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Accumulation of *bldA*-Specified tRNA Is Temporally Regulated in *Streptomyces coelicolor* A3(2)

B. K. LESKIW,[1,2]* R. MAH,[1] E. J. LAWLOR,[2]† AND K. F. CHATER[2]

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9,[1] and John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom[2]

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Deletion of the *bldA* gene of *Streptomyces coelicolor* A3(2), which encodes the only tRNA for the rare UUA codon, had no obvious effects on primary growth but interfered with aerial mycelium formation and antibiotic production. To investigate the possible regulatory role of *bldA*, its transcription start point was identified, and time courses were determined for the appearance of its primary transcript, the processing of the primary transcript to give a mature 5′ end, and the apparent efficiency of translation of *ampC* mRNA, which contains multiple UUA codons. The *bldA* promoter was active at all times, but processing of the 5′ end of the primary transcript was comparatively inefficient in young cultures. This may perhaps involve an antisense RNA, evidence of which was provided by promoter probing and in vitro transcription. The presence of low levels of the processed form of the tRNA in young cultures followed by increased abundance in older cultures contrasted with the pattern observed for accumulation of a different, presumably typical tRNA which was approximately equally abundant throughout growth. The increased accumulation of the 5′ processed form of *bldA* tRNA coincided with more-efficient translation of *ampC* mRNA in older cultures, supporting the hypothesis that in at least some physiological conditions, *bldA* may have a regulatory influence on events late in growth, such as morphological differentiation and antibiotic production.

Streptomycetes are mycelial gram-positive soil bacteria that have received much attention because of their ability to produce a wide array of secondary metabolites, including many antibiotics. Their growth on agar medium is characterized in the late stages by differentiation to form spore-bearing aerial hyphae. The activation of antibiotic production, sometimes involving many different pathways in the same organism, often coincides with the onset of aerial growth. However, relatively little is understood to date about the mechanisms by which multiple, distinct antibiotic biosynthetic gene sets are activated coordinately, either with each other or with morphological differentiation. These mechanisms are both biologically interesting and potentially relevant to the efficient and cost-effective industrial production of antibiotics.

In *Streptomyces coelicolor* A3(2), genetically the most studied of streptomycetes, the existence of various classes of mutants pleiotropically defective in both morphological differentiation and antibiotic production implies that these processes have common regulatory elements (8, 25). Among the genes identified by these mutants, the best understood is *bldA*, which encodes a leucyl tRNA that recognizes the UUA codon (35, 37). This codon is rare in DNA from *Streptomyces* spp., which is particularly rich in G+C (ca. 73 mol%). The fact that although *bldA* point mutations prevent the formation of normal aerial hyphae and block antibiotic production, they have no apparent effect on vegetative growth of *S. coelicolor* led to the prediction that genes needed for primary growth of *S. coelicolor* should be devoid of TTA codons. Indeed, when the sequences of 100 known *Streptomyces* genes were scanned, the occurrence of TTA codons was shown to be nonrandom with respect to the class of genes concerned (36). Most of the TTA-containing genes specify antibiotic-resistance proteins or antibiotic-pathway-specific regulators associated with the production of antibiotics, including actinorhodin (15), bialaphos (40), streptomycin (14), and spiramycin (19). Such products would be expected to be required only under conditions associated with secondary metabolism, such as the cessation of rapid growth and the absence of repressing conditions such as excessive phosphate, nitrogen, or carbon (12). In addition to this circumstantial evidence, the suggestion that the *bldA* gene product plays a positive regulatory role in morphological differentiation and antibiotic production came from RNA dot blot analysis showing accumulation of *bldA*-specific RNA only in older cultures (35) and from the fact that expression of structural genes for actinorhodin, undecylprodigiosin, and methylenomycin A is transcriptionally blocked in *bldA* mutants (3, 21, 47a). Taken together, these observations have led to the suggestion that *bldA* represents a developmental regulatory mechanism that operates at the translational level through regulatory proteins, such as the transcriptional activator for actinorhodin biosynthesis (15), that require the *bldA*-encoded tRNA for their translation. Evolution of such a regulatory mechanism is intriguing in view of the striking contrast between the predicted pattern of *bldA* expression and the expected stringent-response-mediated down-regulation of most tRNAs during nutritional stress (5), but it is worth noting that this global picture is not without exceptions even in *Escherichia coli*, in which the leucyl $tRNA_1$ preferentially accumulates during starvation for methionine (11). A possible wider occurrence of *bldA*-like translational regulatory devices is suggested by the discovery that *Clostridium acetobutylicum* mutants lacking the tRNA for a rare threonine codon are viable but deficient in sporulation and secondary metabolism (43) (*Clostridium* spp. contain 30 to 40 mol% G+C in their DNA).

Here, we show that accumulation of the active form of *bldA* tRNA is temporally regulated during liquid and surface

* Corresponding author.
† Present address: SmithKline Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey RH3 7AJ, United Kingdom.

growth in a rich culture medium, in a pattern that contrasts with that of lysine tRNA (taken to be typical of most tRNAs). We also provide evidence to suggest that under the conditions used, translation of UUA codons in the transcript of a TTA-containing reporter gene, *ampC*, is impaired in very young cultures.

MATERIALS AND METHODS

Chemicals and enzymes. All electrophoresis chemicals were ultrapure grade. Restriction endonucleases and DNA-modifying enzymes were from Boehringer Mannheim. S1 nuclease was from Sigma. Nitrocefin was from Oxoid Unipath Limited, Basingstoke, England, and thiostrepton was a gift from S. Lucania, Squibb Institute for Medical Research, Princeton, N.J.

Bacterial strains, culture conditions, and transformation procedures. *S. coelicolor* A3(2) strains were J1501 (*hisA1 uraA1 strA1 pgl* SCP1 SCP2 [10]) and its derivatives J1700 (*bldA39* [39]) and J1681 (Δ*bldA*; this study). *Streptomyces lividans* 66 TK24 (*str-6* SLP2$^-$ SLP3$^-$ [27]) was the host for promoter-probing studies with pIJ486 and pIJ487 (see below). *E. coli* JM101 (41) was the host for pUC plasmids and M13 phages. For some purposes, *Streptomyces* cultures were grown on cellophane discs (45) on the surface of R2YE (26) agar. Yeast extract-malt extract (YEME) medium (26) was used for *Streptomyces* liquid culture. Protoplasting and transformation of *Streptomyces* strains were as described previously (26). *E. coli* strains were cultured and transformed by using standard procedures (41). Kanamycin gradient plates were prepared as described elsewhere (47).

Plasmids and phages. *E. coli* plasmid pIJ5606 (a pUC18 derivative containing *ermE*; generously provided by C. Khosla) was the source of *ermE*, which confers resistance to erythromycin and lincomycin (13), and pIJ580 (0.87-kb *Bgl*II-*Pst*I fragment containing *bldA* [35] inserted into the *Bam*HI and *Pst*I sites of pUC18 [48]) was the template for the polymerase chain reaction (PCR) amplification of *bldA*. *Streptomyces* plasmids pIJ486 and pIJ487 (47) were used as cloning and promoter probe vectors, and pJAS14-P15 (a gift from M. Forsman and B. Jaurin; see reference 37) was used as a source of *ampC* (34). A high-copy-number plasmid derivative containing *ampC* (named pAU2) was constructed by subcloning *ampC* from pJAS14-P15 (as a ca. 6-kb *Eco*RI-*Bgl*II fragment) into *Eco*RI-*Bam*HI-digested pIJ486. Plasmids pIJ583 to pIJ588 (Fig. 1) were pIJ486 or pIJ487 derivatives containing all or part of the previously sequenced 873-bp *bldA*-containing *Bgl*II-*Pst*I fragment (35). The inserts in the pIJ486 derivatives pIJ583 and pIJ584 were obtained by *Bgl*II digestion of pIJ582, consisting of the pUC18 derivative pIJ2921 (31) containing the *Bgl*II-*Pst*I fragment from pIJ580 inserted between its *Bam*HI and *Pst*I sites. The inserts in pIJ585 to pIJ588 were all from pIJ581, a pIJ580 derivative with the *Nco*I site upstream of *bldA* converted to a *Bgl*II site by filling in and ligation to a *Bgl*II linker. The final constructions were as follows: pIJ585, *Eco*RI-*Bgl*II (formerly *Nco*I) fragment from pIJ581 inserted into *Bam*HI-*Eco*RI-cleaved pIJ486; pIJ586, the same fragment inserted into *Bam*HI-*Eco*RI-cleaved pIJ487; pIJ587, a *Bgl*II (formerly *Nco*I)-*Hin*dIII fragment from pIJ581 inserted into *Bam*HI-*Hin*dIII-cleaved pIJ486; and pIJ588, the same fragment inserted into *Bam*HI-*Hin*dIII-cleaved pIJ487. *Streptomyces* plasmids were maintained by selection for thiostrepton resistance (26).

Construction of the *bldA* deletion mutant J1681. A ca. 1.7-kb *ermE*-containing, blunt-ended fragment from pIJ5606 was inserted into pIJ581 that had been digested with *Bgl*II and *Sac*II and blunt ended, to replace the *bldA*-encoded tRNA sequence (remaining flanking sequences available for recombination were 540 bp upstream and 160 bp downstream of the inserted *ermE*-containing fragment). The recombinant plasmid was isolated after transformation of *E. coli* JM101, and the insert was subcloned as an *Eco*RI-*Pst*I fragment into M13mp19 (48). Single-stranded DNA isolated from a 1.5-ml culture (41) was used without quantitation to transform approximately 10^9 protoplasts of *S. coelicolor* J1501 (23). After 16 h of incubation, the plates were flooded with 1 ml of an aqueous solution of lincomycin (2 mg/ml). A few lincomycin-resistant transformants, potentially resulting from a double-crossover event (Fig. 1A), were obtained. Replacement of the *bldA* gene by *ermE* in the chromosome of J1681 was verified by Southern analysis of chromosomal DNA digests (41).

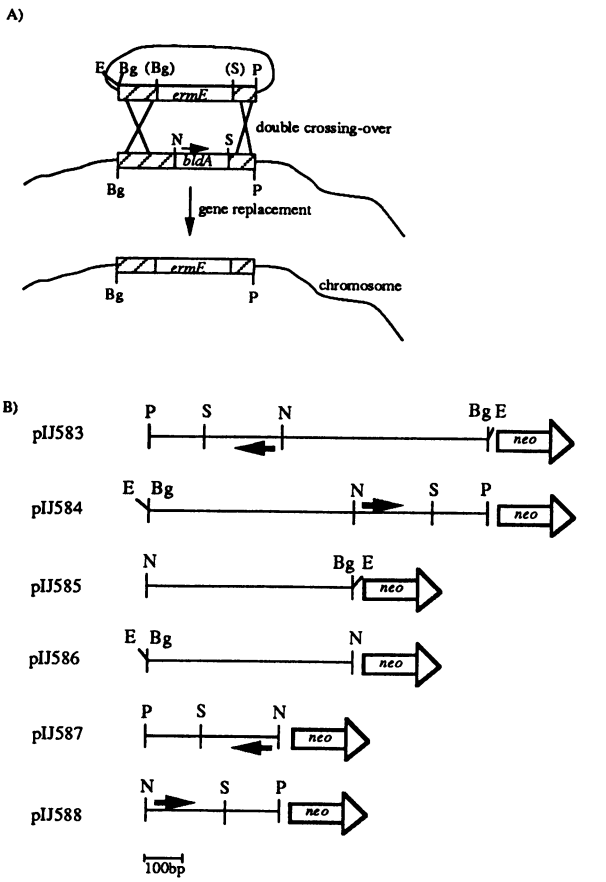

FIG. 1. Genetic manipulations involving *bldA*. The position and orientation of *bldA* are shown by an arrowhead. (A) Strategy for construction of a *bldA* deletion mutant. The replacement by *ermE* of the *Bgl*II-*Sac*II fragment containing *bldA*, which involved destruction of the *Bgl*II (Bg) and *Sac*II (S) sites in pIJ581, is described in Materials and Methods (brackets denote destroyed sites). (B) Fragments inserted next to the *neo* reporter gene of pIJ486 or pIJ487. Details of the constructions are given in Materials and Methods. E, *Eco*RI (located in the multiple cloning site of pUC18); P, *Pst*I; N, *Nco*I (in pIJ581 this site was converted to a *Bgl*II site [see Materials and Methods]).

Preparation of cell extracts. Cultures of *S. coelicolor* J1501 (*bldA*$^+$) containing plasmid pAU2 were grown at 30°C and 250 rpm in 25 ml of YEME medium (in a 250-ml flask)

containing thiostrepton at 5 μg/ml. A large volume of medium was inoculated to a final concentration of 4.6×10^6 spores per ml and then dispensed into the flasks to standardize the inoculum (enough flasks were inoculated to serve as a source of culture for RNA isolation as well [see below]). At 15, 18, 24, 30, 36, 42, 48, and 54 h after incubation, samples of the cultures were collected in duplicate, and cell extracts were prepared as previously described (18), except that lysozyme was not used. The final resuspension volume varied from 0.25 to 1.0 ml, depending on the culture density. The amounts of protein were determined by the method described by Bradford (2). Extracts were also prepared from *S. coelicolor* J1700 (*bldA*) containing pAU2 and from J1501 containing pIJ486, except that the cultures were sampled at 12-h intervals beginning at 24 h. *S. coelicolor* J1501 containing pAU2 was also grown on the surface of cellophane discs on R2YE agar plates, and extracts were prepared, in duplicate, as described above after the mycelium was scraped into 1.5-ml microcentrifuge tubes (as with the liquid cultures, enough plates were inoculated for RNA isolation).

Detection and assay of β-lactamase activity. β-Lactamase activity was determined spectrophotometrically with nitrocefin as a substrate as described previously (18). Specific β-lactamase activity is expressed as milliunits per milligram of protein.

DNA procedures. Standard procedures were used for both *S. coelicolor* (26) and *E. coli* (41).

In vitro runoff transcription. In vitro runoff transcription was performed either with (prebinding) or without (free-start) equilibration of RNA polymerase and the DNA template as described by Buttner et al. (4) and Janssen et al. (32), respectively. RNA polymerase that had been purified from exponentially growing *S. coelicolor* A3(2) but not fractionated by promoter specificity was generously provided by M. J. Buttner. Purified DNA templates were prepared from pIJ580 and included three fragments: *Sma*I-*Hin*dIII, *Sma*I-*Pst*I, and *Eco*RI-*Pst*I (see Fig. 2A). The *Sma*I-*Hin*dIII fragment extends 12 bp to the right, and the *Eco*RI-*Pst*I fragment extends 18 bp to the left of the ends of the *Sma*I-*Pst*I fragment.

RNA isolation. RNA was extracted essentially as described elsewhere (26), except that liquid cultures were diluted with ice water, harvested by centrifugation at 4°C, and washed once with ice-cold distilled water, and mycelium from surface-grown cultures was scraped directly from cellophane discs into modified Kirby mix. For the *S. coelicolor* J1501/pAU2 liquid and surface culture time courses, YEME medium and R2YE agar plates were inoculated and harvested at various times as described above (see preparation of cell extracts). RNA was also isolated at 15, 24, 36, 48, and 60 h from surface cultures of *S. coelicolor* J1501 without pAU2 and at 36 and 48 h from *S. coelicolor* J1681.

High-resolution S1 nuclease mapping. The probe for S1 nuclease mapping of *bldA* was generated by PCR amplification of a 206-bp fragment with pIJ580 as template. The primers were an 18-mer synthetic oligonucleotide (5′ CGG AGCCGGACTTGAACC 3′) corresponding to a sequence near the 3′ end of the *bldA*-encoded tRNA and a 28-mer synthetic oligonucleotide (5′ CATGGATCCACCCGGTAA CTGATGCACC 3′) corresponding to a region upstream of the *bldA* promoter and containing a 9-nucleotide (nt) nonhomologous extension (designed by M. J. Bibb [1a]). The amplified DNA was purified from excess oligonucleotides by Sephadex G-75 chromatography. The 5′ ends of the amplified DNA (about 5 pmol) were labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. The probe, labeled at both ends, was used without further treatment, since the nonhomologous extension would be removed by S1 nuclease treatment and would not result in the appearance of labeled, protected fragments (this was verified in trials with PCR-amplified probe prepared after end labeling of the 18-mer oligonucleotide only). A sequence ladder was generated by the dideoxy chain termination method (42) with the 18-mer oligonucleotide as primer and, as template, M13mp18 single-stranded DNA containing the cloned DNA from pIJ580. For each S1 nuclease reaction, 20 to 40 μg of RNA was hybridized to 50,000 Cerenkov cpm of the probe in formamide buffer as described elsewhere (26), except that the hybridizations were carried out overnight and that glycogen (Boehringer Mannheim) replaced the carrier tRNA. Controls included yeast tRNA (Sigma) and RNA extracted from the *bldA* deletion mutant J1681. To eliminate cross-protection by partially homologous, highly abundant tRNA species, stringency was adjusted by varying the hybridization temperature at approximately 5°C increments until no signal was detected with the RNA isolated from the *bldA* deletion mutant. The samples were run under standard conditions on a 6% polyacrylamide sequencing gel.

Northern (RNA) blot analysis. For Northern blot analysis of both the *lysT*α-specified tRNA (44) and the *bldA*-specified tRNA, total RNA (5 μg) isolated from surface-grown cultures of *S. coelicolor* J1501 (containing pAU2) was separated in an 8% polyacrylamide gel containing 8 M urea. RNA isolated from a 36-h *S. lividans* culture grown in liquid medium (generously provided by Astrid Petrich) was a positive hybridization control for the *S. lividans lysT*α-specified tRNA. Negative controls included yeast tRNA and, for *bldA*-specific hybridization, RNA isolated from J1681. The RNA was denatured for 3 min at 90°C in formamide dye mix (41) before loading. Plasmid pBR322 (150 ng), digested with *Hpa*II, was treated in a similar manner and used as a size standard. Transfer to Hybond-N (Amersham) was done by electroblotting with the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) for 30 min at 3 mA/cm^2. The filter pieces containing the molecular-weight markers were then excised and treated separately. For detection of the *lysT*α-specified tRNA, hybridization analysis was performed at 37°C according to procedure B described in reference 26 with the [γ-^{32}P]ATP-labeled oligonucleotide 5′-CCAGGGACTCGAACCCCG, complementary to the 3′ end of the *lysT*α tRNA, as a probe (44). For detection of the *bldA*-specified tRNA, the same blot was stripped of the *lysT*α probe and hybridized, under the same conditions, with the [γ-^{32}P]ATP-labeled oligonucleotide 5′-TTAAGCTCGCCGTGTCT (complementary to the region between the D-loop and anticodon loop of the *bldA* tRNA). The size standards were detected by hybridization with an [α-^{32}P]dATP, random primer-labeled probe of pBR322 DNA.

Dot blot analysis of *ampC* transcripts. Total RNA (8 μg) was denatured as described previously (47) and applied to nitrocellulose with a dot blot apparatus. After 2 h of baking at 80°C in a vacuum oven, hybridization was performed at 47°C according to the procedure described by Ward et al. (47) with an [α-^{32}P]dATP, random primer-labeled probe of a 410-bp *Hin*dIII-*Xho*I fragment internal to the *ampC* gene. After hybridization, the nitrocellulose filter was washed as described elsewhere (47). To ensure that the signals resulted from hybridization to RNA transcripts, one sample was treated with DNase-free RNase. After autoradiography, RNA accumulation was quantified by densitometry of the exposed films with a BioImage XRS 3cx scanner (Millipore).

RESULTS

Phenotype of a *bldA* deletion mutant. The entire *bldA* gene was replaced in the *S. coelicolor* J1501 chromosome by *ermE* to give strain J1681 (Fig. 1A). The resulting phenotype during growth on agar plates was indistinguishable from that conferred by previously studied *bldA* point mutations (35, 38): vegetative growth on minimal or rich medium was unimpaired, but no red or blue pigment developed on prolonged culture (indicating absence of undecylprodigiosin and actinorhodin antibiotic production, respectively), and aerial mycelium did not form, except when mannitol replaced glucose as the carbon source in minimal medium. These observations strengthen the hypothesis that the *bldA* gene product plays no important role in vegetative growth but is essential under these conditions for antibiotic production and differentiation. To investigate this hypothesis further, we carried out a series of experiments addressing *bldA* transcription and the processing and activation of its gene product, $tRNA^{Leu}_{UUA}$, at different growth stages.

Promoter probing reveals apparent bidirectional transcription proceeding through a region just upstream of *bldA*. A conveniently located *Nco*I site a few base pairs upstream of *bldA* provided a junction point for fusion with a transcriptional reporter gene, *neo*, present in the *Streptomyces* plasmids pIJ486 and pIJ487 (Fig. 1B). The regions to the left and right of this site, extending to a *Bgl*II site and a *Pst*I site, respectively, as well as the whole 873-bp *Bgl*II-*Pst*I fragment containing *bldA*, were tested in both orientations. The plasmids were introduced into *S. lividans* 66 (strain TK24), the usual host for promoter-probing experiments with pIJ486 and pIJ487 [*S. lividans* 66 is very closely related to *S. coelicolor* A3(2), and *bldA* mutants of both have similar phenotypes (37)]. The resulting strains were streaked on kanamycin gradient plates to detect promoter activity (data not shown). Although the inserts all gave at least slightly increased resistance, the effect was most marked with pIJ586 and pIJ587; these allowed growth at up to about 10 to 20 μg of kanamycin per ml, compared with less than 1 μg/ml for the vector pIJ487. These results indicated that, surprisingly, there was transcription in both directions through the *Nco*I site: rightward, presumably from the *bldA* promoter (pIJ586) and—perhaps slightly less strongly—leftward (pIJ587) to generate antisense RNA to the primary *bldA* transcript. Apart from providing evidence of promoter activity per se, these results indicated that the promoters were probably active even in young cultures, in order to allow resistant growth to take place.

Transcript mapping of the *bldA* promoter region. To localize the *bldA* sense and antisense promoters, in vitro runoff transcription was performed with purified but unfractionated RNA polymerase from *S. coelicolor* (Fig. 2A). By using templates differing in length at one end or the other, potential runoff transcripts could be recognized and their directions could be deduced by the occurrence of predictable shifts in electrophoretic mobility. Some bands were not accountable in this way and were considered to be artifactual. However, three kinds of potential runoff transcripts (a, b, and c in Fig. 2) were observed (alternative explanations invoking run-on transcription initiated at the end of the template and terminating within the template are possible but less likely in view of the evidence of promoter activity from promoter-probing experiments). One of the transcripts initiated approximately 70 bp upstream of the *bldA* gene and extended rightward, consistent with its being the *bldA* transcript, while the other two were transcribed leftward and included one (presumably

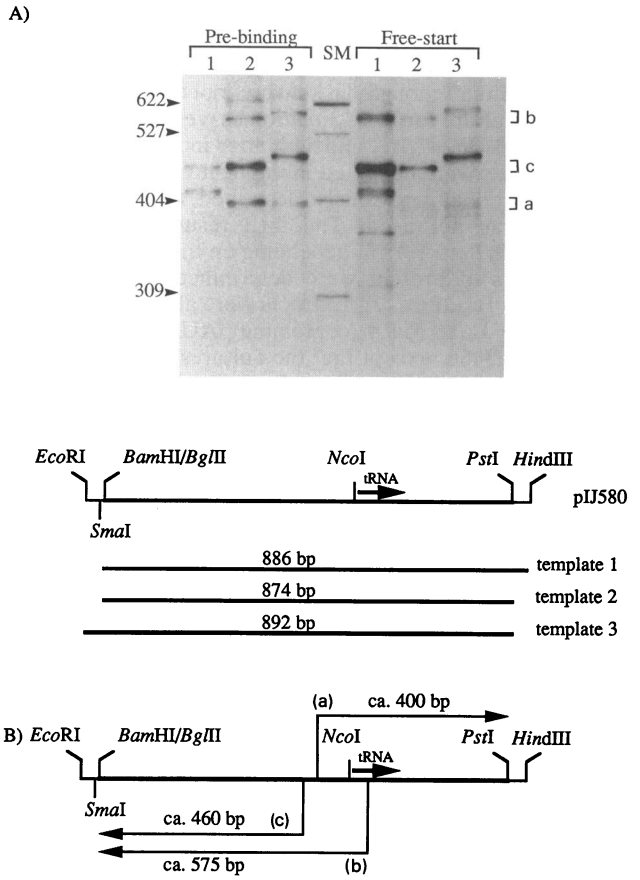

FIG. 2. Localization of the *bldA* transcription start point by in vitro runoff transcription. (A) Transcripts observed by using templates 1 (lane 1), 2 (lane 2), and 3 (lane 3). The cloned *Bgl*II-*Pst*I fragment carrying the *bldA* gene inserted into the polylinker region of pIJ580 and the purified templates used for in vitro runoff transcription studies are shown below the autoradiograph. Three fragments, *Sma*I-*Hin*dIII, *Sma*I-*Pst*I, and *Eco*RI-*Pst*I, are templates 1, 2, and 3, respectively. Template 1 extends 12 bp to the right, and template 3 extends 18 bp to the left of template 2. SM, end-labeled *Hpa*II-digested pBR322 size markers (the sizes [in base pairs] of the marker bands are shown to the left of the autoradiograph); Pre-binding, RNA polymerase and DNA template preequilibrated prior to initiation of transcription; Free-start, no equilibration of RNA polymerase and DNA template (see Materials and Methods). (B) Schematic diagram showing the approximate length and direction of transcription deduced for each of three transcripts (a, b, and c) observed in panel A. The locations of the bands corresponding to the transcripts are marked in panel A.

from the antisense promoter) that initiated approximately 25 to 30 nt downstream of the first nucleotide of the mature *bldA* tRNA (Fig. 2B).

The apparent start site for *bldA* sense transcription was further localized by high-resolution S1 mapping, with RNA isolated from cultures of *S. coelicolor* J1501 grown for 15 h on the surface of R2YE agar (Fig. 3). RNA samples were hybridized with a 206-bp PCR-generated fragment labeled at the 5′ ends (see Materials and Methods for explanation of why the fragment was not uniquely labeled at one end only, prior to S1 nuclease treatment). Two major protected fragments were detected. The smaller, 80 nt long, corresponds to the predicted 5′ end of the mature form of the *bldA*-encoded tRNA (35); the larger, 149 to 150 nt long, corresponds to a

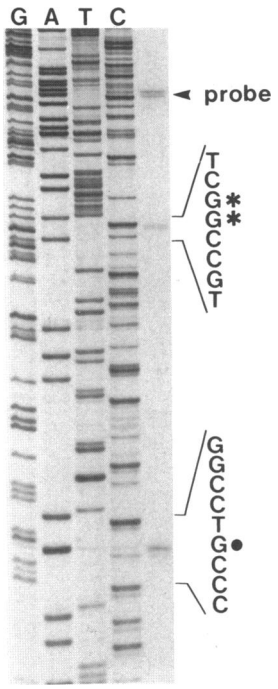

FIG. 3. S1 nuclease mapping of the *bldA* primary transcript and mature tRNA in an RNA sample (40 μg) isolated at 15 h from surface-grown cultures of *S. coelicolor* J1501. A dot and asterisk indicate 5′ ends of S1 nuclease-resistant fragments corresponding, respectively, to the mature tRNA and a transcript previously observed (Fig. 2) by in vitro runoff transcription studies (primary *bldA* transcript). A sequence ladder, which was generated with the 18-mer oligonucleotide used for probe preparation, is shown. The doublet of bands corresponding to full-length probe results from strand separation in the denaturing polyacrylamide gel.

FIG. 4. DNA sequence of the *bldA* region. Primers used for PCR amplification of a 206-bp probe for S1 nuclease mapping are shown. IVT and S1*, the locations of the 5′ ends of transcripts observed by in vitro transcription studies and by S1 nuclease mapping, respectively. Closed circles indicate the locations of the 5′ ends deduced for bands whose appearance and intensity varied in different experiments (see Fig. 5 and 8). The potential −10 and −35 regions discussed in the text are indicated.

potential in vivo transcription start point 69 to 70 nt upstream of the 5′ end of the mature tRNA. There was no evidence of *bldA* promoter activity from upstream of the probe, since this would have generated protected DNA 9 nt shorter than the bands due to reannealed probe. The DNA sequence in the region of the presumptive transcription start site for the *bldA* gene is shown in Fig. 4. Upstream of the location of the 5′ end of the largest S1-protected fragment, the only −10 promoter-like sequence is the TAAAAT hexamer separated by an unusually large interval, 11 nt, from the transcription start point. This hexamer is separated by 16 bp from a CTGATG hexamer, which might act as a −35 sequence for RNA polymerase interaction (see Discussion). The putative promoter sequence shows similarity (4 of 6 nt in the −35 region and 6 of 6 nt in the −10 region) to a previously identified *E. coli*-like *Streptomyces* promoter, SEP2 (33). It should be noted that SEP2 is also separated by a long interval, 10 nt, from its transcription start point. An additional series of bands 21 to 23 nt upstream of the beginning of the tRNA (shown by the open circle in Fig. 5 and 8A, B, and C) was sometimes seen, but the presence and intensity of these bands in identical RNA samples varied from experiment to experiment and with various concentrations of S1 nuclease (data not shown), suggesting that they do not represent an in vivo RNA 5′ end. Nevertheless, they must represent *bldA*-specific transcripts, the extra 5′ ends in Fig. 5 and 8 corresponding to sites exposed to S1 nuclease attack. We could not confidently identify any sequence features that would strongly suggest secondary structures affecting this region, but it is possible to envisage complex multiple interactions among sense RNA, antisense RNA, and DNA probe that could account for the bands.

The abundance of the *bldA* tRNA is temporally regulated in surface-grown cultures. To investigate in more detail the indication that *bldA* tRNA accumulates relatively late in growth (35), S1 nuclease protection studies were performed with RNA isolated at various times from surface-grown cultures of *S. coelicolor* J1501 (without pAU2). Experiments were carried out to define hybridization conditions in which the signals observed would definitively represent *bldA* rather than cross-hybridization to related leucyl tRNA species. RNA isolated from the *bldA* deletion mutant, J1681 (harvested at 36 h), was included as a control, and duplicate RNA samples were hybridized at different temperatures in formamide-containing hybridization buffer (Fig. 5). With the 50°C experiment (Fig. 5A), a signal could be seen with RNA from J1681, and the intensity of the band corresponding in size to that expected for the 5′ end of the mature *bldA* tRNA was high in RNA isolated from *S. coelicolor* J1501 at early time points and gradually decreased in the 48- and 60-h samples. However, a different pattern was observed when the hybridization temperature was raised to 56°C (Fig. 5B). The intensity of all of the bands corresponding to the size of the mature tRNA decreased slightly, as might be expected for a relatively short hybrid at higher stringency, but the proportion of the signal lost from young J1501 cultures (15 and 24 h) appeared to be greater than that seen with the RNA samples from later time points. Even prolonged exposure did not reveal a signal with RNA from the *bldA* deletion mutant J1681. In these J1501 cultures aerial mycelium was first visible at 36 h. We interpret these results to indicate that at 50°C much of the signal in the earlier time samples was due to other (perhaps leucyl) tRNA species, which were presumably maximally abundant during the period of most-rapid growth; this nonspecific signal was eliminated at higher stringency, revealing a temporal increase in abundance of the mature *bldA*-encoded tRNA. The signals corresponding to the 5′ end of the primary transcript were unaffected by the increase in stringency. These results were reproducible in separate experiments, so all subsequent S1 nuclease protec-

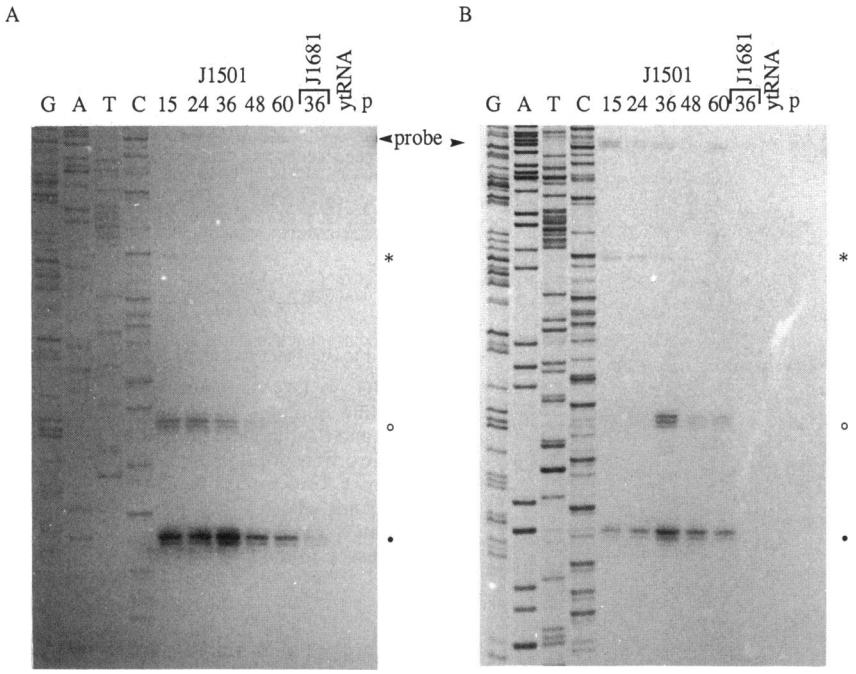

FIG. 5. Selective hybridization to determine the temporal pattern of abundance of *bldA*-specific signals. Duplicate sets of RNA samples (40 μg) isolated at 15, 24, 36, 48, and 60 h from surface-grown cultures of *S. coelicolor* J1501 and at 36 h from *S. coelicolor* J1681 were hybridized with the PCR-generated probe at 50°C (A) and 56°C (B). *, protected fragment corresponding to the primary transcript; ●, 5′ end of mature tRNA; ○, a series of bands whose presence and intensity in identical RNA samples varied from experiment to experiment and with various concentrations of S1 nuclease; probe, location of full-length probe. Controls included yeast tRNA (ytRNA) and probe (p) hybridized in the absence of RNA and treated with S1 nuclease.

tion studies employed the more-stringent hybridization conditions.

Expression of a tRNA for an abundant codon is not temporally regulated. The temporal pattern of accumulation of the *bldA*-specified tRNA, which recognizes a very rare codon in *Streptomyces* mRNA, appeared to be opposite, under these conditions, to that expected for tRNAs in general, which are expected to be abundant during rapid growth. To provide independent evidence of this observed pattern and to verify that it was not typical of all tRNA species, the accumulation of a tRNA recognizing an abundant codon was compared with that of the *bldA*-specified tRNA by Northern blot analysis. The *lysTα* tRNA from *S. lividans*, which recognizes the most abundant lysine codon, AAG (44), was chosen for comparison. Total RNA samples isolated from surface cultures of *S. coelicolor* J1501 containing pAU2 (these samples were used for the S1 nuclease studies shown in Fig. 8C) were subjected to Northern blot analysis. The probes were end-labeled oligonucleotides complementary to the *lysTα* tRNA and the *bldA* tRNA sequences (see Materials and Methods). As seen in Fig. 6, a band corresponding in size to the *lysTα* tRNA was approximately equally abundant throughout growth. In contrast, when the same blot was probed with the *bldA*-specific probe, a clear temporal pattern of accumulation of the *bldA*-specified tRNA was seen. Under the conditions used, no hybridization between the *bldA*-specific probe and RNA isolated from J1681 was seen. As in the studies by Vold et al. (46), bands corresponding to tRNA precursors were not detected by Northern blot analyses.

Translation of a UUA-containing mRNA and the abundance of the mature *bldA* gene product are low in early growth. The S1 nuclease and Northern blot analysis of RNA from surface-grown cultures extended earlier observations suggesting that the mature *bldA* tRNA is most abundant relatively late in growth in *S. coelicolor* (35) at a time that would be consistent with a role in antibiotic production and morphological differentiation. To investigate the temporal relationship of *bldA* tRNA abundance with the efficiency of translation of UUA codons, the expression of a TTA-containing reporter gene, *ampC* of *E. coli*, was studied by measuring the transcript abundance and activity of the enzyme specified by the transcript. The *ampC* gene specifies a readily assayable enzyme, a β-lactamase, and contains 7 TTA codons (34). In previous plate tests, *ampC*-specified β-lactamase production was undetectable in a *bldA* mutant (37). To provide quantitative information and to verify that the *bldA* dependence of β-lactamase production was also true for the liquid culture conditions used here, pAU2 (pIJ486 carrying *ampC* fused to a 300-bp promoter-active fragment from *Streptomyces lavendulae* [37]) was introduced into J1501 and its *bldA39* derivative J1700, and β-lactamase activity was assayed in cell extracts from YEME-grown liquid cultures at different times (*ampC* β-lactamase is not secreted from *Streptomyces* species [22]). The J1501/pAU2 culture produced activities of up to 4 mU/mg of protein (Fig. 7A), but the activity in the *bldA* mutant was at the limit of detection and was the same as that in a control culture of J1501 carrying the vector pIJ486 only (0.04 to 0.09 mU/mg of protein). Thus, effective expression of *ampC* in the *bldA* mutant was no more than 1 to 2% of that in the *bldA*$^+$ parental strain.

Since *ampC*-specified β-lactamase production showed such a strong dependence on *bldA*, it was used as an in vivo

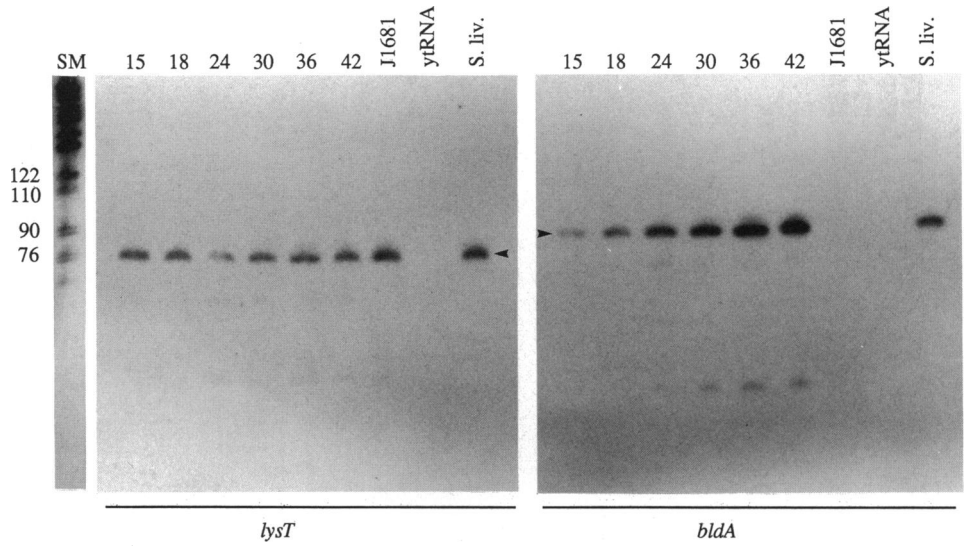

FIG. 6. Northern blot analysis of *lysTα* tRNA and *bldA* tRNA in RNA samples (5 μg) isolated from surface-grown *S. coelicolor* J1501. RNA was isolated from *S. coelicolor* J1501 at various times (hours postinoculation) as indicated (the same RNA samples were analyzed in Fig. 8C). The gene to which the oligonucleotide probe was complementary is indicated below the autoradiograph (see Materials and Methods for details). Controls included yeast tRNA (ytRNA), RNA isolated from a 36-h *S. lividans* liquid culture (S. liv.), and RNA isolated at 48 h from surface-grown J1681. The arrowheads on each autoradiograph indicate the bands corresponding to the expected sizes of the mature *lysTα* tRNA (77 nt) and the mature *bldA* tRNA (87 nt). SM, *Hpa*II-digested pBR322 size markers detected with labeled pBR322 DNA as probe.

reporter of *bldA*-specific tRNA activity. Beginning at 15 h and then at 6-h intervals from 18 to 54 h, cell extracts were prepared, in duplicate, from both liquid- and surface-grown cultures and analyzed by nitrocefin assay for β-lactamase activity. RNA samples were also isolated from the same cultures and analyzed by S1 nuclease protection assays for the appearance of the *bldA* tRNA; however, in this case

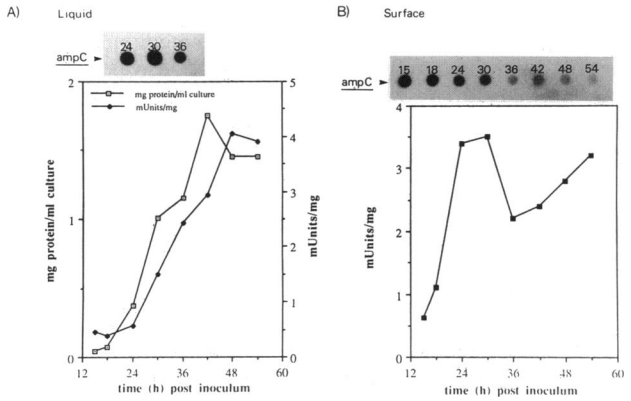

FIG. 7. Time course of β-lactamase production and abundance of *ampC* transcripts in *S. coelicolor* J1501 cultures containing pAU2. (A) Specific activity of *ampC* β-lactamase (milliunits per milligram) in liquid culture compared with growth as measured by protein concentration (milligrams per milliliter). The graph represents data averaged from duplicate sets of cultures. (B) Specific activity of *ampC* β-lactamase (milliunits per milligram) in surface-grown culture. The graph represents data averaged from duplicate sets of samples isolated from the same plates. The resulting signals from dot blot analysis of *ampC* transcripts in the corresponding RNA samples isolated from 24-, 30-, and 36-h liquid cultures (the same RNA samples as those used in Fig. 8B) and from all of the time points during surface-grown culture (the same RNA samples as those used in Fig. 8C) are shown above the graphs.

RNA samples were not isolated in duplicate from the surface cultures (some of the samples were also analyzed for the presence of other RNA species—see below).

Under the liquid culture conditions employed, *S. coelicolor* J1501 appeared to undergo a reproducible brief period of slower growth at 30 h after inoculation, as measured by protein concentration (Fig. 7A). A reproducible (although even shorter) bimodality of growth was previously reported for *Streptomyces hygroscopicus* (24). After 42 h in YEME medium, visible lysis of the culture was accompanied by a drop in the protein concentration. When *ampC* was present on a high-copy-number plasmid, β-lactamase production, although detectable in very young liquid cultures, remained at a relatively constant low level until after 24 h, when it began to rise sharply (Fig. 7A). The pigmented antibiotic undecylprodigiosin was first visible at 36 h.

Surface-grown cultures grew vigorously as vegetative mycelium and began to show visible signs of aerial hyphae and slight orange-red pigment at 24 h; spores were present by 42 h. Sporulation was abundant by 54 h, making it difficult to extract protein and RNA. As with the liquid culture samples, β-lactamase expression was relatively low in early samples (15 and 18 h; Fig. 7B). There was a sharp increase in β-lactamase activity at 24 h, and then the activity dropped and subsequently increased again. A similar pattern with *S. lividans* liquid cultures containing pJAS14-P15 was previously attributed in part to effects of the culture pH on β-lactamase stability and in part to the possible effect of a β-lactamase inhibitor (17); however, factors affecting enzyme stability were not addressed in the present study.

The delay in *ampC* expression could have been due to temporally regulated transcription of the *S. lavendulae* promoter driving the *ampC* gene. To investigate this possibility, RNA isolated from liquid- and surface-grown culture was subjected to dot blot analysis with an internal fragment of *ampC* as probe. RNA samples isolated at 24, 30, and 36 h from liquid culture and at 15, 18, 24, 30, 36, 42, and 54 h from

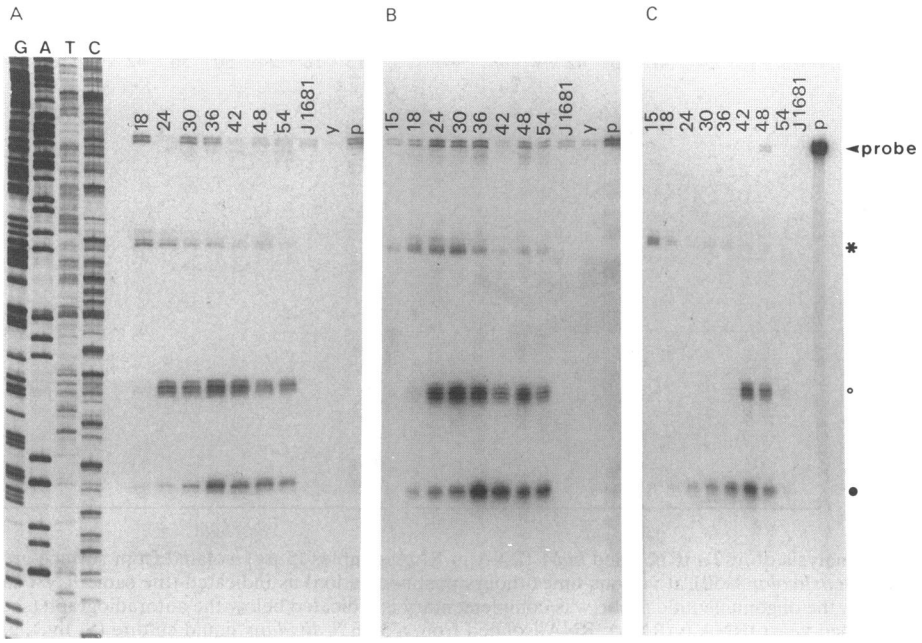

FIG. 8. (A) Nuclease S1 protection analysis of *bldA* transcripts and mature tRNA from RNA samples (20 μg) isolated from duplicate sets of *S. coelicolor* J1501 cultures grown in liquid medium (A and B) and on the surfaces of agar plates (C). *, protected fragment corresponding to the primary transcript; ●, 5′ end of mature tRNA; ○, a series of bands whose presence and intensity in identical RNA samples varied from experiment to experiment and with various concentrations of S1 nuclease; probe, the location of full-length probe. Controls included RNA isolated at 48 h from surface-grown *S. coelicolor* J1681, yeast tRNA (y), and probe (p) hybridized in the absence of RNA and treated with S1 nuclease (in panel C, the probe was untreated).

surface-grown cultures were tested. That the signals resulted from hybridization to RNA transcripts was verified for the 36-h samples by loss of the signal after digestion with DNase-free RNase. Abundant *ampC* transcripts were detected in RNA samples isolated from 24-h liquid cultures, despite the relatively low β-lactamase level at this time point (Fig. 7A). Although the level of *ampC* transcripts approximately doubled in the 30-h RNA samples (the increase was reproducible in the duplicate set of RNA samples isolated from liquid cultures [data not shown]) and *ampC* β-lactamase showed a corresponding 2.6-fold increase in activity at the same time, the level of *ampC* transcripts in the subsequent 36-h RNA samples was virtually the same as that at 24 h, while the level of β-lactamase activity at 36 h was 4.2-fold higher than that seen at 24 h. In RNA samples from surface-grown cultures, *ampC* transcripts were most abundant at 15 and 18 h, with somewhat reduced levels (about half) at 24 h (Fig. 7B). In contrast, the β-lactamase activity detected in the 24-h sample was 5.4-fold higher than at 15 h and 3-fold higher than at 18 h. Thus, between 15 and 24 h, the apparent translatability of *ampC* mRNA increased by about 10-fold. These results suggest that the delay in β-lactamase activity is not attributable to a delay in accumulation of *ampC*-specific RNA.

To verify that the apparent delay in β-lactamase activity was correlated with a delay in expression of the *bldA* tRNA, as was predicted from the earlier time course experiment (Fig. 6), the RNA samples that had been used for detection of *ampC* transcripts were monitored by S1 nuclease protection analysis (Fig. 8). In RNA samples from both liquid- and surface-grown cultures, the mature *bldA* tRNA was present only at low concentrations during early growth and then clearly accumulated with culture age. The increase between 24 and 36 h in the liquid culture time courses (Fig. 8A and B) correlated with the increase in β-lactamase activity between these time points, suggesting that the *bldA* tRNA may have to reach a threshold level before efficient translation of UUA codons occurs. In both sets of RNA samples from the liquid cultures, *bldA* tRNA abundance peaked at 36 h, when red undecylprodigiosin was first visible in the medium. (It should be noted that undecylprodigiosin production was monitored only visually.) The *bldA* tRNA levels in the 24-h RNA sample from surface-grown cultures were also consistent with the sharp increase in β-lactamase activity in the corresponding cell extracts. This time course for the accumulation of the *bldA* tRNA from surface-grown cultures was also seen when the same RNA samples were analyzed by Northern blotting (Fig. 6).

A notable feature of the results from the S1 nuclease studies of RNA samples from both the liquid- and the surface-grown cultures is the apparent inverse relationship between the accumulated levels of primary transcript and the mature form of the *bldA*-specified tRNA. The band corresponding to the primary transcript was abundant in RNA samples isolated early in growth, as would be expected from a vegetatively expressed promoter, and then clearly decreased with culture age. In contrast, the mature form of the tRNA showed the opposite pattern, accumulating with culture age.

DISCUSSION

Previous studies with *bldA* point mutants had indicated that *bldA* function was dispensable for growth. This was verified by the construction of a *bldA* deletion mutant, which showed apparently normal growth but defective morphogen-

esis and secondary metabolism, a phenotype indistinguishable from that of the point mutants. Thus, the point mutations—as anticipated by their locations in positions likely to be critical for tRNA structure or function—were shown to inactivate all significant tRNA activity, and the crucial and specific requirement for *bldA* in morphological and physiological differentiation was established. To investigate further the possible role of *bldA* in differentiation, we undertook studies of the regulation of *bldA* transcription, the efficiency of removal of the 5′ end of the primary transcript to generate the 5′ end of the mature tRNA, and the relatedness of these processes to the translation of mRNA from a reporter gene that contains seven TTA codons.

When *S. coelicolor* spores contained a plasmid in which the *bldA* promoter region was fused with the *neo* gene, they could germinate and grow in the presence of relatively high levels of kanamycin. This suggested that the *bldA* promoter was appreciably active at all growth stages. This was verified by the demonstration by S1 nuclease protection that transcription—from a start point 69 to 70 nt upstream of the 5′ end of the mature *bldA*-specified tRNA—was active in the youngest cultures tested. This transcription start point was authenticated by the detection of the expected runoff transcript after in vitro transcription with *S. coelicolor* RNA polymerase, opening the way for future analysis of the form of RNA polymerase involved.

Although the primary *bldA* transcript was relatively abundant early in growth, the mature tRNA accumulated markedly only relatively late in growth. This time course of accumulation contrasted with that of a lysine tRNA presumed to be active and essential during growth-associated protein synthesis. This differential accumulation seems, at least in part, to be due to an increase in the efficiency with which the primary *bldA* transcript is processed late in growth. It is attractive to consider that an antisense transcript emerging from the *bldA* gene, which was detected by in vitro transcription and by promoter probing, might act as an inhibitor of processing. The promoter-probing study showed that antisense transcription was active from the start of growth. It is possible that a reduction in antisense transcription later in growth might account for the increased ratio of processed to unprocessed *bldA* transcript (a possibility that will be the subject of future investigations).

The peculiar time course of accumulation of the mature *bldA*-specific tRNA strengthened the proposition that it may regulate the translation of UUA-containing mRNAs that encode functions important for morphological and physiological differentiation. A prediction of this model is that UUA-containing mRNA should be more efficiently translated when the cognate tRNA becomes more abundant (at least, over a certain range of abundance). This was apparently true for the *ampC* mRNA, which contains seven UUA codons. (It should be noted, however, that our experiments did not address the possibility that the *ampC* gene product is especially stable in older cultures or that its mRNA is more efficiently translated in older cultures for unknown reasons unconnected with translation of UUA codons.)

The increase in both mature *bldA*-specified tRNA accumulation and expression of *ampC*, in liquid cultures, appeared to coincide with a transient depression in the growth rate. Such a coincidence was also observed, with greater precision, in *S. hygroscopicus* cultures, between a hesitation in the growth rate and the accumulation of proteins involved in the production of the secondary metabolite bialaphos (24). The expression of genes for bialaphos production is regulated in part by a pathway-specific regulatory gene, *brpA*, that contains a TTA codon, and so may be a target for *bldA*-dependent regulation. Accumulation of the *bldA*-specified tRNA during a period of growth rate reduction may represent an inversion of the typical stringent-response-mediated down-regulation of tRNA accumulation by ppGpp, since, at least in *S. hygroscopicus*, ppGpp levels increased during the transient period of growth rate reduction (24).

Recently, Gramajo et al. (20) have reported a related series of experiments comparing *bldA* expression with the translation of UUA-containing or non-UUA-containing mRNA, using a translational fusion of the *ermE* reporter gene to a part of the *actII*ORF4 gene that regulates actinorhodin synthesis in *S. coelicolor*. Their experiments mostly used strains and culture conditions different from ours, and their results showed some disparity with those described here. In particular, they did not observe such a pronounced increase in the mature *bldA* tRNA during growth, and they could find no effect of UUA codons on translatability at different stages of culture. Although there may be a technical explanation for the differing observations, we think it at least likely that they reflect biological differences. It is quite likely that critical (but unidentified) differences in culture conditions cause different regulatory elements to predominate in determining the onset of secondary metabolism: it is perfectly clear that many different parameters can be involved, such as repression by phosphate, glucose, or ammonium; choice of alternative developmental fate; stringent response and GTP levels; and production of extracellular autoregulatory molecules. Moreover, at least 10 genes that exert pleiotropic regulatory effects on secondary metabolism are known (1, 6, 7, 9, 16, 28–30). It would not be surprising if the physiological roles of any regulatory patterns detected in these experiments are difficult to explain in teleological terms, particularly in liquid culture, considering that *S. coelicolor* is a mycelial soil organism, closely adapted to life on nutritionally unpromising surfaces such as plant remains.

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