Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2461–2465, March 1991
Genetics

TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants

[tRNA/sporulation/*Streptomyces coelicolor* A3(2)/*Streptomyces lividans* 66/translational regulation]

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Communicated by Sydney Brenner, November 19, 1990 (received for review July 27, 1990)

ABSTRACT **In *Streptomyces coelicolor* A3(2) and the related species *Streptomyces lividans* 66, aerial mycelium formation and antibiotic production are blocked by mutations in *bldA*, which specifies a tRNA^{Leu}-like gene product which would recognize the UUA codon. Here we show that phenotypic expression of three disparate genes (*carB*, *lacZ*, and *ampC*) containing TTA codons depends strongly on *bldA*. Site-directed mutagenesis of *carB*, changing its two TTA codons to CTC (leucine) codons, resulted in *bldA*-independent expression; hence the *bldA* product is the principal tRNA for the UUA codon. Two other genes (*hyg* and *aad*) containing TTA codons show a medium-dependent reduction in phenotypic expression (hygromycin resistance and spectinomycin resistance, respectively) in *bldA* mutants. For *hyg*, evidence is presented that the UUA codon is probably being translated by a tRNA with an imperfectly matched anticodon, giving very low levels of gene product but relatively high resistance to hygromycin. It is proposed that TTA codons may be generally absent from genes expressed during vegetative growth and from the structural genes for differentiation and antibiotic production but present in some regulatory and resistance genes associated with the latter processes. The codon may therefore play a role in developmental regulation.**

Streptomycetes are mycelial, Gram-positive bacteria with (G+C)-rich DNA. Their sporulation (on specialized aerial hyphae) often coincides with antibiotic production (1). Among morphological mutants of *Streptomyces coelicolor* A3(2) devoid of normal aerial mycelium (*bld*, for "bald" mutants), some are also pleiotropically defective in production of several quite distinct antibiotics (2, 3). In *bldA* mutants the morphological defect, but not the loss of antibiotic production, is overcome by replacing glucose in the medium with certain alternative carbon sources, such as mannitol (2). Five *bldA* mutations were located by DNA sequencing within a region of cloned DNA deduced to specify a tRNA-like gene product (4, 5). The proposed tRNA would recognize the UUA (leucine) codon, which is expected—and observed—to occur rarely in *Streptomyces* mRNA, which is rich in G + C (6). In this paper we present genetic evidence that the *bldA* gene product is indeed the principal means by which UUA codons are translated in *S. coelicolor* and its close relative, *Streptomyces lividans*, even though *bldA* is not needed for growth. This provides a particularly extreme example of the specialized use of a rare codon.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, Phages, and Plasmids. *S. coelicolor* J1501 (7) and *S. lividans* 1326-9 (8) were used as *bldA*$^+$ hosts. *bldA* mutants included *S. coelicolor* J1700 (*bldA39*) and J1702 (*bldA16*), both J1501 derivatives, and *S. lividans* J1725 (*bldA39*) and J1726 (*bldA16*), both 1326-9 derivatives. Agar media were R2 or R2YE (R2 with yeast extract) and minimal medium (MM), in some cases with mannitol (0.5%) as carbon source instead of glucose (7). Yeast extract/malt extract medium (YEME) was used for liquid cultures. Conditions for culture, transformation, transfection, and lysogen isolation were as described (7). Antibiotic gradient plates (9) were prepared in square bioassay dishes (Nunc; ref. 10). *Escherichia coli* JM101 was the host for pUC plasmids and M13 phages (11). *Streptomyces* plasmids and phages (Table 1) were manipulated as described (7). Double lysogens carrying PM8, which is *att*-deleted, were constructed by using ϕC31 *c1* as the *att*-proficient prophage (7). In all cases except those of pJOE829 and pIJ4517 (when hygromycin B at 200 μg/ml was used), primary selection of transformants or lysogens was by thiostrepton resistance (7). Site-directed mutagenesis (22) of the two TTA codons within *carB* (to obtain pIJ4500) was performed using the 260-base-pair (bp) *Sst* I–*Bam*HI fragment (Fig. 1) of pIJ589 subcloned in M13mp18 (17), as template. Fusions of *hyg* to the *ermE* promoter [*ermE*p(1) + *ermE*p(2) (ref. 18)] were made by first ligating the full-length (*Nru* I–*Bam*HI fragment) or 5′-deleted (*Eco*RI–*Bam*HI fragment) *hyg* gene (Fig. 1) from pIJ963 into pIJ2925 followed by ligating the same fragments (now with *Bgl* II ends) into the *Bam*HI site of pIJ4070. For introduction into *Streptomyces* strains, the fused products were subcloned as *Bgl* II fragments in the *Bam*HI site of pIJ487, to give pIJ4515 and pIJ4511, respectively. The GTG (valine) at codon 42 of *hyg* (Fig. 1) was converted to GTA (valine) by using a mutagenic oligonucleotide as one primer in a polymerase chain reaction (23) with pJOE829 PL$^-$ as template. From the 102-bp amplified fragment the *Sst* I–*Bcl* I fragment containing the mutation was isolated and ligated with the two largest *Sst* I–*Bcl* I fragments of pJOE829 PL$^-$ to construct, in pIJ4517, a full-length mutant *hyg* gene. The selectable *tsr* gene [isolated after cloning the 1085-bp *Bcl* I fragment from pIJ702 (24) in *Bam*HI-cut pIJ2925, followed by oligonucleotide adaptor-mediated conversion of the *Bgl* II sites to *Pst* I sites] was then inserted into the *Pst* I site of pIJ4517 to give pIJ4519.

Detection and Assay of Enzymes. For β-lactamase detection, cultures of R2YE plates were sprayed with a fresh solution of the chromogenic cephalosporin nitrocefin (20) to give a red-brown diffusible pigment in positive tests. For detecting β-galatosidase in plaques, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) was incorporated into top layers (12). Spectrophotometric assay of hygromycin phosphotransferase was as in ref. 25 except that hygromycin B (0.25 mM) was used as the substrate. Activity was also

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Table 1. *Streptomyces* phages and plasmids

Plasmid or phage	Comments* and refs.
KC659	ϕC31 derivative with *lacZ* (12)
KC855	ϕC31 KC304 (13) derivative with *aad* (C. J. Bruton, personal communication)
PM8	ϕC31 derivative with *hyg* (14)
pIJ486/7	pIJ101-derived general-purpose and promoter-probe vector (10)
pIJ589	pIJ486 with a 2.8-kilobase (kb) *carB*-containing *Bam*HI (partial digest) fragment from pOJ159 inserted between its *Bgl* II sites (unique remaining *Bam*HI and *Sst* I sites flank the *carB* segment containing two TTA codons) (this study)
pIJ941	SCP2 derivative with *hyg* and *tsr* (15)
pIJ963	pUC18 derivative with *hyg* (16)
pIJ2925	pUC18 (17) with an altered polylinker flanked by *Bgl* II sites (G. R. Janssen, personal communication)
pIJ4070	pIJ2925 with *ermE* promoter fragment between its *Kpn* I and *Bam*HI sites (18)
pIJ4500	pIJ589 with the TTA codons of *carB* replaced by CTC codons (this study)
pIJ4501	Obtained in the same experiment as pIJ4500 but its TTA codons had escaped change (this study)
pIJ4511	pIJ487 with 5'-deleted *hyg* fused to *ermE* promoter (this study)
pIJ4515	pIJ487 with full-length *hyg* fused to *ermE* promoter (this study)
pIJ4517	pJOE829 PL$^-$ with modified *hyg* lacking a potential internal translation start site (this study)
pIJ4519	pIJ4517 with *tsr* at the unique *Pst* I site (this study)
pIJ4520	pJOE829 PL$^-$ with *tsr* at the unique *Pst* I site (this study)
pJAS7	SLP1.2 containing *ampC* with an up-promoter mutation strongly expressed in *S. lividans* (19)
pJAS14	SLP1.2-based promoter-probe with promoterless *ampC* (20)
pJAS14-P15	pJAS14 derivative with 300-bp promoter-active insert from *Streptomyces lavendulae* (M. Forsman and B. Jaurin, personal communication)
pJOE829	pIJ101 derivative with *hyg* (J. Altenbuchner, personal communication)
pJOE829 PL$^-$	pJOE829 with polylinker deleted from between *Pst* I sites (this study)
pOJ159	pIJ101 derivative with *carB* and *tsr* (21)

*The copy numbers of the plasmid replicons are estimated at 4–5 for SLP1.2, 1 for SCP2, and 30–300 for pIJ101 (7).

detected nonquantitatively by using paper chromatography and autoradiography to separate and detect [^{32}P]phosphohygromycin B produced in the presence of cell-free extracts, hygromycin, and [γ-^{32}P]ATP (26). Cell-free extracts were prepared by sonication of cultures grown in YEME for 42 hr at 30°C.

RESULTS

The possibility that *bldA* specifies a tRNA recognizing the rare leucine codon UUA (4) was addressed by testing the expression of genes containing TTA codons in isogenic *bldA*$^+$ and *bldA*$^-$ strains. Two *bldA* mutations were used: *bldA39*, in which the presumptive anticodon was changed from UAA to UGA, corresponding to the serine codon UCA; and *bldA16*, in which the D stem has only two base pairs (4). The isogenic strains were constructed by homogenization (27) in derivatives of *S. coelicolor* A3(2) (strain J1501) and the closely related *S. lividans* 66 (strain 1326-9, a mutant of *S. lividans* lacking endogenous β-galactosidase activity). We

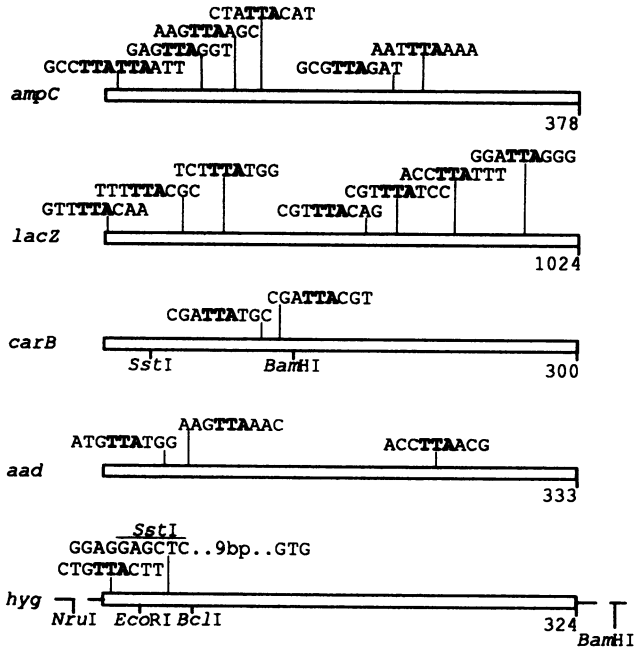

FIG. 1. Locations of TTA codons (as well as surrounding nucleotides to show context) within the five TTA-containing genes used in this study, and features used in altering the genes. The boxes represent the coding region of each gene with its number of codons shown to the right. Sequences flanking *hyg* are indicated by broken lines.

discuss separately those TTA-containing test genes that show severe *bldA* dependence and those that are only partially dependent on *bldA* for phenotypic expression.

Some TTA-Containing Genes Are Severely Dependent on *bldA* for Phenotypic Expression. (*i*) *ampC*. The *E. coli ampC* (β-lactamase) gene contains seven TTA codons (28) (Fig. 1). The *ampC* gene in pJAS14-P15 and pJAS7 is expressed in *S. lividans* (M. Forsman and B. Jaurin, personal communication; ref. 20). This was prevented by the *bldA39* mutation (Fig. 2). The tests with the *bldA*$^+$ strains were positive only with older (2 or 3 day) patches; no activity was detected after 1 day.

(*ii*) *lacZ*. The *E. coli lacZ* gene contains seven TTA codons (29) (Fig. 1). Although *lacZ* could not be stably maintained in those *Streptomyces* plasmids tested (12), it could be introduced into ϕC31 vectors, giving phages such as KC659 which expressed β-galactosidase activity in plaques on *S. lividans* 1326-9 (12). No β-galactosidase activity was seen in KC659 plaques on *bldA16* and *bldA39* mutants of 1326-9 (J1726 and J1725, respectively). Phages isolated from the plaques on the *bldA* mutants retained a functional *lacZ* gene: they expressed β-galactosidase on *S. lividans* 1326-9.

(*iii*) *carB*. There are two TTA codons in *carB*, a carbomycin-resistance gene from *Streptomyces thermotolerans*, which confers resistance to various macrolide, lincosamide, and streptogramin B-type antibiotics by methylating 23S ribosomal RNA (21). In *S. coelicolor* or *S. lividans* strains containing pIJ589 or pOJ159, *carB* conferred strong lincomycin resistance on *bldA*$^+$ strains but little or none on *bldA39* mutants in tests done on MM plus mannitol (Fig. 3) or on the rich medium R2YE. The same result was seen on MM plus mannitol with low phosphate (0.04 mM), conditions in which one of the *S. coelicolor* antibiotic pathways (for undecylprodigiosin synthesis) is expressed in the *bldA39* mutant J1700 (30).

In summary, severe *bldA* dependence was shown for three diverse genes containing TTA codons. Conditions selectively reversing aspects of the *bldA* phenotype (mannitol as carbon source, or reduced phosphate concentration) did not affect

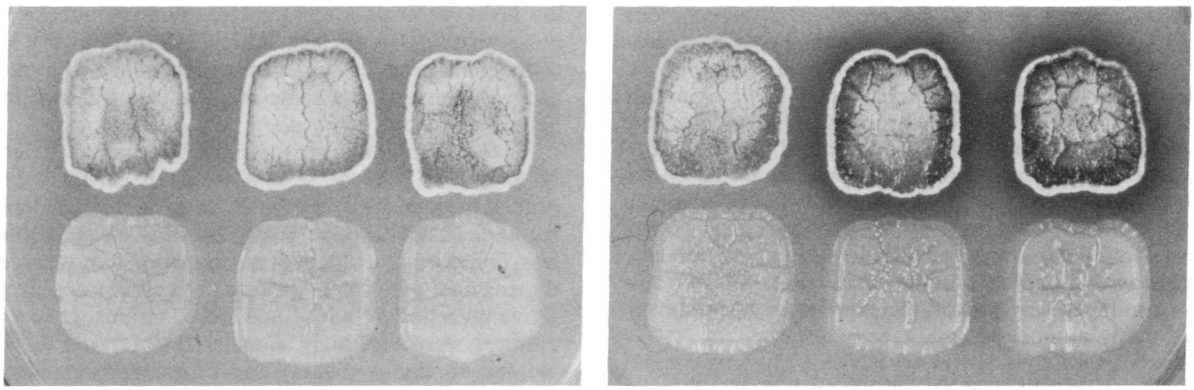

FIG. 2. *bldA*-dependent expression of *ampC*. The patches (3-day cultures on medium R2YE) shown are before (*Left*) and after (*Right*) spraying with the chromogenic cephalosporin nitrocefin as a substrate for the *ampC*-determined β-lactamase. Dark haloes indicate β-lactamase activity. Upper row, *S. lividans* 1326-9 (*bldA*$^+$) containing, from left to right, pJAS14 (containing a promoterless *ampC*), pJAS7, and pJAS14-P15 (in both of which *ampC* is transcribed from promoters active in *S. lividans*). Lower row, *S. lividans* J1725 (*bldA39*) containing the same three plasmids as described above.

the level of *bldA* dependence of *carB* (or of *hyg* and *aad*, which are discussed below).

Site-Directed Alteration of TTA Codons to Synonymous Codons Renders Expression of *carB* Independent of *bldA*. The above data suggested that *bldA* specifies the principal $tRNA^{Leu}_{UUA}$ in *S. coelicolor* and *S. lividans*. To rule out alternative explanations, the two TTA codons in the internal *Sst* I–*Bam*HI fragment of *carB* were converted to CTC and the mutated fragment was ligated back into the *carB* gene of pIJ589. The ligation mixture was used to transform J1725 (*S. lividans*, *bldA39*). Many of the thiostrepton-resistant transformants were lincomycin resistant, and so presumptively contained the mutated gene. The rest were sensitive, and presumptively contained a regenerated wild-type *carB* gene. Plasmid DNA from one resistant (pIJ4500) and one sensitive (pJ4501) colony was used to transform *bldA*$^+$ and *bldA*$^-$ derivatives of *S. coelicolor* and *S. lividans*. Lincomycin resistance was conferred on the *bldA*$^+$ strains by both plasmids, but the *bldA*$^-$ strains became resistant only when transformed by pIJ4500 (Fig. 3). DNA sequencing confirmed that only pIJ4500 contained the mutated *carB*, and thus that the *bldA* dependence of *carB* was attributable to its two TTA codons.

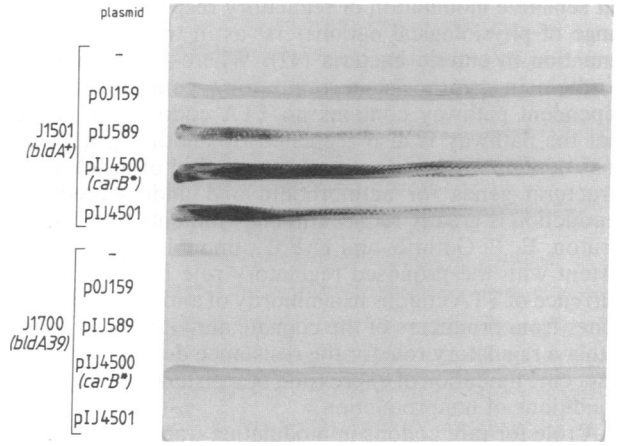

FIG. 3. Expression of *carB* and *carB** in *bldA*$^+$ and *bldA*$^-$ *S. coelicolor* strains. The plate contained MM plus mannitol and a gradient (0–400 μg/ml) of lincomycin. All the plasmids contained *carB*, in the case of pIJ4500 in a mutant form (*carB**) in which the TTA codons were replaced by CTC codons. pIJ589 and pIJ4501 are identical to each other, and they differ from pIJ4500 only at these TTA codons. pOJ159 contains *carB* in the same replicon (pIJ101) but with different flanking sequences. For further details of plasmids, see Table 1.

Some TTA-Containing Genes Are Only Partially Dependent on *bldA* for Phenotypic Expression. (*i*) *aad*. The *aad* gene, from R-plasmid R538-1, encodes spectinomycin adenyltransferase and contains three TTA codons (31) (Fig. 1). It was introduced into *S. coelicolor* and *S. lividans* strains on KC855 prophage. Strong spectinomycin resistance was observed in the *bldA*$^+$ strains grown on MM with glucose or mannitol as carbon source, but the *bldA39* mutants were markedly less resistant (Fig. 4*a*; the same result was obtained with *bldA16* mutants). However, in contrast to *carB*, resistance on the richer medium R2YE was equally high in the *bldA*$^+$ and *bldA* mutant strains (not shown).

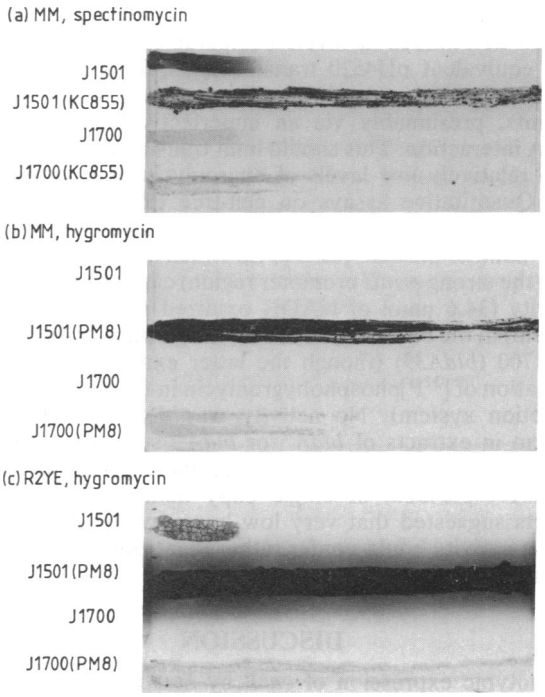

FIG. 4. Expression of *aad* and *hyg* in *S. coelicolor bldA*$^+$ and *bldA*$^-$ strains. (*a*) Spectinomycin gradient (0–200 μg/ml in MM plus mannitol). (*b* and *c*) Hygromycin gradient (0–100 μg/ml in MM plus mannitol; 0–400 μg/ml in R2YE). KC855 and PM8 are φC31 phage derivatives carrying *aad* and *hyg*, respectively. For further details of plasmids and phages, see Table 1. Note that the apparently weaker overall growth of *bldA* stains compared with *bldA*$^+$ strains was also seen in the absence of antibiotic, and it probably reflects the reduced opacity consequent on failure to sporulate or produce pigments.

(*ii*) *hyg*. The *hyg* gene of *Streptomyces hygroscopicus* encodes hygromycin phosphotransferase and confers hygromycin resistance (32). Its seventh codon is a TTA (Fig. 1). Hygromycin resistance of *S. coelicolor* and *S. lividans* strains containing *hyg* on either PM8 prophage (Fig. 4*b*) or the low-copy-number plasmid pIJ941 was tested. On mannitol-containing MM, perceptible resistance was conferred on the *bldA39* mutants by *hyg*, but it was markedly lower than with the equivalent *bldA*$^+$ strains (Fig. 4*b*). However, just as with *aad* (see above), resistance on R2YE medium was equally high in the *bld*$^+$ and *bldA* mutant strains (Fig. 4*c*). *hyg* also gave *bldA*-independent phenotypic expression when present on a high-copy-number plasmid (see below).

Explanation for Expression of *hyg* in a *bldA* Mutant Probably Involves Translation of UUA by a Noncognate tRNA. Expression of *hyg* in a *bldA* mutant might result either from translation of the UUA codon—perhaps involving a poorly matched codon–anticodon interaction—or from the use of a GUG, the 42nd codon in *hyg* mRNA, as an alterative translation start site bypassing the UUA codon (5, 32) (Fig. 1). Two experiments eliminated the latter possibility. In one, a 5′-deleted *hyg* gene retaining the potential alternative translation start site, but lacking both its normal translation start and the TTA codon, was fused, on a high-copy-number plasmid, to the strong constitutive *ermE* promoter fragment (Fig. 1) (18) and reintroduced as pIJ4511 into *S. lividans* and *S. coelicolor bldA*$^+$ and *bldA*$^-$ derivatives. No hygromycin resistance was detectable. [In a control experiment, pIJ4515 with the *ermE* promoter region driving expression of the intact *hyg* gene (Fig. 1) conferred equally strong hygromycin resistance on all the strains, including *bldA*$^-$, tested on MM plus mannitol.] In the second experiment, the potential alternative start codon was changed from GTG to GTA, a valine codon that is not expected to specify translation initiation. The resulting plasmid (pIJ4519) conferred hygromycin resistance on *S. lividans* and *S. coelicolor bldA*$^+$ and *bldA39* derivatives at levels comparable to those obtained with equivalent pIJ4520 transformants. Translation of the UUA codon in *hyg* mRNA must therefore occur in *bldA* mutants, presumably via an imperfectly matched codon-tRNA interaction. This should limit translation and therefore yield relatively low levels of hygromycin phosphotransferase. Quantitative assays on cell-free extracts of cultures grown in the rich medium YEME confirmed this: the high-copy-number plasmid pIJ4515 (in which *hyg* was transcribed from the strong *ermE* promoter region) caused a high specific activity (34.6 nmol of NADH oxidized per min per mg of protein) in the *bldA*$^+$ strain J1501, but no measurable activity in J1700 (*bldA39*) (though the latter extract catalyzed the formation of [^{32}P]phosphohygromycin in the nonquantitative detection system). No activity was detectable by either system in extracts of *bldA*$^+$ or *bldA*$^-$ strains carrying *hyg* under the control of its natural promoter, whether at high (pIJ4519, pIJ4520) or single copy number (PM8). These results suggested that very low hygromycin phosphotransferase activity could confer rather high resistance to hygromycin.

DISCUSSION

Phenotypic expression of *carB* by *S. coelicolor* and/or *S. lividans* is dependent on *bldA*. This dependence is abolished by changing the two TTA codons to alternative leucine codons. This provides compelling genetic evidence that *bldA* does indeed specify a $tRNA^{Leu}_{UUA}$ that is active in protein synthesis. The severe *bldA* dependence of *lacZ*, *ampC*, and *carB* shows that the *bldA* gene product is the only efficient means of translating at least one of the UUA codons in mRNA for each of these genes. The phenotypic expression of *hyg* and *aad* detectable in *bldA* mutants is probably due to a low level of translation of the UUA codons in these genes by noncognate tRNA species. At least for *hyg*, a very low level of the gene product confers high resistance to hygromycin, and we infer that the same may be true for spectinomycin resistance conferred by *aad*.

Since *bldA* mutants grow rapidly on minimal or rich media, TTA codons may be absent from all genes essential for normal growth. This is difficult to prove, in the light of the incomplete dependence of *hyg* and *aad* on *bldA*. Nevertheless, supporting evidence comes from two sources: two-dimensional gel protein patterns of cell extracts from vegetative mycelium of *bldA*$^+$ and *bldA*$^-$ strains, grown in liquid culture, were strikingly similar (B.K.L. and C. Laurent-Winter, unpublished data); and TTA codons are absent from all but 4 of 63 sequenced *Streptomyces* genes (19,397 codons) of different known or inferred function (including *hyg* and *carB*) compiled by M. J. Bibb and F. Wright (personal communication). The other two TTA-containing genes in that compilation are for streptomycin resistance in *Streptomyces glaucescens* (33), and the regulation of streptomycin biosynthesis and resistance in *Streptomyces griseus* (34). More recently, TTA codons have also been found in pathway-specific regulatory and resistance genes for actinorhodin synthesis in *S. coelicolor* (M. A. Fernandez-Moreno and J. L. Caballero, personal communication) and in a gene needed for differentiation in an *S. griseus* strain (35). Although these cases are all regulatory or resistance genes associated with antibiotic production and morphological differentiation, not all such genes contain TTA codons: in *S. coelicolor* the codon is absent from the pleiotropically acting *afsR* and *bldB* genes (refs. 36 and 37; M. Harasym and J. M. Piret, personal communication), the pathway-specific *redD* regulatory gene for undecylprodigiosin synthesis (38), the methylenomycin-resistance gene (39), and the aerial sporulation genes *whiG* (40), *whiE* (41), and *whiB* (N. K. Davis and K.F.C., unpublished data). Sequencing in various laboratories of structural genes for diverse *Streptomyces* antibiotic pathways has so far revealed no TTA codons in at least 30 kb of DNA.

Together, these data suggest a general model in which morphological differentiation and antibiotic production in *Streptomyces* are positively controlled by TTA-containing regulatory genes. Such a *bldA*-dependent system could permit sensitive modulation of regulatory responses by a wider range of physiological parameters, as in transcriptional attenuation in enteric bacteria (42). Where—as for undecylprodigiosin synthesis—a regulatory gene for a *bldA*-dependent pathway contains no TTA codons, we propose that the pathway is also regulated by another gene, which does contain TTA codon(s). Transcription of at least some structural genes for actinorhodin and undecylprodigiosin production is greatly reduced in *bldA* mutants (ref. 30; C. J. Bruton, E. P. Guthrie, and K.F.C., unpublished data), consistent with the proposed regulatory role of *bldA*. The occurrence of TTA codons in a minority of antibiotic-resistance genes from producers of the cognate antibiotics could imply either a regulatory role for the resistance determinant (43) or a specific limitation of expression of resistance during certain conditions of nonproduction.

A role for rare codons in modulating translation has often been discussed (e.g., ref. 44), and at least in the case of the AGG/AGA arginine codons in *E. coli*, the codons can limit gene expression in a growth-stage specific manner (45, 46). The use of the TTA codon in a potentially regulatory capacity may reflect, in part, its unsuitability for reading via "wobble" rules (47). Despite this, the studies of *hyg* expression suggest that a noncognate tRNA may sometimes translate UUA codons. The likelihood of an unusual extension of wobble rules is difficult to assess, because the literature appears to

contain no precedent for the complete absence of a cognate tRNA for a particular codon.

Earlier experiments (4, 48) showed that *bldA* gene product is principally accumulated late in colony growth (as might be expected if it regulates late events). In line with this, *ampC*-specified β-lactamase activity in *S. lividans* containing pJAS7 or pJAS14-P15 was very low in the first 24 hr in liquid cultures, but it usually increased sharply during the second or third day (ref. 20; M. Forsman and B. Jaurin, personal communication); we observed a similar lag on plates. Similarly, when the *luxAB* reporter (which contains many TTA codons) was driven by presumptively vegetatively expressed promoters in *S. coelicolor*, the youngest cultures showed little light emission (48). On the other hand, in order for *S. coelicolor* containing *carB*, *aad*, or *hyg* to grow in the presence of the relevant antibiotic, the *bldA* gene product, the relevant gene product, or (for *carB*) modified ribosomes must be present at the earliest stages of growth. This could be due to the persistence of one or more of these in spores, or exposure to the antibiotics might itself induce premature *bldA* expression.

We thank Drs. Mervyn Bibb, Mark Buttner, Tobias Kieser, Chaitan Khosla, and David Hopwood for very helpful comments on the manuscript. pOJ159 and prepublication information on the carB sequence were kindly provided by Dr. Brigitte Schoner (Lilly Research Laboratories, Indianapolis, IN). The presence of TTA codons in *ampC* was pointed out to us and *ampC*-containing plasmids were generously provided by the late Dr. Bengtåke Jaurin, to whose memory this paper is dedicated. This work was supported by grants from the Alberta Heritage Foundation for Medical Research to B.K.L., from the John Innes Foundation to E.J.L., and from the Ministerio de Educación y Ciencia of Spain to J.M.F.-A.

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