Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2)

Elizabeth J. Lawlor, Howard A. Baylis, and Keith F. Chater

Agricultural and Food Research Council Institute of Plant Science Research, John Innes Institute, Norwich, NR4 7UH, UK

In *Streptomyces coelicolor*, *bldA* mutants are defective in antibiotic production and the development of aerial hyphae and spores. Subcloning analysis showed that sequences spanning an *NcoI* site in cloned *bldA*⁺ DNA were needed to allow complementation of a *bldA* mutant. Nucleotide sequencing revealed a tRNA-like sequence 9 bp downstream from the *NcoI* site. Five independent *bldA* mutations all fell in a 16-bp region in the tRNA-like sequence, one of them changing the putative anticodon. In RNA dot-blot analysis, hybridization was detected with a probe specific for the tRNA-like transcript but not with a probe for "anti-tRNA-like" transcripts. The transcripts detected were all in the salt-soluble RNA fraction and accumulated relatively late in growth. It is postulated that *bldA* specifies a tRNA that would recognize the codon UUA (for leucine). This codon is very rare in *Streptomyces* genes [which generally contain >70 mole% (G + C)], suggesting a possible role for *bldA* in translational control of development.

[Key Words: Differentiation; translational regulation; secondary metabolism; sporulation]

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The genus *Streptomyces* comprises Gram-positive soil bacteria that grow as a branching vegetative mycelium, on which aerial, sporogenous hyphae develop, presumptively in response to nutritional limitation. Numerous antibiotics, including many that are valuable to man, are produced by this group of bacteria, often at a time coinciding with the formation of aerial hyphae.

In different *Streptomyces* species, studies of morphological mutants have variously implicated arginine metabolism (Meade 1985), guanosine nucleotides (Ochi 1986), and extracellular diffusible factors (Khokhlov 1986) in both sporulation and antibiotic production (reviewed by Chater 1984). In the genetically well-characterized *S. coelicolor*, *bldA* mutants are one of several classes of mutants defective in different stages of development while being apparently unimpaired in vegetative growth (Merrick 1976). On minimal agar medium containing glucose or cellobiose as a carbon source, *bldA* mutant colonies produce no recognizable aerial hyphae or spores; instead, the surface hyphae undergo frequent septation and fragmentation, which may be an aberrant form of the sporulation process (Chater and Merrick 1979). In addition, production of four quite different antibiotics (two of them pigmented) made by the wild-type strain is abolished. On other carbon sources, such as mannitol or maltose, aerial mycelium development and sporulation appear to occur normally, but antibiotic production is not restored (Merrick 1976). The *bldA* gene

was recently cloned on a *Streptomyces* temperate phage vector as a 5.6-kb *PstI* fragment (Piret and Chater 1985). Here, we present the nucleotide sequence of a part of this fragment containing the *bldA* gene. The location of five *bldA* mutations in this sequence, coupled with analysis of *bldA* RNA, leads us to suggest that *bldA* specifies a tRNA for the rare leucine codon UUA and that use of this codon may allow translational control of development.

Results and discussion

bldA mutations lie in a potential tRNA gene

Subcloning analysis showed that a *BglII*–*PstI* fragment of approximately 870 bp (fragment A, Fig. 1), derived from the 5.6-kb *PstI* fragment originally cloned by Piret and Chater (1985), gave *bldA* complementation results identical to those obtained with the larger fragment (J.M. Piret and E.J. Lawlor, unpubl.). Following the observation that cloning the 5.6-kb *PstI* fragment at high copy number did not disrupt differentiation (J.M. Piret, pers. comm.), further subcloning and complementation tests were carried out using a multicopy plasmid vector. The results suggested that sequences spanning an *NcoI* site (Fig. 1) were required for *bldA* expression because fragments B and C failed to complement a *bldA* mutation (*bldA62*). Occasional wild-type recombinant colonies were obtained with fragment C, indicating that the

Figure 1. Fragments used for the localization, sequencing, and transcription analysis of *bldA*. Colonies obtained by transformation of *S. coelicolor* J395 and *S. coelicolor* J668 with a plasmid carrying fragment A all showed the *Bld*⁺ phenotype; those obtained with plasmids carrying either fragment B or C generally retained the *bldA* mutant phenotype. Fragment D, inserted in M13mp19, was used to generate two radioactively labeled single-stranded probes (ss probes 1 and 2; see Materials and methods) for transcription analysis (Fig. 4).

bldA62 mutation lay to the right of the *Nco*I site. Fragments B and C also failed to complement a second *bldA* mutation (*bldA39*); no wild-type recombinant colonies were seen among the few transformants obtained with this particularly poorly transformable strain.

The nucleotide sequence of fragment A was determined (Fig. 2). Using the computer program "Frame" (Bibb et al. 1984), only one (truncated) potential open reading frame (ORF) was identified, beginning at a GTG triplet 69 bp from the left-hand end of fragment A, and reading leftward through the *Bgl*II site. The finding that *bldA62* lay to the right of the *Nco*I site (see above) made it unlikely that this ORF was *bldA*. However, further computer analysis of the nucleotide sequence using the program of Staden (1980) revealed a potential tRNA gene reading rightward from a position 9 bp to the right of the *Nco*I site (Fig. 3). If this tRNA were the *bldA* gene product, cleavage at the *Nco*I site would prevent complementation of *bldA* mutants, because it would presumably separate the tRNA gene (and any genes downstream of it) from its promoter and from any transcribed leader sequences that might be important for correct processing of the transcript to give a mature tRNA. This, combined with the apparent absence of other potential genes from the sequenced DNA, suggested that the potential tRNA gene was indeed *bldA*.

The nucleotide sequences of DNA fragments carrying five independent *bldA* mutations [including four previously shown to be alleles (Piret and Chater 1985)] were determined. The 870-bp fragment A (Fig. 1) from each mutant differed by only 1 bp from the wild-type se-

quence. Each mutation was in the potential tRNA gene (Fig. 3).

The bldA gene specifies a small RNA that accumulates late in growth

Although the double-stranded sequence potentially encoding a tRNA was indeed *bldA* (or a part of it), it remained possible that *bldA* was transcribed from right to left through this sequence. Using primed synthesis on single-stranded templates containing each orientation of the 160-base fragment D (Fig. 1), probe 1, specific for a tRNA-like transcript, and probe 2, specific for an anti-tRNA-like transcript, were obtained. In Southern blot analysis of total *S. coelicolor* DNA, using probe 1, only fragments of the sizes predicted from the restriction map of the *bldA* region were detected (results not shown). The probes were therefore specific for *bldA* transcripts and were used to analyze RNA that had been isolated at different growth stages from a *bldA*⁺ strain and fractionated by precipitation in 3 M sodium acetate (in which RNA larger than about 6S is preferentially precipitated). Hybridization was detected only with probe 1 and only with the soluble (sRNA) fraction that contains smaller RNA species such as 5S RNA and tRNA (Fig. 4). It was strongest in RNA isolated from 36-hr cultures (just before aerial hyphae appeared). Thus, only the potential tRNA gene (and not its complementary strand) is detectably transcribed, and its product accumulates as a small RNA at a time compatible with its role in differentiation and antibiotic production.

Bgl II

AGATCTTGGAAAGCTCCGTGGCGCGCGCGCCACGAGGGTGGACCGGTGGGCGATGGGTG
TCTAGAACCTTTTCGAGGCACCGCGCGCGCGCGTCTCCACCTGGCCAGCCGCTACCCAC 60

CGGTGGTCACGGGAAGGGCGCTCCTGTGCGGTACGGCGTGTGCGGTGGGCACGGTGGGTGC
GCCACCACTGCCCTTCCCGCAGGACAGCCATGCCGCACACGCCAGCCGTGCCACGCACG 120

GGTGGCACGGTGTGTGCTGGGCACGGTGGGTGCTGGGCACGGTGGGTGCGGTGGGCACGG
CCACCGTGGCACACACGACCCGTGCCACGCACGACCCGTGCCACGCACGCCAGCCGTGCC 180

TGCGTGCTCGGGGACGTTGTCAATCGTCCCGGTGCAACGGCGCCCTGTAGTCAGCCGCTG
ACGCACGAGCCCCCTGCAACAGTTAGCAGGGCCAGCTTGCCCGGGGACATCAGTCGGCGAC 240

TTCCGGTTCAGGGGGGCGACTTCGGTGGACGGGCGGGCGGGTGTGCATACCTGCGGATG
AAGGCCAAGTCCCCCGCTGAAGCCAGCCTGCCCGGGCGGGCCACAGTATGGACGCCTAC 300

ACGGCGGACCCCGACGGCGGGGGCGGGTGAGAACGGGGCGGGCGCCCTTGTGAGACGGGTG
TGCCGGCTGGGGCTGCCGCCCCGCCACTCTTGCCCCGCGCGGGAACAGCTCTGCCAC 360

AAATCCCGTCATTCCGCATACAGCGCGGGCGGTGCGACGCAAGGCTGTTGGCATGTGCTG
TTTAGGGCAGTAAGGCGTATGTGCGCCCCGCCAGCGTGGCGTTCCGACAACCGTACACGAC 420

TTGGCCTGACCCGGTAAGTGTATGCACCTCAGCGCGCAATAAAATAAGACAACATCGGCC
AACCAGACTGGGCCATTGACTACGTGGGAGTCCCGCGTTATTTTATTCTGTTGTAGCCGG 480

GTCCCGCCCGAGGCGGGAGGAGCGTCCCGATAGGGTCAAGGCGCGCGAAGCGGAGCCCA
CAAGCGGGGCTCCGGCCCTCCTCGCAGGGCTATCCAGTTCCGCGCGCTTCGCCTCGGGT 540

↓ tRNA start

TGGCCTGCCCGGATGGTGGAAATGCAGACACGGCGAGCTTAAACCTCGCTGCCCTTCGAG
ACCGGACGGGCGCTACACCTTACGTCTGTGCCGTGCAATTTGGAGCGACGGGGAAGCTC 600

↓ tRNA stop

GGCGTGCCGGTTCAAGTCCGGCTCCGGGCACCACTACTGTACGCTGCGTGACCATCGGT
CCGCACGGCCAAGTTACAGCCGAGGCCGTGGTGTGATGACAGTGGCAGCACTGGTAGCCA 660

Sac II

GAAGGTTCCACGGGGCGGGCCATTGCCCTGCCCGGGCTGGTTGCGACTCAGCGGCCCTG
CTTCCAAGGTGCCCCGCGCGGTAACGGGACGGGCGCGACCAACCGCTGAGTCGCCGGGAC 720

CTGGCGCTCCCCGCGCTCGCGCCCTGGCGACCTGGGTATGGGCTTTCGTCCTGCCGGCC
GACCGCGAGGGGCGGGAGCGCGGGACCGCTGGACCCATACCCGAAAGCAGGACGGCCGG 780

GGCAGAGGGGGCGGAGTCGCGAACGGCGGCTTCACGGCCGGACACGTGATGATCGGACTG
CCGCCTCCCCCGCTCAGCGCTTGCCGCGCGAAGTGCCGGCCTGTGCACTACTAGCCTGAC 840

Pst I

GCCCTCGTGTGCACCTGTCTGATCGGACTGCAG
CGGGAGCACACGTGGACAGACTAGCCTGACGTC 873

Figure 2. Nucleotide sequence of fragment A. The boxed nucleotides form an incomplete ORF, and the black bars indicate the sequences of the 20-base oligonucleotide primers used to sequence *bldA* mutant alleles (Fig. 3). The latter experiments confirmed the sequence across the *Nco*I site shown here.

How tRNA-like would the proposed *bldA* gene product be?

These results provide strong evidence that *bldA* specifies a tRNA-like product. How tRNA-like would it be? The stems and loops of its proposed secondary structure are consistent with those of tRNAs, and it has a typical "cloverleaf" form (Gauss et al. 1979). Ignoring modified bases (which cannot be deduced from the DNA sequence), the potential tRNA contains all the invariant and semiinvariant residues present in almost all tRNAs (excluding initiator tRNAs) involved in protein synthesis (Rich and RajBhandary 1976), except the highly conserved GG pair at nucleotides 18 and 19 in the D loop. The absence of one G of this pair is striking, but

there are examples of tRNAs where both G residues are absent (Roberts 1972). The anticodon would recognize the codon UUA, and so the *bldA*-gene product can be designated tRNA^{Leu}_{UUA}. It showed highest homology (65%) to a *Bacillus subtilis* tRNA^{Leu}_{UUA} (Wawrousek et al. 1984) and greater homology to leucyl tRNAs with nonhomologous anticodons [for example, *Escherichia coli* tRNA^{Leu}_{CTC} (57%) (Sprinzl et al. 1985)] than to nonleucyl tRNAs [for example, *B. subtilis* tRNA^{Ser}_{TCC} (39%) (Wawrousek et al. 1984)].

What effects might the sequenced *bldA* mutations have on tRNA structure and function? The *bldA39* mutation changes the anticodon to UGA, which would recognize the UCA (serine) codon. The *bldA16*, *bldA62*, and *bldA1* mutations result in mismatched base pairs in

Figure 3. Deduced secondary structure of the potential tRNA and the positions of five *bldA* point mutations. The base change for each *bldA* mutation is shown, together (in the upper part of the diagram) with the allele number in parentheses.

either the D stem or the anticodon stem, reducing the stability of the secondary structure. The *bldA34* mutation lies at N₃₈ in the anticodon loop where the (often highly modified) residues play a part in anticodon-codon recognition (Bouadloun et al. 1986; Ericson and Björk 1986). This mutation may therefore prevent an important modification.

How could the *bldA*-gene product influence differentiation?

Although a variety of nontranslational roles of tRNA-like molecules have been reported (see the end of this section), we first discuss the most obvious hypothesis for *bldA* action—that its product might play a crucial role in differentiation simply by influencing translation, for example, by changes in its abundance or degree of modification.

In *B. subtilis* the tRNA profile changes at least

slightly during sporulation, and these changes are thought to be due to post-translational modification (Vold 1985; Henner and Steinberg 1979), but it is not known whether they influence the course of sporulation. The high G + C content of *Streptomyces* DNA might allow (or even result from) the use of A + T-rich codons as regulatory signals. In a compilation of 17 sequenced *Streptomyces* genes provided by M.J. Bibb (pers. comm.), there was only one example of a TTA codon, in the *hyg* gene of *S. hygroscopicus* (Zalacain et al. 1986). In addition, recent sequence analysis has shown that the *carB* gene of *S. thermotolerans* (Epp et al. 1987) contains two TTA codons and that the *sph* gene of *S. glaucescens* (Vögtli and Hütter 1987) contains one TTA codon. Overall, this represents a frequency of usage for this codon of less than 1%, CTG and CTC being preferentially used to encode leucine. Thus, it is possible that limited availability of the *bldA* tRNA^{Leu} in *S. coelicolor* restricts the translation of mRNA species containing UUA codons and that efficient translation of at least one such mRNA is necessary for normal development. This hypothesis requires that there should be no other tRNA capable of translating UUA codons efficiently. Although Southern blotting (results not shown) indicated that *S. coelicolor* DNA contains no other sequences at least 70% homologous to *bldA*, this does not exclude the possibility of a less homologous tRNA with the same anticodon. Although constrained by the lack of information concerning modified residues, consideration of the “wobble hypothesis” as originally formulated (Crick 1966) indicates that only tRNAs with the anticodon UAA are able to read UUA codons. However, wobble has also been reported at the first position of the codon, with pairing occurring between G and U residues (Lin et al. 1986 and references therein). This potentially allows leucyl tRNAs with the anticodon UAG to translate UUA codons.

Alternatively, the *bldA* tRNA could be involved in a process other than ribosome-mediated protein synthesis. In other organisms, tRNAs have been implicated in nonribosomal amino-terminal addition of amino acids to proteins (Soffer and Horinishi 1969), donation of the lysyl group of lysyl-tRNA in the formation of O-L-lysyl phosphatidylglycerol in *Staphylococcus aureus* (Lennarz

Figure 4. Hybridization between 3 µg of the salt-soluble RNA fractions and probe 1 (Fig. 1), indicating that a *bldA* tRNA-like transcript accumulates relatively late in growth. No signals were detected between probe 1 and the salt-insoluble RNA fractions or between probe 2 (Fig. 1) and either the salt-soluble or salt-insoluble RNA fractions.

et al. 1971], and peptidoglycan synthesis in staphylococci (Steward et al. 1971). Moreover, there are requirements for an arginine tRNA for DNA replication (Mullin et al. 1984; Garcia et al. 1986) and a serine tRNA for cell division (Tamura et al. 1984) in *E. coli*. It is not clear whether these latter effects are exerted through the translation machinery or via some other activity of these particular tRNA species.

Elucidation of both the precise mode of action of tRNA^{Leu} and the mechanism of temporal control of *bldA* expression will provide a basis for a number of interesting future experiments. These matters will also be of some relevance to the commercial production of antibiotics by *Streptomyces* fermentations.

Materials and methods

Subcloning of *bldA*

Fragments A, B, and C (Fig. 1) were ligated with the multicopy plasmid vectors pIJ486 and pIJ487 (Ward et al. 1986), cleaved at appropriate sites in their oppositely oriented polylinker sequences, and introduced into *Streptomyces lividans* TK24 by transformation (Hopwood et al. 1985). For the easier subcloning of fragments B and C, the *Nco*I site was converted to a *Bgl*II site by end filling with the Klenow fragment of DNA polymerase I and attachment of a synthetic *Bgl*II linker (Maniatis et al. 1982). Plasmids of the correct structure were identified by restriction enzyme analysis and introduced into *S. coelicolor* J395 (*bldA62*) (Piret and Chater 1985) and *S. coelicolor* J668 (*bldA39*) (Merrick 1976) by transformation. (Note that *bldA* mutants were generally transformed at relatively low frequencies.)

Cloning of *bldA* mutant alleles

The *bldA* mutations analyzed were cloned from the following strains: J668 (*bldA39*) and J673 (*bldA16*) (Merrick 1976); and J406 (*bldA1*), J394 (*bldA34*), and J395 (*bldA62*) (D.A. Hopwood and K.F. Chater, unpubl.). The mutations were cloned in vivo by recombination between ϕ C31 KC603, carrying the cloned *bldA* gene as a 5.6-kb *Pst*I fragment, and the *bldA* region of the respective *bldA* mutant chromosome as described by Piret and Chater (1985). The 870-bp *Bgl*II-*Pst*I fragment A (Fig. 1) from each mutant was ligated with *Pst*I- and *Bam*HI-cleaved M13mp19 (Norlander et al. 1983) and introduced into *E. coli* JM101 (Messing 1979) by transformation (Bankier and Barrell 1983).

DNA sequence analysis

The DNA sequences of both strands of fragment A carrying the wild-type *bldA* allele were determined by the chemical degradation method (Maxam and Gilbert 1980), using fragments uniquely labeled at the *Bgl*II, *Nco*I (in some cases after its conversion to a *Bgl*II site), and a vector *Hind*III site adjacent to the *Pst*I site of the cloned fragment. The DNA sequence of one strand of fragment A was determined for each *bldA* mutant allele by the chain-terminating procedure (Bankier and Barrell 1983) using the synthetic oligonucleotide primers shown in Figure 2.

Preparation of probes

The 5' single-stranded ends generated by *Nco*I and the 3' single-stranded ends generated by *Sac*II of the 160-bp fragment

D (Fig. 1) were respectively filled in or digested with the Klenow fragment of DNA polymerase I (Maniatis et al. 1982) and ligated with *Sma*I-cleaved M13mp19 and introduced into *E. coli* JM101 by transformation. Derivatives of M13mp19 containing each orientation of fragment D were detected by sequence analysis (Bankier and Barrell 1983) and were used to prepare ³²P-labeled single-stranded DNA probes by primed synthesis (Biggin et al. 1984). Labeled DNA was separated from unincorporated nucleotides by passage through a Sephadex G-100 column (Hopwood et al. 1985) and denatured at 90°C for 10 min prior to use. Probe 1 was prepared from template containing fragment D oriented such that the *Sac*II end of the fragment was next to the primer site for in vitro DNA synthesis, and probe 2 from template containing fragment D in the opposite orientation.

Southern blots

Southern blots were performed as described in Hopwood et al. (1985). The filters were washed in 3 × SSC/0.1% SDS or 0.2 × SSC/0.1% SDS at 70°C, according to the degree of homology being sought (70% and 90%, respectively, in the two sets of conditions).

Dot blots

RNA was isolated from cultures of *S. coelicolor* J1507 (*bldA*⁺) (Chater and Bruton 1983), grown for different lengths of time on the surface of cellophane sheets laid on R2YE medium (Hopwood et al. 1985). The RNA was size-fractionated by two precipitations in 3 M sodium acetate in which RNA larger than 6S precipitates (Kirby et al. 1967). The fractionation was checked by agarose gel electrophoresis. RNAs (1.5, 3, and 6 µg) were "dotted" onto nitrocellulose filters and hybridized with probes 1 and 2, as described in Hopwood et al. (1985).

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