-grown cultures were scraped from cellophane-covered R2YE plates. Approximately 500 mg of mycelium from each culture was resuspended in 1 ml of Complete protease inhibitor buffer (Roche Diagnostics) in a 1.5-ml Eppendorf tube, and samples were sonicated at half power for four cycles of 10 s, with cooling on ice in between. Cell debris was removed by centrifugation at 14,000 rpm for 2 min, and the protein concentration of the supernatant was estimated by using Bradford reagent (Bio-Rad). Samples (20 μg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, transferred to Hybond-C nylon membrane (Amersham Pharmacia Biotech), and probed with a 1:2,500 dilution of rabbit anti-BldN antibody. Horseradish peroxidase-coupled secondary antibody (Amersham Pharmacia Biotech) was used at a 1:5,000 dilution and detected by chemiluminescence by using ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

C-terminally His-tagged σ^{BldN}. A 5′-truncated allele of *bldN* carrying seven histidine codons at the 3′ end was generated by PCR by using pIJ6714 (1) as the template and the oligonucleotides 5′-GCCGGATCCGCGCGGCTTCGTCCCC ACCGC-3′ and 5′-CGGGGATCCTCAGTGGTGGTGGTGGTGGTGGTGG CGGGCGTCGTCGGGGAGCAG-3′. The PCR conditions consisted of 20 cycles of 96°C for 1 min, 60°C for 50 s, and 72°C for 50 s, followed by 72°C for 10 min. The resulting PCR fragment was cut with *Bam*HI and cloned into *Bam*HI-cut pIJ6650 to create pIJ6729. The sequence of the *bldN* allele was confirmed in pIJ6729, which was introduced by conjugation into *S. coelicolor* J1915, selecting for apramycin resistance. A single crossover event between the full-length (chromosomal) and truncated copies of *bldN* to give strain J2178 was confirmed by PCR with an oligonucleotide complementary to the His tag sequence (5′-TCA GTGGTGGTGGTGGTGGTG-3′) and one within the *bldN* gene, upstream of the truncated version of *bldN* (5′-GCTACGACGGGTGAATGGTTC-3′).

Construction of a SCO2260 (ORF6 on cosmid C75A) null mutant. A SCO2260 (putative metalloprotease gene) null mutant derivative of J1915, a plasmid-free,

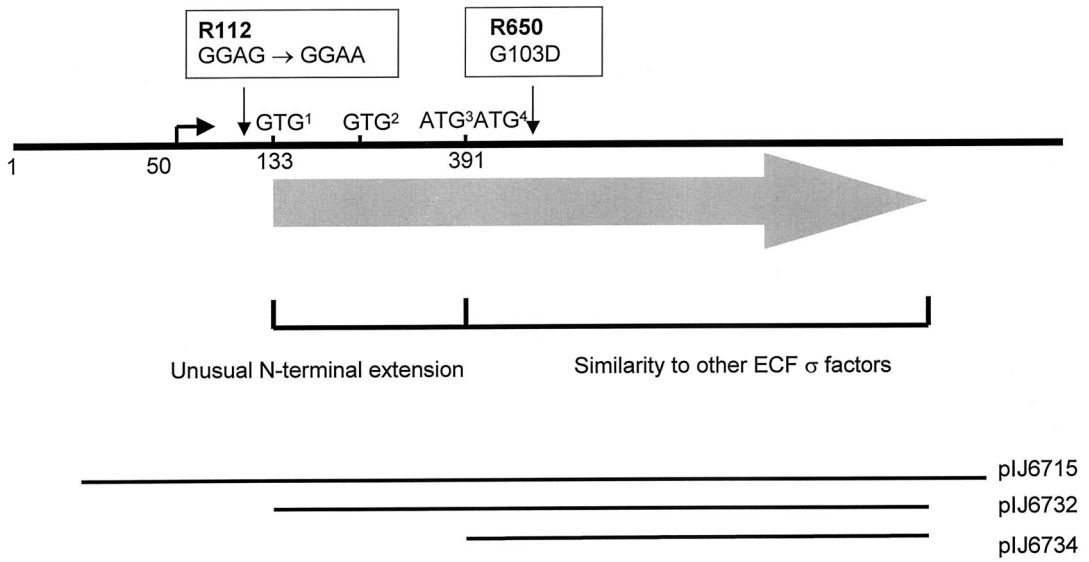

FIG. 1. Genetic organization of the *bldN* locus, showing the four in-frame potential start codons, the nature and positions of the two point mutations (R112 and R650) that originally defined the *bldN* locus, and the extent of DNA carried in various *bldN* clones. The numbering of the nucleotides is taken from Bibb et al. (1). pIJ6715 is pSET152 carrying a *Fok*I fragment containing *bldN*; pIJ6732 and pIJ6734 are pET11a-derived constructs expressing *bldN* from GTG^1 and ATG^4, respectively.

glkA derivative of the wild-type strain, was constructed by using the method of Buttner et al. (4).

A 2.8-kb *Sma*I fragment (nucleotides 7275 to 10058 from the Sanger Centre sequence) carrying SCO2260, isolated from cosmid C75A, was cloned into *Sma*I-digested pUC19 to give pIJ6735, and a 1.8-kb *Bgl*II *hyg* cassette (conferring resistance to hygromycin) (42) was cloned into the unique *Bcl*I site (at nucleotide 8515) internal to SCO2260 to give pIJ6736. pIJ6736 was digested with *Nde*I, end filled, digested with *Hin*dIII, and cloned into *Eco*RV- and *Hin*dIII-digested pIJ6650 to give pIJ6737.

pIJ6737 was introduced into *S. coelicolor* J1915 (Δ*glkA119*) by conjugation from *E. coli* and exconjugants in which the plasmid had presumptively integrated at the SCO2260 locus by single-crossover homologous recombination were selected with 50 μg of apramycin/ml. After we checked for apramycin and hygromycin resistance and 2-deoxyglucose sensitivity, one such isolate, J2179, was grown nonselectively through four rounds of sporulation and putative SCO2260::*hyg* mutants in which the delivery plasmid had been lost were selected on minimal medium containing 100 mM 2-deoxyglucose and 50 μg of hygromycin/ml. The structure of one null mutant was confirmed by PCR (using the oligonucleotides 5′-TGTCCACCGCCACCGCCCGTC-3′ from SCO2260 and 5′-GCGACGGTGTACGCCACAGCTTG-3′ from the *hyg* cassette) and by Southern hybridization, and the strain was designated J2180.

Construction of J2183 and J2184 (*bldN*::*aadA*). *bldN*-null mutants, in which the entire *bldN* (SCO3323) coding sequence was precisely replaced by *aadA* (conferring resistance to both spectinomycin and streptomycin), were constructed by the PCR-targeted method of Gust et al. (B. Gust, G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater, unpublished data). In this method, *S. coelicolor* genes carried on cosmids in *E. coli* are replaced with a selectable marker generated by PCR with primers with 39-nucleotide gene-specific extensions. The selectable marker cassette also includes an *oriT* which permits the direct transfer of the mutagenized cosmid into *S. coelicolor* by conjugation (Gust et al., unpublished). Cosmid E68 was introduced into *E. coli* BW25113 (9), and *bldN* was disrupted by electroporation with an *oriT*/*aadA* cassette that had been amplified with oligonucleotides that carried *bldN*-specific extensions: 5′-CGTACTGCACGTGATG GAAGCTCTGCCTCTGGGAGTCCCATTCCGGGGATCCGTCGACC-3′ (forward) and 5′-CTTGGGGAACACGAAGGGTGAGCGCCTCTGTGGCG TCTCTGTAGGCTGGAGCTGCTTC-3′ (reverse). The resulting cosmid was introduced into *S. coelicolor* M145 and J1501 by conjugation. Three *bldN*-null mutant derivatives of each strain, generated by double crossing over, were identified by their spectinomycin-resistant, kanamycin-sensitive, and bald phenotypes. Their structures were confirmed by Southern hybridization, and representative *bldN*-null mutant derivatives of M145 and J1501 were designated J2183 and J2184, respectively.

Construction of a SCO5695 (ORF19 on cosmid 5H4) null mutant. A SCO5695 (putative metalloprotease gene) null mutant was constructed by the PCR-directed method of Gust et al. (unpublished), by using an *oriT*/*apr* (apramycin resistance) cassette amplified with oligonucleotides with SCO5695-specific extensions: 5′-GGCGGCACAGACGGCGGAGGCCCGTGCATGACGACCCT GATTCCGGGGATCCGTCGACC-3′ (forward) and 5′-GTCCGGAGGGACA GGCTCTCCGGATCGGGCTTCTCGTCGTGTAGGCTGGAGCTGCTTC-3′ (reverse). The resulting SCO5695::*apr* derivative of cosmid 5H4 was introduced into *S. coelicolor* M145 by conjugation from *E. coli*, and two SCO5695 null mutants, generated by double crossing over, were identified by their apramycin-resistant, kanamycin-sensitive phenotype. After their structures were confirmed by Southern hybridization, one was designated J2182. A SCO5695::*apr* SCO2260::*hyg* double mutant (J2188) was constructed by repeating the disruption of SCO5695 in J2180.

Expression of σ^{BldN} in *E. coli*. A truncated form of σ^{BldN} (starting at ATG^3/Met-87) was overexpressed in *E. coli* by using the pRSET derivative pIJ6722 (1) and purified as described previously (1). Then, 2 mg of this protein was used to raise a polyclonal antiserum in rabbit (Genosys). In addition, two derivatives of pET11a were constructed to express *bldN* from GTG^1 (Met-1) or ATG^4 (Met-88). Pairs of abutting oligonucleotides were used to amplify the entire pIJ6723 plasmid, simultaneously introducing appropriately positioned *Nde*I sites. The PCR conditions were as described above (PCR-based site-directed mutagenesis), and the oligonucleotides used were as follows: 5′-CATATGTACCCACACGT CGGGGTTG-3′ and 5′-GGGACTCCCAGAGGCAGAGC-3′ (GTG^1/Met-1) and 5′-ATGGAGCTGGTCGAGCGGGCC-3′ and 5′-ATGCGGGCGCTGTC GCTGTCC-3′ (ATG^4/Met-88). The resulting plasmids, pIJ6731 and pIJ6733, were confirmed by sequencing, and the *bldN* alleles were removed as *Nde*I-*Bam*HI fragments and ligated to *Nde*I-*Bam*HI-digested pET11a to create pIJ6732 (GTG^1/Met-1) and pIJ6734 (ATG^4/Met-88).

RESULTS AND DISCUSSION

The cloning and characterization of *bldN* raised important questions about its translation (1). *bldN* has four in-frame ATG or GTG potential start codons (GTG^1, GTG^2, ATG^3, and ATG^4; Fig. 1), only one of which, GTG^1, is preceded by a credible ribosome-binding site (GGAG). Translation from GTG^1 would give rise to a σ factor with an unusual N-terminal extension of ca. 86 amino acids that is absent from the other σ

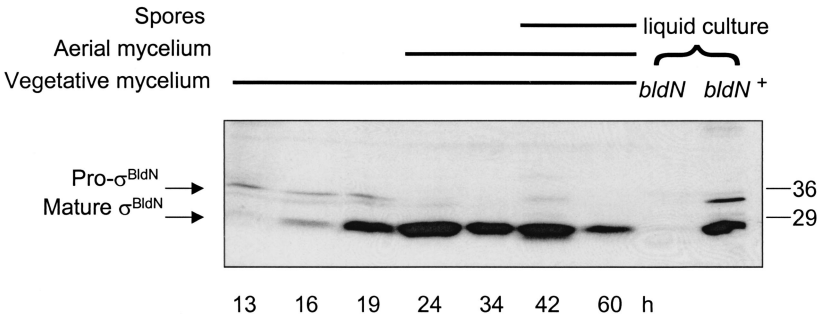

FIG. 2. Immunoblot analysis of σ^{BldN} expression during a developmental time course of *S. coelicolor* M145 grown on R2YE solid medium. The time points at which mycelium was harvested, and the presence of vegetative mycelium, aerial mycelium, and spores, as judged by microscopic examination, are indicated. Control extracts from the *bldN*-null mutant (J2177) and its congenic *bldN*$^+$ parent (J1915), both grown in YEME liquid medium, are shown. The positions of the molecular mass markers are indicated on the right in kilodaltons.

factors. The discovery that R112, the more severe of the two point mutants that originally defined the *bldN* locus, had a wild-type protein coding sequence but carried a GGAG→GGAA mutation in the putative ribosome-binding site strongly implied that at least some *bldN* translation initiated at GTG[1] (1). However, although the two adjacent ATG codons lack a recognizable ribosome-binding site, translation initiation at either codon would give rise to a σ factor approximately co-N-terminal with other σ factors (Fig. 1) (1). Moreover, these two ATG codons are conserved within *adsA*, the orthologue of *bldN* in *S. griseus* (40). These considerations raised the possibility that there might be two independent *bldN* primary translation products, differing in length by 86 or 87 amino acids.

Two proteins of 28 and 35 kDa are encoded by *bldN*. To examine *bldN* translation experimentally, a polyclonal antiserum was raised against purified protein expressed in *E. coli*, initiating at ATG[3], and used in immunoblots against *S. coelicolor* extracts. A time course taken during differentiation of the wild type on R2YE solid medium (Fig. 2) showed early production of a cross-reacting species of apparent molecular mass of 35 kDa and the subsequent appearance of a second species of apparent molecular mass of 28 kDa, which was concomitant with aerial mycelium formation and the disappearance of the larger species. In liquid culture, conditions under which *S. coelicolor* does not sporulate, the 35-kDa species was more persistent (Fig. 2).

The presence of σ^{BldN} was also investigated in a variety of different genetic backgrounds. Immunoblots against single, late time points, in which only the 28-kDa species was present in the *bldN*$^+$ parent strain J1915 (Fig. 3, lane 2), showed that the 28-kDa species was absent from the constructed *bldN*-null mutant, J2177 (Fig. 3, lane 4), and was barely detectable in R112, the *bldN* strain carrying the point mutation in the RBS associated with GTG[1] (Fig. 3, lane 3). In R650, a second *bldN* point mutant carrying a G103D substitution in region 2.1 (Fig. 1) (1), the 35-kDa species was readily detected, despite the late time point (Fig. 3, lane 1; the persistence of the 35-kDa species in R650 was reproducible). Complemention of the null mutant (J2177) with the *bldN* clone pIJ6715 restored normal levels of the 28-kDa species (Fig. 3, lane 5).

The previously observed transcriptional dependence of *bldN* on *bldG* and *bldH* (1) was confirmed and extended at the protein level. σ^{BldN} protein was absent in the *bldH* mutant, WC109 (Fig. 3, lane 6), and was only faintly detectable in the *bldG* mutant, WC103 (Fig. 3, lane 8). In contrast, the 28-kDa species was readily detectable in J1501, the congenic parent of both these strains (Fig. 3, lane 7).

To confirm that both the 28-kDa and the 35-kDa cross-reacting species were products of the *bldN* locus, a strain expressing C-terminally His-tagged BldN was constructed. pIJ6729, a suicide plasmid carrying a 5′-truncated version of *bldN* with a 3′ tag of seven histidine codons, was introduced into M145 by conjugation. Homologous recombination between the two copies of *bldN* resulted in a strain (J2178) in which transcription from the *bldN* promoter gave rise to expression of C-terminally His-tagged BldN. The chromosomal structure of J2178 was confirmed by PCR and sequencing. Immunoblot analysis showed that the His tag caused a de-

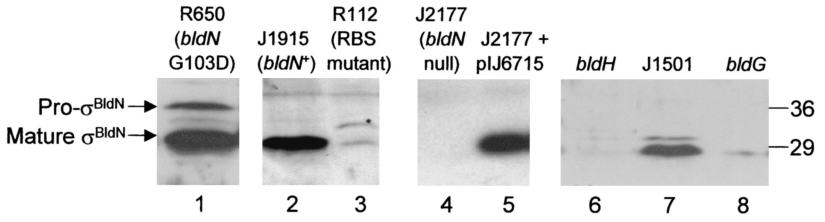

FIG. 3. Immunoblot analysis of σ^{BldN} expression in different genetic backgrounds. The following strains were grown on R2YE solid medium, and late (48 h) samples of mycelium were harvested for analysis: the *bldN*-null mutant J2177 (lane 4); its congenic *bldN*$^+$ parent, J1915 (lane 2); the *bldN*-null mutant J2177 complemented with pIJ6715 (lane 5); the *bldN* point mutants, R112 (lane 3) and R650 (lane 1); the *bldG* (lane 8) and *bldH* (lane 6) mutants and their congenic parent, J1501 (lane 7). The positions of the molecular mass markers are indicated on the right in kilodaltons.

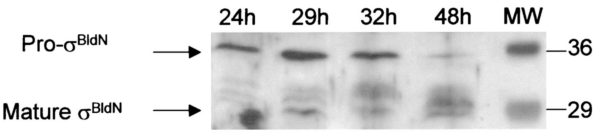

FIG. 4. Immunoblot analysis of σ^{BldN} expression during growth in YEME liquid medium of *S. coelicolor* carrying *bldN* on a multicopy plasmid (pIJ6739). Molecular weight markers (MW) are shown in the rightmost lane in kilodaltons.

crease in mobility of both the 28- and 35-kDa cross-reacting species, confirming that they are *bldN* encoded and co-C-terminal (data not shown). J2178 showed reduced aerial mycelium formation relative to M145, implying that the His tag interfered with σ factor function.

The 35-kDa species was detected only transiently in early time points during differentiation on plates but was more persistent in samples isolated from liquid culture (Fig. 2). To better visualize this species, *bldN* was cloned into the multicopy vector pIJ487 to give pIJ6739. When pIJ6739 was introduced into *S. coelicolor*, it caused only moderate overexpression. Despite this, not only was the 35-kDa species readily detectable in liquid cultures, it was the predominant species in early time points (Fig. 4). The presence of multicopy *bldN* had no obvious phenotypic consequences.

σ^{BldN} is synthesized as a proprotein. The 28-kDa cross-reacting species could have resulted from posttranslational processing of the 35-kDa species or from translation initiation at a second, independent start site. To distinguish between these two possibilities, site-directed mutagenesis was used to remove each potential start codon in turn and to introduce internal stop codons within *bldN*. Each of the resulting *bldN* alleles was cloned into the *attP*$^+$ integrative vector, pSET152, to generate a series of plasmids (pIJ6724-6728) identical to pIJ6715 (carrying wild-type *bldN*) except for the mutation indicated in Fig. 5A.

Each construct was introduced by conjugation into the *bldN*-null mutant, J2177. Although the phenotypes of J2177 carrying pIJ6724-6728 were unambiguous after the primary introduction of the plasmids, on repeated subculture the isolates carrying noncomplementing plasmids began progressively to take on a morphologically wild-type appearance. Because the allele carried by the null mutant J2177 replaced a 66-bp *Xho*I fragment internal to *bldN* with a *hyg* cassette (1) but left the remainder of the gene present (including GTG^1, GTG^2, ATG^3, and ATG^4), we speculated that the progressive change in phenotype might be caused by homogenotization. To prevent homogenotization, a new null mutant (J2184) was constructed in which the entire *bldN* coding sequence was replaced by an *aadA* cassette. The phenotypes of J2184 carrying pIJ6724-6728 were stable, suggesting that homogenotization was indeed the cause of the phenotypic instability associated with J2177 carrying pIJ6724-6728.

pIJ6725, carrying a mutation of GTG^2 to GTC, fully complemented the *bldN*-null mutant J2184 (Fig. 5B) and restored production of the 28-kDa species (Fig. 5C), demonstrating that GTG^2 is not an alternative initiation codon. In contrast, mutation of GTG^1 to GTC (pIJ6724) completely abolished complementation of the *bldN*-null mutant phenotype (Fig. 5B) and no σ^{BldN} protein was detectable by immunoblot (Fig. 5C). Moreover, introduction of a TGA stop codon, either between GTG^1 and GTG^2 or between GTG^2 and ATG^3, also resulted in clones (pIJ6726 and pIJ6727, respectively) that could no longer complement the *bldN*-null mutant (Fig. 5B) and produced no detectable σ^{BldN} protein (Fig. 5C). The absence of σ^{BldN} protein in these strains suggested either (i) that the 28-kDa σ^{BldN} species arose from posttranslational processing of a 35-kDa pro-σ^{BldN} species, or (ii) that both the mutation of GTG^1 and the introduced stop codons were blocking translation initiation at ATG^3 or ATG^4 through polar effects. To distinguish between these two alternatives, ATG^3 and ATG^4 were mutated to two alanine codons (GCGGCG), resulting in pIJ6728 (Fig. 5A). pIJ6728 fully complemented the *bldN*-null mutant (Fig. 5B) and restored wild-type levels of the 28-kDa σ^{BldN} species (Fig. 5C). These data are therefore consistent with the 28-kDa species (mature σ^{BldN}) arising by proteolytic processing of the 35-kDa species (pro-σ^{BldN}).

Pro-σ^{BldN} processing. Translation initiation at GTG^1 would give rise to a primary translation product of predicted molecular mass of 28.6 kDa, whereas translation initiation at ATG^4 would give rise to one of 20.16 kDa. However, because many σ factors have highly aberrant mobilities on SDS-polyacrylamide gels, appearing larger than their actual size, it was not immediately possible to correlate predicted primary translation products with the experimentally observed *bldN*-encoded 28- and 35-kDa apparent molecular masses of the species. Two forms of σ^{BldN} were therefore expressed in *E. coli* and then purified, and their mobilities were compared with the species detected in *S. coelicolor*.

σ^{BldN} protein expressed from GTG^1 (pIJ6732) had the same mobility as the large (35-kDa) species observed in *S. coelicolor*, confirming that pro-σ^{BldN} arises from translation initiation at GTG^1 (Fig. 6). σ^{BldN} protein expressed from ATG^4 (pIJ6734) had an apparent mass of ~26 kDa, 2 kDa smaller than the mature (28-kDa) σ^{BldN} species observed in *S. coelicolor* (Fig. 6). This result suggested that the processing event that gives rise to mature σ^{BldN} occurs close to, but on the N-terminal side of, Met-87 (corresponding to ATG^4; 2 kDa would correspond to ca. 18 residues). Repeated attempts to use nickel affinity chromatography to purify sufficient C-terminally His-tagged, mature σ^{BldN} to determine its N-terminal sequence were unsuccessful.

Attempts to identify the gene encoding the pro-σ^{BldN} processing enzyme. SpoIIGA and SpoIVFB, the proteases in *B. subtilis* responsible for processing pro-σ^E and pro-σ^K, respectively, have been characterized (25, 29, 30, 32). Database searches of the *S. coelicolor* genome failed to identify full-length homologues of these enzymes. However, Rudner et al. (33) and Yu and Kroos (41) assigned SpoIVFB and Site-2 protease (S2P), the enzyme responsible for cleavage of mammalian SREBP at site 2 (31), to a novel family of metalloproteases containing two signature motifs, HEXXH and NXXPXXXXDG. A search of the *S. coelicolor* genome database (http://www.sanger.ac.uk/Projects/S_coelicolor/) revealed two putative metalloproteases containing both motifs: SCO2260 (ORF6 from cosmid C75A) and SCO5695 (ORF19 from cosmid 5H4). To investigate the possibility that one or both of these proteins might be responsible for pro-σ^{BldN} processing, SCO2260 and SCO5695 were disrupted to yield J2180

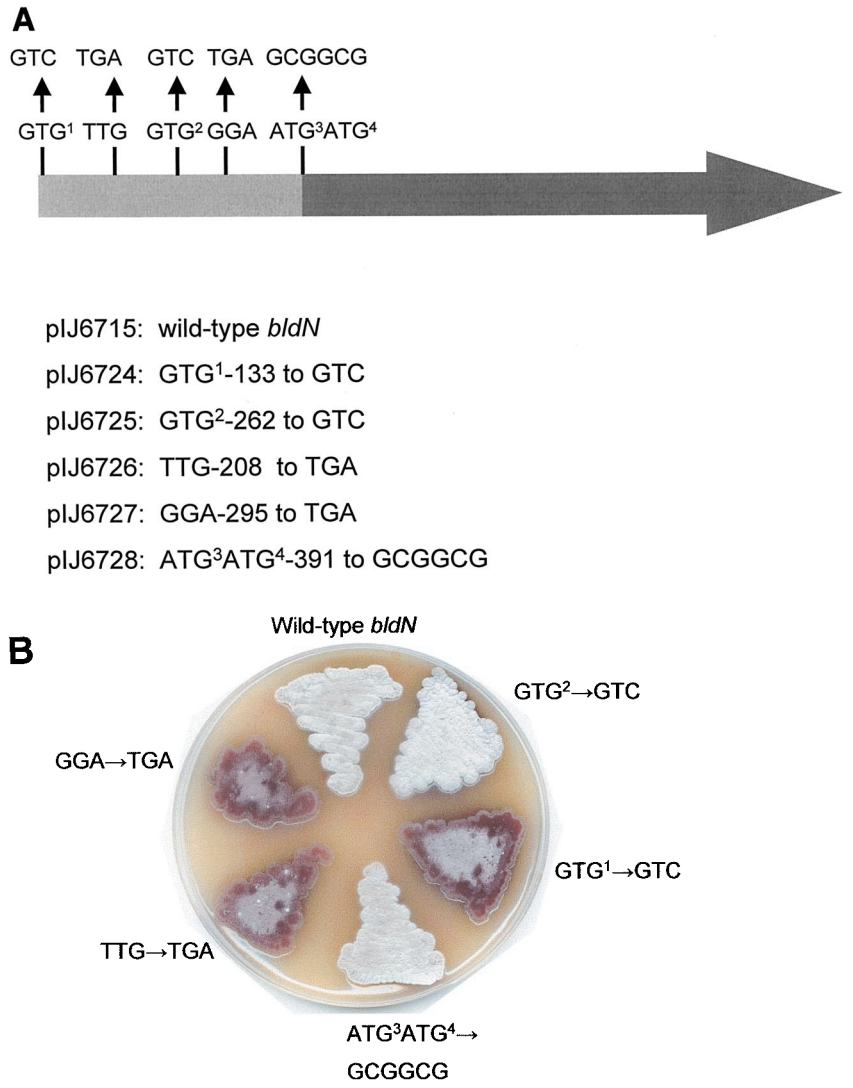

FIG. 5. Mutational analysis of *bldN* translation. (A) Positions of the mutations introduced into *bldN* and the numbers of the plasmids carrying each allele. The numbering of the nucleotides is taken from Bibb et al. (1). (B) Phenotype of the *bldN*-null mutant (J2184) after the introduction of each *bldN* allele, grown on R2YE. (C) Immunoblot analysis of σ^{BldN} expression in the *bldN*-null mutant (J2184) after the introduction of each *bldN* allele. Mycelium was harvested at 48 h from cultures grown on R2YE solid medium. Molecular weight markers (MW) are shown in the leftmost lane in kilodaltons.

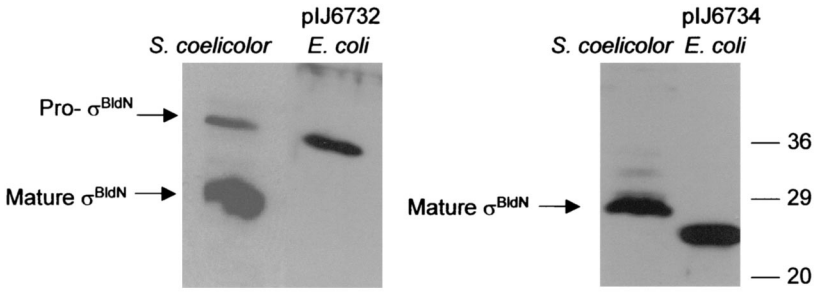

FIG. 6. Immunoblot comparison of the pro-σ^{BldN} and mature σ^{BldN} species observed in *S. coelicolor* with recombinant σ^{BldN} protein expressed in *E. coli* from GTG[1] (pIJ6732) or ATG[4] (pIJ6734). The positions of the molecular mass markers are indicated on the right in kilodaltons.

and J2182, respectively, and a double mutant, J2188, was also constructed. All three strains were unaffected in pro-σ^{BldN} processing and were morphologically wild type (data not shown).

Conclusions. Using a combination of site-directed mutagenesis and immunoblot analysis, we have provided evidence that all *bldN* translation arises from initiation at GTG[1], and that the primary translation product is a proprotein (pro-σ^{BldN}) that is proteolytically processed to mature σ^{BldN} by removal of most of an unusual ~86-residue N-terminal extension. In the absence of the N-terminal sequence of the 28-kDa species (mature σ^{BldN}), it is still possible that the change in SDS-polyacrylamide gel migration could reflect intein processing or a chemical modification that dramatically alters the mobility of the primary translation product, although we consider both of these possibilities unlikely. During differentiation on solid medium, pro-σ^{BldN} is detected in early time points and mature σ^{BldN} appears subsequently, concomitant with aerial mycelium formation and the disappearance of pro-σ^{BldN}. Thus, σ^{BldN} is developmentally regulated at at least two levels: transcription initiation and posttranslational processing. This is the first report of pro-σ factor processing in a bacterium outside of the genus *Bacillus*.

Placing *bldN* on a multicopy plasmid caused only mild overexpression of the protein, perhaps implying titration of the factors under the control of *bldG* and *bldH* that are required for activation of its transcription. Despite this, the low level of overexpression caused pro-σ^{BldN} to accumulate, suggesting that the processing enzyme is readily saturated. Pro-σ^{BldN} also persisted in the *bldN* point mutant R650, showing that the σ^{BldN} G103D substitution in this strain has a weak negative influence on pro-σ^{BldN} processing. Growth in liquid culture, conditions under which *S. coelicolor* does not sporulate, also led to increased persistence of pro-σ^{BldN}.

We identified two *S. coelicolor* genes encoding putative members of a family of metalloproteases that includes SpoIVFB and S2P, the proteases responsible for cleavage of pro-σ^{K} and SREBP Site-2, respectively. Both genes were dispensable for differentiation and pro-σ^{BldN} processing. However, the proteases involved in the regulated proteolysis of transcription factors are likely to be diverse, since SpoIVFB and S2P are not related to the pro-σ^{E} processing enzyme, SpoIIGA. There are no homologues of SpoIIGA encoded by *S. coelicolor*.

In other systems, transcription factor processing serves to coordinate transcription factor activity with other events. It will be interesting in the future to identify mutants that are blocked in pro-σ^{BldN} processing and to use these mutants to begin to understand how pro-σ^{BldN} processing is controlled during differentiation.

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