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REVIEW

The Use of the Rare UUA Codon to Define "Expression Space" for Genes Involved in Secondary Metabolism, Development and Environmental Adaptation in *Streptomyces*

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In *Streptomyces coelicolor*, *bldA* encodes the only tRNA for a rare leucine codon, UUA. This tRNA is unnecessary for growth, but is required for some aspects of secondary metabolism and morphological development, as revealed by the phenotypes of *bldA* mutants in diverse streptomycetes. This article is a comprehensive review of out understanding of this unusual situation. Based on information from four sequenced genomes it now appears that, typically, about 2~3% of genes in any one streptomycete contain a TTA codon, most having been acquired through species-specific horizontal gene transfer. Among the few widely conserved TTA-containing genes, mutations in just one, the pleiotropic regulatory gene *adpA*, give an obvious phenotype: such mutants are defective in aerial growth and sporulation, but vary in the extent of their impairment in secondary metabolism in different streptomycetes. The TTA codon in *adpA* is largely responsible for the morphological phenotype of a *bldA* mutant of *S. coelicolor*. AdpA-dependent targets include several genes involved in the integrated action of extracellular proteases that, at least in some species, are involved in the conversion of primary biomass into spores. The effects of *bldA* mutations on secondary metabolism are mostly attributable to the presence of TTA codons in pathway-specific genes, particularly in transcriptional activator genes. This is not confined to *S. coelicolor* – it is true for about half of all known antibiotic biosynthetic gene sets from streptomycetes. Combined microarray and proteomic analysis of liquid (and therefore non-sporulating) *S. coelicolor bldA* mutant cultures revealed effects of the mutation during rapid growth, during transition phase, and in stationary phase. Some of these effects may be secondary consequences of changes in the pattern of ppGpp accumulation. It is argued that the preferential accumulation of the *bldA* tRNA under conditions in which growth is significantly constrained has evolved to favour the expression of genes that confer adaptive benefits in intermittently encountered sub-optimal environments. The evolution of this system may have been a secondary consequence of the selective pressure exerted by bacteriophage attack. Some biotechnological implications of *bldA* phenomenology are considered.

***Keywords*:** codon usage, comparative genomics, antibiotic production, bacterial development, horizontal gene transfer, ecological adaptation

The discovery of *bldA* and elucidation of its molecular function

Mycelial bacteria of the genus *Streptomyce*s are the richest natural source of antibiotics and other pharmacologically active molecules for human and veterinary medicine and agriculture. Antibiotic production appears often to be associated with developmental complexity, which in streptomycetes is manifested as the formation of an aerial mycelium bearing long chains of spores. This association has a genetic basis. For example, in an early review of *Streptomyces* genetics, Hopwood (1967) described the genetic mapping of a morphological locus then called S48 on the chromosome of the model streptomycete, *S. coelicolor* A3(2). Both aspects of the mutant phenotype co-segregated (absence of blue pigment, and failure to form an aerial mycelium or spores; Fig. 1), showing that S48 identified a genetic element common to secondary metabolism (blue pigment production) and morphological differentiation. Subsequently, S48 was renamed *bldA* (Merrick, 1976), one of more than 20 known genes needed for aerial mycelium formation but not for vegetative growth of *S. coelicolor* (Kelemen and Buttner, 1999; Chater, 2001). Mutants in most of the *bld* genes are also defective in the production of both the blue pigment, which is a polyketide antibiotic called actinorhodin, and a second pigmented antibiotic, the red prodiginine complex generally called "Red". Both of these are determined by chromosomal gene clusters, but most *bld* mutants also fail to produce a colourless antibiotic, methylenomycin, specified by genes carried on the SCP1 plasmid of *S. coelicolor* (Champness and Chater, 1994).

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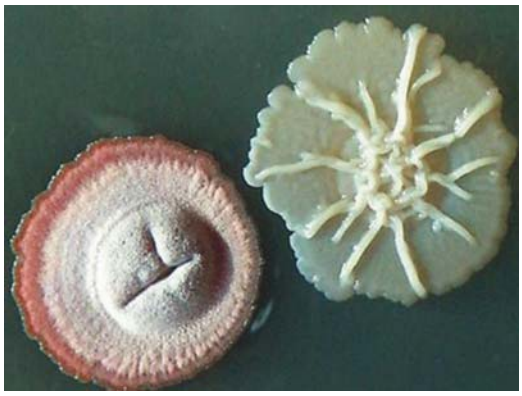

Fig. 1. Colonies of *S. coelicolor* strains differing only in their possession of an intact *bldA* gene. Left, wild-type colony; right, *bldA* deletion mutant. Photograph courtesy of Dr. T. Kieser.

This review focuses on *bldA*, which was the first *Streptomyces* developmental gene to be cloned by complementation (Piret and Chater, 1985). The *bldA* sequence, which was determined for the wild-type allele and several mutant alleles, revealed that it specified a tRNA specific for the leucine codon UUA (Lawlor *et al*., 1987). Genome sequencing (Bentley *et al*., 2002), coupled with codon-anticodon recognition rules (Crick, 1966), indicates that no other tRNA in the genome should be able to read the UUA codon. Indeed, *bldA* mutants are markedly defective in the expression of several TTA-containing test genes, and changing the TTA to a synonymous codon in these genes resulted in their full expression in a *bldA* mutant (Leskiw *et al*., 1991a). There is some apparent variation in the extent to which introduced genes and "native" *Streptomyces* genes containing a UUA codon are expressed in a *bldA* mutant (Leskiw *et al*., 1991a, and see below). There is still no experimentally-based explanation for this.

The last review specifically addressing this unusual role for a tRNA was published some 14 years ago (Leskiw *et al*., 1991b). The purpose of this article is to re-examine the role of *bldA* in the light of information from genome sequencing and functional genomics.

Evidence that increased abundance of *bldA* tRNA late in growth influences the translation of UUA-containing mRNAs

The readily observable effects of *bldA* mutations are all on processes that take place after the main period of rapid growth is over. Indeed, the *bldA*-specified tRNA is more abundant in stationary phase than during growth (Leskiw *et al*., 1993; Trepanier *et al*., 1997). This differs from the growth-associated expression typically expected for tRNA genes in response to the need for high rates of protein synthesis (the production of tRNAs, like that of other components of the protein synthesis machinery, is usually subject to "stringent control" by ppGpp, production of which is sensitive to the availability of nutrients: Strauch *et al*., 1991; Chakraburtty and Bibb, 1997). For example, in control experiments, a lysyl tRNA and another leucyl tRNA were most abundant during growth of *S. coelicolor* (Leskiw *et al*., 1993; Trepanier *et al*., 1997). The relative abundance of the *bldA* tRNA is at least partly determined by the rate of processing of the precursor tRNA (Leskiw *et al*., 1993). There is no other published information about the control of *bldA* expression, nor any about the isolation and characterisation of the tRNA or the nature, kinetics and significance of any base modifications such as are typical of mature tRNAs (Rozenski *et al*., 1999).

Three papers have provided evidence that the presence of a UUA codon in a *Streptomyces* mRNA causes delays in the expression of the mRNA as a translated gene product in relation to culture age. Leskiw *et al.* (1993) found that when the *ampC* gene (two TTA codons) was used as a reporter in *S. coelicolor,* its mRNA was detectable earlier in culture than the activity of the gene product (β-lactamase). A more complex experiment in *S. globisporus* likewise indicated a lag between transcription of a TTA-containing gene and translation of the UUA-containing mRNA (Rebets *et al*., 2006, further described in the next section). Thirdly, the change of TTA codons to alternative leucine codons in a *gfp* reporter gene resulted in earlier and spatially less specific fluorescence in *S. lividans* colonies (Kataoka *et al*., 1999). On the other hand, one paper reported that varying the number of TTA codons in the pathway-specific regulator for actinorhodin synthesis from none to three made no perceptible difference in the time of onset of actinorhodin biosynthesis (Gramajo *et al*., 1993).

Does *bldA* have a similar role in other streptomycetes? Phenotypically comparable *bldA* mutants have been described in *Streptomyces lividans*, which is closely related to *S. coelicolor* (Leskiw *et al*., 1991b), and in *Streptomyces clavuligerus* (Trepanier *et al*., 2002), *Streptomyces avermitilis* (Tao *et al*., 2007) and *Streptomyces griseus* (Kwak *et al*., 1996), three strains that belong to a phylogenetic group whose last common ancestor with *S. coelicolor* probably lived about 220 million years ago (A. Ward, personal communication).

The influence of *bldA* on production of several antibiotics in *S. coelicolor* is mediated via TTA-containing pathway-specific regulatory genes

Using transcriptional fusions of biosynthetic genes to the reporter gene *xylE*, it was shown that the failure of a *bldA* mutant of *S. coelicolor* to produce actinorhodin, Red or methylenomycin is a transcription-level effect (Guthrie and Chater, 1990; Bruton *et al*., 1991; A. Wietzorrek and KFC, unpublished, cited by Champness and Chater, 1994). This suggested that *bldA* acted on the pathways via a transcriptional regulatory mechanism. Indeed, TTA-containing pathway-specific regulatory genes are present in each of the three biosynthetic gene clusters. Thus, one of two regulatory genes in the *act* cluster, *actII-ORF4*, contains a TTA codon and is the key activator of most or all of the biosynthetic pathway genes (the other regulatory gene, *actII-ORF1*, encodes a repressor of two adjacent genes involved in actinorhodin export: one of the latter also has a TTA codon) (Fernandez-Moreno *et al*., 1991). When the TTA codon in *actII-ORF4* was changed to an alternative leucine codon, actinorhodin biosynthesis was restored to a *bldA* mutant, confirming that the effect of *bldA* mutations on actinorhodin biosynthesis was attributable to this TTA codon (Fernandez-Moreno *et al*., 1991). The key role of *actII-ORF4* in the *bldA*-dependence of actinorhodin

production was also suggested by the finding of Passantino *et al*. (1991) that the presence of multiple copies of *actII-ORF4* on a plasmid led to actinorhodin production by a *bldA* mutant. This observation was paradoxical, since the translation of the *actII-ORF4* mRNA should remain *bldA*-dependent regardless of its abundance. Clearly, some degree of *bldA*-independent translation of the UUA codon in *actII-ORF4* mRNA was possible.

Likewise, multiple copies of *redZ*, a positively acting regulatory gene that contains the only TTA codon in the entire *red* cluster, permitted Red production by a *bldA* mutant (White and Bibb, 1997), and a base change in the *redZ* promoter, probably causing *redZ* overexpression, allowed a *bldA* mutant to produce Red (Guthrie *et al*., 1998). Because all these effects were most marked on the rich, high osmolarity medium R2YE, there may be increased UUA mistranslation on this medium compared with minimal medium (White and Bibb, 1997). UUA mistranslation may also be enhanced on minimal medium when phosphate levels are lowered, since Red was produced by a *bldA* mutant under these conditions (Guthrie and Chater, 1990; White and Bibb, 1997).

The *bldA* dependence of methylenomycin production results from the presence of TTA codons in two genes involved in the pathway-specific activation of the *mmy* biosynthetic genes (Bentley *et al*., 2004). Methylenomycin production was restored to a *bldA* mutant by changing both of these codons to alternative leucine codons (O'Rourke, 2003; Chater, 2006).

In *Streptomyces griseus*, the dependence of streptomycin production on *bldA* involves global regulation as well as pathway-specific effects

Bald, streptomycin non-producing *S. griseus* mutants include *bldA* mutants (Kwak *et al*., 1996) and mutants defective in production of the extracellular signalling γ-butyrolactone molecule A-factor (reviewed by Horinouchi, 2002 and Chater and Horinouchi, 2003). The activity of A-factor depends on the diffusion of the factor back into cells, where, at sufficient concentration, it binds to a receptor protein, ArpA, which is a repressor of the gene *adpA*. Binding of A-factor to ArpA releases repression, and *adpA* becomes actively transcribed. Failure to express *adpA* is by itself responsible for the entire pleiotropic phenotype of A-factor deficient mutants (Kato *et al*., 2004). Since *adpA* mRNA contains a UUA codon (Ohnishi *et al*., 1999; Chater and Chandra, 2006), *bldA* is presumably needed at this point for AdpA protein to be made. AdpA also represses its own synthesis (Kato *et al*., 2005b), so we may predict that any limitation in translation of *adpA* mRNA due to a lack of the *bldA* tRNA would tend to lead to increased *adpA* transcription. Thus, relief of this deficiency, for example by developmental upregulation of *bldA* expression, would suddenly permit the translation of the over-abundant *adpA* mRNA, potentially giving rise to a rapid burst of AdpA protein (Hesketh *et al*., 2007).

The substantial regulon of genes activated by AdpA (Ohnishi *et al*., 2005: see also below) includes *strR*, the pathway-specific activator gene for streptomycin biosynthesis (Ohnishi *et al*., 1999). The 30th triplet in *strR* is a TTA codon, and a TTA codon is at position two in *strN*, the first gene in one of the operons of streptomycin biosynthetic structural genes. These TTA codons are conserved in the orthologous genes of the hydroxystreptomycin producer *Streptomyces glaucescens*, despite the significant degree of sequence and organisational divergence of the clusters from the two organisms (Distler *et al*., 1992). One aspect of the organisational differences is that in *S. griseus* the resistance gene *strA* is cotranscribed with, and downstream of, *strR*, whereas in *S. glaucescens strA* is transcribed independently. Only the *S. glaucescens strA* has a TTA codon (position 39), the absence of the codon from the *S. griseus* gene perhaps being unnecessary because of polar effects of the TTA upstream in *strR* (Distler *et al*., 1992). Thus, streptomycin production in *S. griseus* depends on *bldA* at several levels. In *S. glaucescens*, there is no evidence of control of streptomycin production by an A-factor-like system, so any influence of *bldA* on production would possibly be exerted directly on the cluster.

Investigating the effects of *bldA* and TTA codons on antibiotic biosynthesis in other streptomycetes

Several studies have investigated the effects of TTA codons in the relevant gene clusters on antibiotic biosynthesis in other streptomycetes. For example, one study exploited transcriptional and translational fusions of the landomycin biosynthesis regulatory gene *lndI* of *Streptomyces globisporus* to a TTA-free version of the gene for the fluorescent reporter protein GFP (Rebets *et al*., 2006). Fluorescence was delayed in the strain carrying the translational fusion relative to that of the strain carrying the transcriptional fusion. This delay was alleviated when the TTA codon in *lndI* was replaced by an alternative leucine codon. In another example, Tercero *et al*. (1998) took advantage of the fact that the complete *Streptomyces alboniger* gene set for puromycin biosynthesis can direct puromycin production when subcloned into the surrogate host *S. lividans,* to examine the effect of using a constructed *S. lividans bldA* mutant as host. There was virtually no production, and production was restored by complementation of the *bldA* mutation. Probably, the *bldA*-dependence can be attributed to TTA codons in two of the structural genes in the cluster (*pur6* and *pur10*). In a third example, Tao *et al*. (2007) constructed a *bldA* mutant of *S. avermitilis*, and found that it lost the ability to produce avermectin, probably because of TTA codons in two of the pathway-specific genes, *aveA3* and the regulatory gene *aveR.*

Just one exception to the general rule of *bldA*-dependence of clusters containing a TTA codon has been described. In *Streptomyces clavuligerus* the gene encoding the pathway-specific activator of both cephamycin C and clavulanic acid production, *ccaR* (Perez-Llarena *et al*., 1997), contains a TTA codon. Unexpectedly, a *bldA* null mutation in *S. clavuligerus* constructed with the cloned *S. clavuligerus bldA* region still made the two antibiotics (Trepanier *et al*., 2002) even though it had a typical sporulation defective phenotype. Transcript analysis showed no difference in the levels of *ccaR* transcripts in the wild-type and *bldA* mutant strains, ruling out any effect of elevated levels of the *ccaR* mRNA. Furthermore, when compared to the wild-type strain, the *bldA* mutant showed no differences in the levels of CcaR protein, suggesting that the single UUA codon in *ccaR* mRNA is mistranslated efficiently, at least under fermentation conditions. Presumably, either codon context can affect the level of UUA mistrans-

lation, or mistranslation is higher in the medium used (see above). It is interesting to note that clavulanic acid production, unusually for an antibiotic, is growth-associated.

The occurrence of TTA codons in other gene clusters for antibiotic biosynthesis in streptomycetes

In a database search, TTA codons were found in 110 antibiotic gene clusters from other streptomycetes while only 34 completely sequenced clusters were free of TTA codons (Chater, 2006; Chandra and Chater, in preparation). In 62 cases TTA codons fell in probable regulatory genes. Probably, therefore, control of antibiotic production in many of those cases is *bldA*-dependent, although the situation for clavulanic acid production in *S. clavuligerus* (above) necessitates caution in making this assumption in the absence of experimental analysis. In one case (clorobiocin in *S. roseochromogenes* subsp. *oscitans*) there were TTA codons in 12 genes, five of the genes having more than one TTA. This gene set, which has a markedly lower GC content (66.1%) than nearly all the other clusters analysed (mostly more than 70% GC), may have been relatively recently acquired from another genus in which TTA codons do not have a comparable regulatory role.

***AdpA*, a central regulator of development, largely mediates the effects of *bldA* on morphological development**

The regulatory gene *adpA* mentioned above is present in all (four) sequenced *Streptomyces* genomes to which there is access at the time of writing, and it always has a TTA codon at the same position. Thus, a *bldA-adpA* link appears to be an ancient feature of streptomycetes (Chater and Chandra, 2006). In *S. coelicolor*, the regulatory position of AdpA in relation to both inputs and outputs differs from that outlined above for *S. griseus*: in *S. coelicolor, adpA* does not appear either to be regulated by an extracellular autoregulator such as A-factor, or to regulate any of the antibiotic biosynthetic gene sets analysed so far (Chater and Horinouchi, 2003). However, it is needed for normal development on most laboratory media (it was originally identified genetically as *bldH* in a survey of *S. coelicolor* mutants lacking aerial mycelium: Champness, 1988; Takano *et al*., 2003). The bald phenotype of *bldA* mutants is largely because of the TTA in *adpA,* since changing this codon restored the ability to make aerial mycelium and spores, though without fully restoring wild-type colony morphology (Nguyen *et al*., 2003; Takano *et al*., 2003). Thus, the effects of *bldA* on development, at least in *S. coelicolor,* should involve the failed expression of some part(s) of the AdpA regulon.

Work in the Tokyo laboratory of S. Horinouchi has identified many direct AdpA target genes, based on *in vitro* binding of AdpA to DNA, particularly in *S. griseus* (Ohnishi *et al*., 2005). They include genes for proteases (Kato *et al*., 2002, 2005a; Tomono *et al*., 2005) and a protease inhibitor (Hirano *et al*., 2006); a developmentally significant sigma factor (Yamazaki *et al*., 2000); SsgA, which regulates cell wall biosynthetic activity associated with septation early in aerial growth (Yamazaki *et al*., 2003a, 2003b); and AmfR, which activates genes for the synthesis of a small morphogenetic peptide, AmfS [interestingly, *amfR* contains a TTA codon, though its equivalent gene in *S. coelicolor*, *ramR*, does not: thus, *amfR*, and hence production of AmfS, is dependent on *bldA* for at least two reasons (Yamazaki *et al*., 2003; Ueda *et al*., 2005)]. A consensus AdpA-binding site derived in these studies (Yamazaki *et al*., 2004) was valuable in the interpretation of some of the data from a proteomic analysis of a *bldA* mutant of *S. coelicolor* (Kim *et al*., 2005a): the absence, in the mutant, of a small protein (Sti) that inhibits trypsin-like proteases led to the recognition of two likely AdpA-binding sequences in the *sti* promoter region, suggesting that the *bldA*-dependence of Sti might be medi-

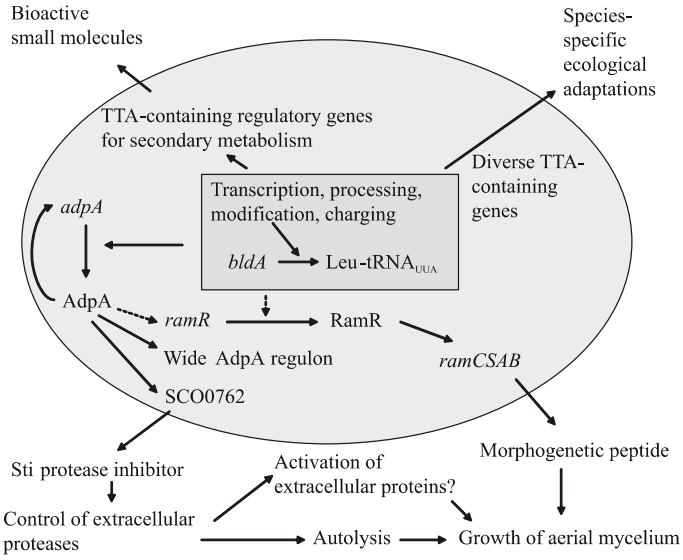

Fig. 2. Extracellular functions dependent on *bldA* in *S. coelicolor.* Dotted arrows indicate a step demonstrated in *S. griseus.* All other aspects are likely to be widespread among streptomycetes. Note that in *S. griseus* the *ram* gene orthologues are known as *amf* genes, and their extracellular end-product is called AmfS peptide (Ueda *et al*., 2002), while the end product of the *ram* genes is called SapB (Kodani *et al.*, 2004).

ated via AdpA. Indeed, the *sti* mRNA was undetectable in an *adpA* mutant. The regulation of protease inhibitors by AdpA in both *S. grieus* and *S. coelicolor* is notable, because protease inhibitors have been implicated in an extracellular cascade of proteases in other streptomycetes, and there is evidence that this cascade plays a significant part in development, apparently as part of the autolytic process by which the vegetative biomass is converted into spores (Kim and Lee, 1995; Chater, 2006; Hirano *et al*., 2006; Manteca *et al*., 2006). As already mentioned, the AdpA-regulated genes of *S. coelicolor* and *S. griseus* include several protease determinants. These may well be important contributors to development, and to the morphological phenotype of *bldA* mutants, though the multiplicity of protease determinants has made it difficult to interpret the results of protease gene knockout experiments (Hirano *et al*., 2006). It remains to be seen whether proteolytic activities are involved in the extracellular signalling among bald mutants described by Willey *et al*. (1993). The end-point of this cascade is the production of certain morphogenetic proteins (SapB and chaplins) that encase aerial hyphae, and whose absence leads to a bald phenotype (Elliot and Talbot, 2004; Willey *et al*., 2006).

Figure 2 summarises the striking extent to which *bldA* is known to be involved in extracellular processes.

Other *bldA*-influenced genes in *S. coelicolor* revealed by combined microarray and proteomic analysis

Recent analyses of the extracellular, membrane-associated and soluble cytoplasmic proteomes of an *S. coelicolor bldA* mutant, augmented by microarray analysis of global gene expression, identified 147 genes whose expression during growth in liquid medium is changed by mutation of *bldA* (Kim *et al*., 2005a, 2005b; Hesketh *et al*., 2007). Only two of these genes contained TTA codons, and no other TTA-containing genes were perceptibly expressed in the wild-type. Most of the genes affected showed reduced expression in the mutant, but a significant minority were over-expressed. There were many examples of *bldA*-dependent changes in the relative abundance of different forms of proteins (as revealed by multiple spots on 2-D gels). Almost nothing is understood of the basis for this, though some of the changes might possibly reflect changes in the protease profile of the *bldA* mutant referred to above.

bldA mutations have some effects that precede stationary phase. Many of the *bldA*-influenced genes studied by Hesketh *et al*. (2007) and Kim *et al*. (2005a, 2005b) were expressed most strongly in stationary phase, consistent with the observed pattern of abundance of the *bldA* tRNA (see above). However, a substantial number were expressed before stationary phase, most notably many ribosomal protein genes. The latter genes normally undergo a brief up-regulation during the transition phase between rapid growth and stationary phase, but this up-regulation was not seen in the *bldA* mutant (Vohradsky and Thompson, 2006; Hesketh *et al*., 2007). In the wild-type, this up-regulation is possibly associated with a sharp increase in levels of the stringent factor ppGpp (Strauch *et al*., 1991; Chakraburtty and Bibb, 1997). Interestingly, the basal level of ppGpp proved to be substantially increased in a *bldA* mutant, leading Hesketh *et al*. (2007) to suggest that this might be responsible for the disturbance of ribosomal protein gene expression. It was further tentatively suggested that the elevated ppGpp levels might result from the activation of ribosome-associated ppGpp synthetase when a small number of ribosomes encounter mRNA containing untranslatable UUA codons (Hesketh *et al*., 2007).

Many of the other *bldA*-influenced genes expressed early in culture are likely to play a part in peripheral aspects of primary and salvage metabolism operating under conditions of nutritional stress-the only central metabolism genes affected were for aromatic amino acid biosynthesis (SCO1496, chorismate synthase; and SCO2115, one of two *aroH*-like genes) and biotin biosynthesis (SCO1244, 1246) (Hesketh *et al.*, 2007).

A surprising and unexplained aspect of the phenotype of the *bldA* mutant studied by Hesketh *et al.* (2007) was its increased growth rate compared to its wild-type parent, an observation confirmed and extended by D.W. Kim and K.J. Lee (personal communication).

Previously unknown effects on secondary metabolism. The list of chromosomally determined *S. coelicolor* secondary metabolism pathways affected by *bldA* grew from the two known previously (actinorhodin and Red; see above) to seven in the global studies of Hesketh *et al*. (2007) and Kim *et al*. (2005a, 2005b). Thus, the only product of the *cda* gene cluster for the lipopeptide antibiotic CDA to be detected (the oxygenase encoded by SCO3236) was significantly reduced in abundance in the *bldA* mutant, while the type III polyketide synthase (SCO7221) for germicidin production (Song *et al*., 2006) was greatly overproduced in the mutant. In neither of these cases is any TTA-containing gene present in or near the genes affected. In another case, mRNA and proteins corresponding to several of the 21 genes of the SCO0381-0401 "deoxysugar/glycosyltransferase" gene cluster were markedly less abundant in the *bldA* mutant. There are two TTA-containing genes (SCO0383 and 0399, neither of regulatory character) in the cluster, and it was suggested that inefficient translation of the corresponding UUA codons in polycistronic transcript(s) can cause both polarity and overall mRNA instability (see also below).

The *bldA* mutant had reduced levels of expression of some, but not all, of the genes for the non-ribosomal peptide iron siderophore coelichelin (SCO0489-0499: Lautru *et al*., 2005), and increased expression of one, but not another, of the genes from the biosynthetic cluster for another iron siderophore complex, desferrioxamines (SCO2782-2785: Bentley *et al*., 2002). Neither of these clusters contains any TTA codons. The effects of *bldA* deletion on the latter two gene sets were manifested before the onset of stationary phase, so perhaps they result from the disturbed pattern of ppGpp abundance referred to above.

A likely operon of unknown function is affected by bldA *through a regulator encoded by a nearby TTA-containing gene*. The expression of a cluster of TTA-free function-unknown genes was particularly strongly dependent on *bldA* (SCO4246, SCO4251-3, SCO4256, and SCO4262; Kim *et al*., 2005b; Hesketh *et al*., 2007). Deletion of a nearby TTA-containing gene, SCO4263, encoding a LuxR-family regulator, abolished expression of the SCO4251-4253 operon (Hesketh *et al*., 2007). No obvious phenotypic change was associated with

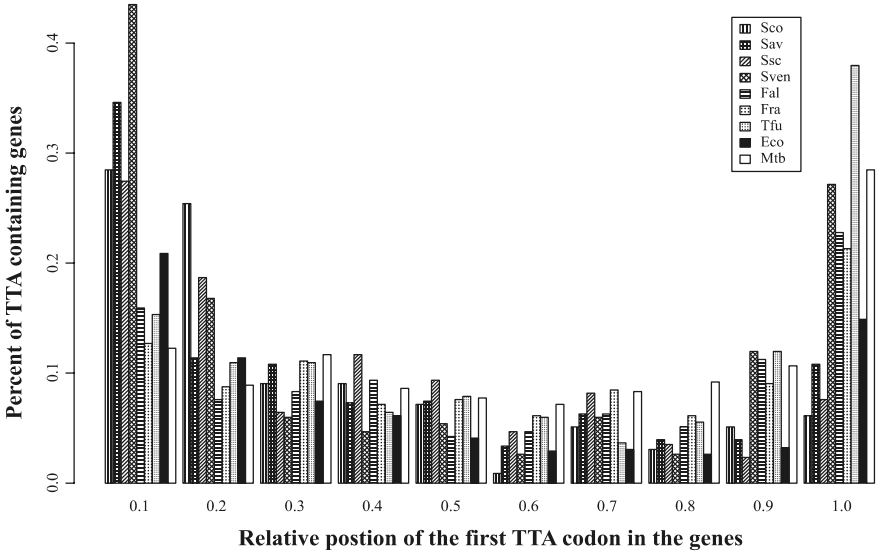

Fig. 3. Relative position of the first TTA codon in genes of various actinomycetes. Note that the four *Streptomyces* species show a conspicuously higher occurrence of TTA codons close to the start of genes than do three closely related actinomycetes with high GC DNA (*Themobifida fusca* and two *Frankia* species) or one (*Mycobacterium tuberculosis*) with moderately high GC DNA. The data for *E. coli*, included for comparative purposes, show a high frequency in the first 10% of genes because the TTA codon occurs several times in most genes, and there is therefore an increased likelihood that the first occurrence will be close to the start. For the sources of genomic information, see Ventura *et al.* (2007).

the deletion of SCO4263.

Polarity effects of UUA codons in polycistronic mRNAs

In some cases, a UUA codon in a polycistronic mRNA may result in polarity effects on the downstream part of the RNA in a *bldA* mutant. A clear case of this was described for a duplicated gene cluster in the SCP1 plasmid, whose encoded gene products include three spore-associated proteins (SapC, D, E) that were absent from a *bldA* mutant, even though the genes contain no TTA codons (Bentley *et al*., 2004). A TTA codon in a putative regulatory gene (SCP1.58c/295) upstream in the same operon was deduced to be responsible for *bldA*-dependent expression: a transcriptional reporter gene inserted downstream of the regulatory gene was *bldA*-dependent, while the same reporter gene inserted upstream of the TTA codon was *bldA*-independent. In another possible example, transcription and translation of a TTA-containing operon of unknown function (SCO6638-6637) were both greatly reduced in the *bldA* mutant (Hesketh *et al*., 2007). In a third case, TTA codons in of the SCO0381-0401 "deoxysugar/glycosyltransferase" gene cluster were presumed to be responsible for reduced levels of mRNA for the entire cluster in a *bldA* mutant in *S. coelicolor*, as measured by microarray analysis (Hesketh *et al*., 2007; see above). It is not excluded for either of the latter two examples that the reduced amounts of mRNA could have resulted from other, less direct, effects.

Bioinformatic analysis of TTA-containing genes in streptomycetes

Consistent with the idea that UUA codons have a special role in streptomycetes, TTA codons are rarer in *Streptomyces* genes than in any other characterised actinobacterial genomes, including those of *Frankia* and *Nocardia farcinica*, which have similarly high GC content in their DNA (Chater and Chandra, 2006; Li *et al*., 2007). In addition, the positions of TTA codons within genes from any of the four available *Streptomyces* genome sequences show a stronger bias towards the beginning of coding sequences (ie within the first 30 codons) than in genes from other actinomycetes (Fig. 3: Leskiw *et al*., 1991b; Fuglsang, 2005; Chater and Chandra, 2006). Similar conclusions can be drawn from analysing TTA-containing genes in secondary metabolism gene clusters from diverse species (Chandra and Chater, in preparation). UUA codons close to the start of the coding sequence of mRNAs may be more effective in eliminating translation when the *bldA* tRNA is at lower abundance levels (Leskiw *et al*., 1991b). However, there are also many cases of TTA codons in the middle of genes or close to their 3' end. At least in certain cases, the codon may fall between the coding sequences for functionally different domains: a good example is the *wblP* gene in the SCP1 plasmid of *S. coelicolor*, whose 5' end encoding a domain homologous with actinobacterial "WhiB-like proteins" is separated by a TTA codon from a domain encoding a presumptive RNA polymerase sigma factor (Bentley *et al*., 2004). This might conceivably permit the expression, in conditions limiting the translation of UUA codons, of a protein consisting only of the more upstream domain (but this would require the operation of some unknown mechanism for the release of the truncated protein). This possibility can be ruled out for some genes containing more-or-less centrally located TTA codons: for example, the products of some of the TTA-containing genes of the clorobiocin biosynthetic pathway referred to above have been characterised structurally, and none of the leucines encoded by the TTA codons fall between deduced func-

tional or folding domains (D.M. Lawson, personal communication).

The chromosome of *S. coelicolor* includes 145-147 TTA-containing genes (Chater and Chandra, 2006; Hesketh *et al.*, 2007; Li *et al*., 2007). The exact number cited varies, because it depends on the allocation of start codons, and many TTA codons occur near the start of genes: in fact, proteomic analysis indicated that the TTA codon in one, SCO6717, is actually upstream of the real translation start site (Hesketh *et al*., 2007). There are also 19 TTA-containing genes in *S. coelicolor* plasmids SCP1 and SCP2. The *S. avermitilis* chromosome has 260 TTA-containing genes, plus eight in plasmid SAP1, and *S. venezuelae* and *S. scabies,* both of which are plasmid-free, have a similar number (Chater and Chandra, 2006; Table 1).

Close to half of the TTA-containing genes of each genome failed to register a hit in reciprocal protein-level BLAST searches of the genomes any of the other three species; and this fraction was increased to about two-thirds when closer inspection of the individual reciprocal hits revealed that they were too diverged to be within the normal range for true orthologues (Table 1; GC and KFC, in preparation). It thus appears that many TTA-containing genes have been acquired by horizontal gene transfer since the last common ancestor of any of these strains. This point is reinforced by the finding that many of the TTA-containing genes of *S. coelicolor* fall within segments of DNA previously identified as being horizontally acquired, using such criteria as possessing atypical base composition, being flanked by tRNA genes (which, in bacteria, are often used as the sites of insertion of DNA adapted for horizontal transfer), or containing genetic information associated with transposition (Jayapal *et al*., 2007; Li *et al*., 2007). For example, the previously mentioned *bldA*-dependent genes SCO4246, SCO4251-3, SCO4256, and SCO 4262, and their TTA-containing regulatory gene SCO4263 are part of an island of DNA absent from other sequenced *Streptomyces* genomes, and one end of this island is at a tRNA gene: two of the genes in the island (SCO4245 and SCO4246) encode products of a kind associated mainly with bacteriophages (Kim *et al*., 2005b; Hesketh *et al*., 2007). In addition, unambiguously laterally transferred elements often contain genes with TTA codons, including the self-transmissible plasmid SCP1 (which has TTA codons in 18 of its 353 genes) and several transposase genes (Bentley *et al*., 2002, 2004).

Out of the total of 846 different (i.e., non-orthologous) genes containing a TTA in at least one of the four genomes, 119 were present in all four (GC and KFC, in preparation). In most of these cases, some of the orthologues are TTA-free. Just three genes have a TTA in all four species. These correspond in *S. coelicolor* to SCO2792 (encoding AdpA – see above), SCO4395 (encoding a possible hydrolase) and SCO7251 (encoding a protein of unknown function). In nearly all examples of omnipresent genes having a TTA codon in at least two of the four genomes, the genes occupy syntenous positions in the various chromosomes. Presumably they were present, and subject to any controls mediated by *bldA*, in a common progenitor of these species, probably at least 220 million years ago (A. C. Ward, cited in Chater and Chandra, 2006), but selection for retention of their function has been stronger than selection for the retention of their TTA codon, and hence of their *bldA*-dependence. We suppose that the 75 genes present in three out of four of the genomes, and having a TTA in at least one genome, were either (a) present early in the lineage, and lost more recently in the line leading specifically to one of the strains, or (b) acquired between the first and second species-specific branch point. The more detailed dissection of the patterns of occurrence of TTA-containing genes and their orthologues among different streptomycetes is beyond the scope of this article, but it may well provide a useful focus for the better understanding of the dynamics of gene acquisition and gene loss during the diversification of the genus.

Even between two strains as closely related as *S. coelicolor* and the still unsequenced *S. lividans*, whose orthologous genes are usually about 99% identical at the DNA sequence level (Chater and Chandra, 2006), there are considerable differences in the complement of TTA-containing genes. Microarray analysis of genomic DNA showed that some 43 (c. 30%) of the *S. coelicolor* TTA-containing chromosomal genes were in groups of genes (islets or islands) absent from *S. lividans* (Jayapal *et al*., 2007). None of the other TTA-containing genes of *S. coelicolor* was reported to be absent from *S. lividans.* Since, overall, 92% of the *S. coelicolor* genome is conserved in *S. lividans* (Jayapal *et al*., 2007), TTA-containing genes are particularly highly enriched among genes for which there is evidence of comparatively recent acquisition or loss.

Table 1. The distribution of reciprocal hits of TTA-containing genes in four *Streptomyces* genomes. The TTA-containing genes of each genome were used as probes in BLAST searches of the other genomes (using the deduced protein sequences). Reciprocal hits were then filtered manually to remove non-orthologous hits. These were defined as those in which the amino acid identity of the gene products was below 50% or the alignment covered less than 80% of the larger of the gene products, except that such cases were not removed if they showed evident synteny. Not all of the orthologous genes contain TTA codons. Sources of genome sequences were: *S. coelicolor*, Bentley *et al.* (2002); *S. venezuelae,* Diversa Corporation, and MJ Bibb, personal communication; *S. scabies*, R. Loria and SD Bentley, pers. comm.. and http://www.sanger.ac.uk/projects/S_scabies/; *S. avermitilis,* Ikeda *et al*. (2003)

TTA-containing genes	*S. coelicolor* genome inc. SCP1	*S. venezuelae* genome	*S. scabies* genome	*S. avermitilis* genome inc. SAP1	Total
Total	165	221	245	268	846*
Strain-specific	101	134	162	156	553

* This is the total after removal of redundant entries from a table summing all the results of reciprocal BLAST analyses using each of the four genomes as the reference. It is therefore not a summation of the total numbers of TTA-containing genes in each genome given in the same row.

The roles of TTA-containing genes

The obvious phenotypic manifestations of *bldA* mutation, namely aerial mycelium deficiency and failure to make pigmented antibiotics, have all been attributed to the TTA codons in regulatory genes (*adpA*, a*ctII-orf4*, *redZ, amfR*; see above for details). It was therefore predicted that mutations in other TTA-containing genes would not generate readily recognisable phenotypes. Some 21 of the *S. coelicolor* set were separately knocked out by Li *et al.* (2007), and indeed none of the mutations apart from *adpA* had any obvious effect on growth and colony morphology on several different media. It is difficult to avoid the conclusion that the great majority of TTA-containing genes are either (a) functionless (not very plausible, because functionless genes tend to be lost quickly), (b) functionally redundant (unlikely, considering the great variety of such genes in different streptomycetes), or – most likely – (c) confer adaptive benefits in particular environments or circumstances that are not usually reproduced in the laboratory (potentially including factors such as the presence of diverse other microorganisms, life close to or even within plants and animals, exposure to unusual physical conditions or chemical antagonists, attack by phages, etc). On this view, the level of *bldA* tRNA would be sensitive to some rather generalised departure from ideal growth conditions-perhaps directly via a nutrient-sensing system such as the stringent response, or a yet-to-be described quorum-sensing system, or indirectly through sensing of growth rate. Any particular TTA-containing gene would probably also be subject to other sensory input. This falls within the idea that checkpoint multiplexes control the progression of *Streptomyces* development (Chater, 2001). In summary, *bldA* may provide an expression space outside of which TTA-containing genes cannot function, and in which most of them exist by a combination of horizontal gene transfer and ecological chance.

How might one find the function of any of these phenotypically cryptic genes? One approach might be through experiments addressing competitiveness directly in quasi-real environments. Another possibility may be through the unexpected discovery by M. Tao (personal communication), which was also suggested by the work of Gehring *et al.* (2000), that *bldA* mutants cannot be constructed in some strains derived from *S. coelicolor* A3(2), including the M145 strain used for genome sequencing.

Evolution of *bldA*

As mentioned earlier, it appears that the ancient progenitor of several modern streptomycetes, living perhaps 220 million years ago, already used the *bldA-adpA* link in development. To explain how the use of the *bldA*-specified tRNA could have have acquired specific ecological/developmental significance, Chater and Chandra (2006) suggested that an ancient drift towards high GC content in early actinomycetes could have reduced the effectiveness of attack by phages with a different codon bias, and, in the line leading to streptomycetes, could eventually have permitted a loss of the UUA-reading tRNA, conferring resistance to any phages that employ this codon. Inevitably, some phages would have countered this by acquiring a gene for a UUA-reading tRNA (tRNA genes are often present in phage genomes). By this means, the tRNA gene might have been reintroduced into a proto-streptomycete as part of a prophage genome. Because of continued selection for phage resistance, the newly acquired tRNA might have evolved to be poorly expressed during vegetative growth (the main phage-sensitive stage of bacteria), but its expression during stationary phase (which is a period generally unfavourable for phage multiplication) would have conferred little disadvantage. This situation could have come to be "exploited" by genes that are specifically useful in circumstances in which free growth is subject to some limitation (Chater, 2006). [We note here that the supposed acquisition of *bldA* would thus be an ancient feature in relation to streptomycetes-this was somewhat obfuscated by Jayapal *et al*. (2007), who interpreted the evolutionary model to imply relatively recent acquisition of *bldA*.]

Possible use of a *bldA* mutant as an expression host for antibiotic biosynthetic genes

The manipulation of antibiotic biosynthetic gene sets for applied or academic purposes is often difficult within their natural hosts, so it has been found beneficial to be able to introduce such gene sets into a surrogate *Streptomyces* host in which genetic manipulation procedures are well-established. The host organisms of choice for this purpose have generally been derivatives of *S. lividans* 66, which is very closely related to *S. coelicolor* A3(2). For example, studies of biosynthetic genes for cinnamycin (Widdick *et al*., 2003), puromycin (Tercero *et al*., 1998), novobiocin and clorobiocin (Eustaquio *et al*., 2005) have made progress through this approach. However, *S. lividans* contains its own sets of antibiotic biosynthetic genes, which may be expressed under some fermentation conditions and compete for metabolic intermediates with the desired end product (as well as potentially complicating the purification or characterisation of the product). It would be of interest to consider the use of a *bldA* mutant of *S. lividans* as a host, even if this would necessitate the change of one or more TTA codons to other leucine codons in the target gene set. An alternative approach would be to eliminate *bldA* in the original host, again requiring the changing of any TTA codons in the cluster of interest.

Concluding Remarks

This article has sought to provide a comprehensive survey of current knowledge about *bldA* and its possible targets. We believe that from this there emerges a great weight of circumstantial evidence in support of the importance of *bldA* for the evolution and adaptation of streptomycetes, even though this role is likely to be manifested mainly in conditions that are not reproduced in normal laboratory experiments. At present, the evidence suggests that this system is confined to *Streptomyces*. If so, one question for the future will be to find out why such a system is not present in other organisms with extreme bias in the base composition of their DNA.

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