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A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*

E. Takano,[1†‡] M. Tao,[1,2‡] F. Long,[2] Maureen J. Bibb,[1] L. Wang,[2] W. Li,[2] M. J. Buttner,[1] Mervyn J. Bibb,[1,3] Z. X. Deng[2,4] and K. F. Chater[1*]

[1]*John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK.*

[2]*Huazhong Agricultural University, Wuhan, China.*

[3]*Diversa Corporation, 4955 Director's Place, San Diego, CA 92121, USA.*

[4]*Bio-X Life Science Research Centre, Shanghai Jiaotong University, Shanghai 200030, China.*

Summary

Streptomycetes are mycelial bacteria that produce sporulating aerial hyphae on solid media. Bald (*bld*) mutants fail to form aerial mycelium under at least some conditions. *bldA* encodes the only tRNA species able to read the leucine codon UUA efficiently, implying the involvement of a TTA-containing gene in initiating aerial growth. One candidate for such a gene was *bldH*, because the *bldH109* mutant of *Streptomyces coelicolor* resembles *bldA* mutants in some aspects. In the work reported here, $adpA_c$, an *S. coelicolor* gene similar to the *Streptomyces griseus* A factor-regulated $adpA_g$, was found to complement the *bldH109* mutant partially at both single and multiple copies. The sequence of $adpA_c$ from the *bldH109* mutant revealed a frameshift. A constructed in frame deletion of $adpA_c$ conferred a bald colony phenotype, and the mutant behaved like *bldA* mutants and *bldH109* in its pattern of extracellular signal exchange. Both $adpA_c$ and $adpA_g$ contain a TTA codon. A TTA-free version of $adpA_c$ was engineered by replacing the TTA leucine codon with a cognate TTG leucine codon. The *adpA*(TTA→TTG) gene could partially restore aerial mycelium formation to a *bldA* mutant when it was followed *in cis* by the gene *ornA*, as in the natural chromosomal arrangement. This indicated that the UUA codon in $adpA_c$ mRNA is the principal target through which *bldA* influences morphological differentiation. It also implied that translational arrest at the UUA codon in $adpA_c$ mRNA caused a polar effect on the downstream *ornA*, and that the poor translation of both genes contributes extensively to the deficiency of aerial mycelium formation in *bldA* mutants. Unlike the situation in *S. griseus*, $adpA_c$ transcription does not depend on the host's γ-butyrolactone signalling system, at least in liquid cultures. In addition, sigma factor BldN, which is the homologue of an *S. griseus* sigma factor AdsA that is absent from $adpA_g$ mutants of *S. griseus*, was present in the constructed $adpA_c$ null mutant of *S. coelicolor*.

Introduction

In *Streptomyces coelicolor*, mutation of *bldA*, which encodes the tRNA for the rare leucine codon UUA, causes pleiotropic deficiencies in both the development of reproductive aerial hyphae and the production of antibiotics on most media (Merrick, 1976; Lawlor *et al*., 1987). Because most genes containing TTA codons are phenotypically very poorly expressed in *bldA* mutants, it is assumed that no essential genes contain this codon (Leskiw *et al*., 1991). (A caveat to this statement arises from the discovery that the UUA-containing mRNA for the pathway-specific regulator of the linked pathways to clavulanic acid and cephamycin C in *Streptomyces clavuligerus* is translated effectively in a *bldA* mutant; Trepanier *et al*., 2002.) Examination of the recently completed genome sequence of *S. coelicolor* (Bentley *et al*., 2002) reveals that only 145 of the 7825 genes in the 8.7 Mb genome contain a TTA codon, and none of these is recognizably a housekeeping gene. The TTA-containing genes include pathway-specific regulatory genes for the biosynthesis of at least two of the antibiotics made by *S. coelicolor* and, in each case, it has been shown that the regulatory gene is the sole and direct means by which *bldA* exerts its effects on production of the cognate antibiotic (Fernandez-Moreno *et al*., 1991; Passantino *et al*., 1991; White and Bibb, 1997; Guthrie *et al*., 1998). Thus, it appears that there is no globally acting *bldA* target gene for secondary metabolism and development. It might be supposed that *bldA* target genes should be found among mutants with developmental defects similar to *bldA* mutants. The literature describes

Accepted 17 July, 2003. *For correspondence. E-mail keith.chater@bbsrc.ac.uk; Tel. (+44) 1603 450 297; Fax (+44) 1603 450 778. †Present address: University of Tuebingen, Institute of Microbiology and Biotechnology, Auf der Morgenstelle 28, 72706 Tuebingen, Germany. ‡These authors contributed equally to this study.

at least 20 genes with mutations that lead to loss of aerial mycelium formation (the bald phenotype, hence the frequent designation of such genes as *bld*; for a list of *bld* genes, see Chater, 2001). DNA complementing most *bld* mutants has been cloned and characterized, but none of the genes identified contains a TTA codon. Thus, until now, *bldA* target genes influencing the development of *S. coelicolor* have remained undiscovered. The purpose of the work described in this paper was to identify such genes.

In extracellular complementation experiments on the rich medium R2YE, Willey *et al*. (1993) established a hierarchy of extracellular signal exchanges among *bld* mutants of *S. coelicolor*. A secreted oligopeptide is taken up via the *bldK*-specified ABC transporter (Nodwell *et al*., 1996; Nodwell and Losick, 1998), and its perception leads to the secretion of a second (unknown) extracellular signal, and so on through three further *bld* gene-dependent steps, until the morphogenetic protein SapB is secreted and allows aerial hyphae to form. Production of the second of the five proposed extracellular signals requires two known genes, *bldA* and *bldH*, which fall between *bldK* and *bldG* in the cascade. Not only do *bldA* and *bldH* have the same extracellular complementation phenotype, they also have a somewhat similar morphological phenotype (although the two previously described *bldH* mutants make pigmented antibiotics on minimal medium with mannitol as carbon source, unlike *bldA* mutants; Champness, 1988). In our search for the target gene(s) through which the *bldA* tRNA exerts its effects on morphological differentiation, we reasoned that *bldH* was a promising candidate. Our results confirm this prediction.

Results

Complementation of bldH*, a potential target of* bldA

In order to clone *bldH*, we attempted to restore aerial mycelium formation to WC109 [the *bldH109* mutant used by Willey *et al*. (1993) in extracellular complementation] with a library of *Sau*3AI fragments inserted into the positive selection high-copy-number plasmid pIJ699 (Kieser and Melton, 1988). Several colonies (five out of ≈ 9000 transformants) were identified in which aerial growth had been partially restored (Fig. 1). All the clones contained overlapping DNA from the same part of the genome, with a common overlap that included three complete genes (SCO2791–2793 in the genome sequence; Bentley *et al*., 2002) (Fig. 1A). Further reduction of the DNA in one of the clones localized the *bldH*-complementing gene(s) to just one open reading frame (ORF), SCO2792, which was indeed one of the 145 TTA-containing *S. coelicolor* genes (Fig. 1C). Moreover, SCO2792 was a close homologue of *adpA*, a gene of *Streptomyces griseus* known to play a key role in development (84.4% identity between the gene products) (Ohnishi *et al*., 1999). The equivalence with *adpA* was further supported by local synteny of gene arrangement around the *S. coelicolor* and *S. griseus* genes (Fig. 1A). For clarity, in this paper, we use the subscripts *c* (*coelicolor*) and *g* (*griseus*) to distinguish between the genes of the two organisms. It is relevant to note that mutations in the gene downstream of the two *adpA* genes, *ornA*, have been shown previously to give rise to a slow-growing and sparse aerial mycelium formation phenotype, and that some $ornA_g$ transcripts emanate from the $adpA_g$ promoter region (Ohnishi *et al*., 2000). It is likely that the oligoribonuclease encoded by *ornA* is involved in mRNA degradation (Ohnishi *et al*., 2000).

WC109 was caused to produce aerial mycelium even by a single copy of $adpA_c$ (Fig. 1C), reinforcing the possibility that the complementation was true, rather than some kind of suppression effect. We therefore sequenced $adpA_c$ from WC109 and found that it contained a frameshift mutation (Fig. 2). The protein resulting from the frameshift was deduced to be terminated at a stop codon only 5 bases downstream from the authentic stop codon, so any potential polar effects on expression of the downstream *ornA* gene were not expected to be severe; but the weak morphological defect remaining in the complemented mutant could have been attributable to slight polarity. Note that $adpA_c$ is separated by about one-third of the genome from the region to which *bldH* had been mapped genetically (Champness, 1988) [indeed, we had initially failed in attempts to complement *bldH109* with a series of 37 overlapping cosmids (p8–1A9; Redenbach *et al*., 1996) encompassing nearly all the *mthB–cysD* interval, based on the genetic map location between *mthB* and *cysD* suggested by Champness (1988)]. In contrast to WC109, the bald phenotype of WC181, previously believed to be a *bldH* mutant (Champness, 1988), was not complemented by DNA containing $adpA_c$, and sequencing revealed no mutations in the $adpA_c$ region of WC181 DNA, from 500 bp upstream of the start codon to 14 bp downstream of the stop codon (data not shown).

A constructed $adpA_c$ *mutant shows a bald colony phenotype*

An in frame deletion of $adpA_c$ was constructed by polymerase chain reaction (PCR) targeting. The resulting mutant, M851, showed a bald phenotype when grown on R2 (Fig. 3), R5 or SMMS media. However, there was no obvious mutant phenotype on MM mannitol or MS (data not shown). Unlike *bldA* mutants, and like *bldH* mutants, the colonies produced red pigment on certain media. This pigment might have been either of two known antibiotics made by *S. coelicolor.* In a simple test to discriminate between them, fuming of the plates with ammonia

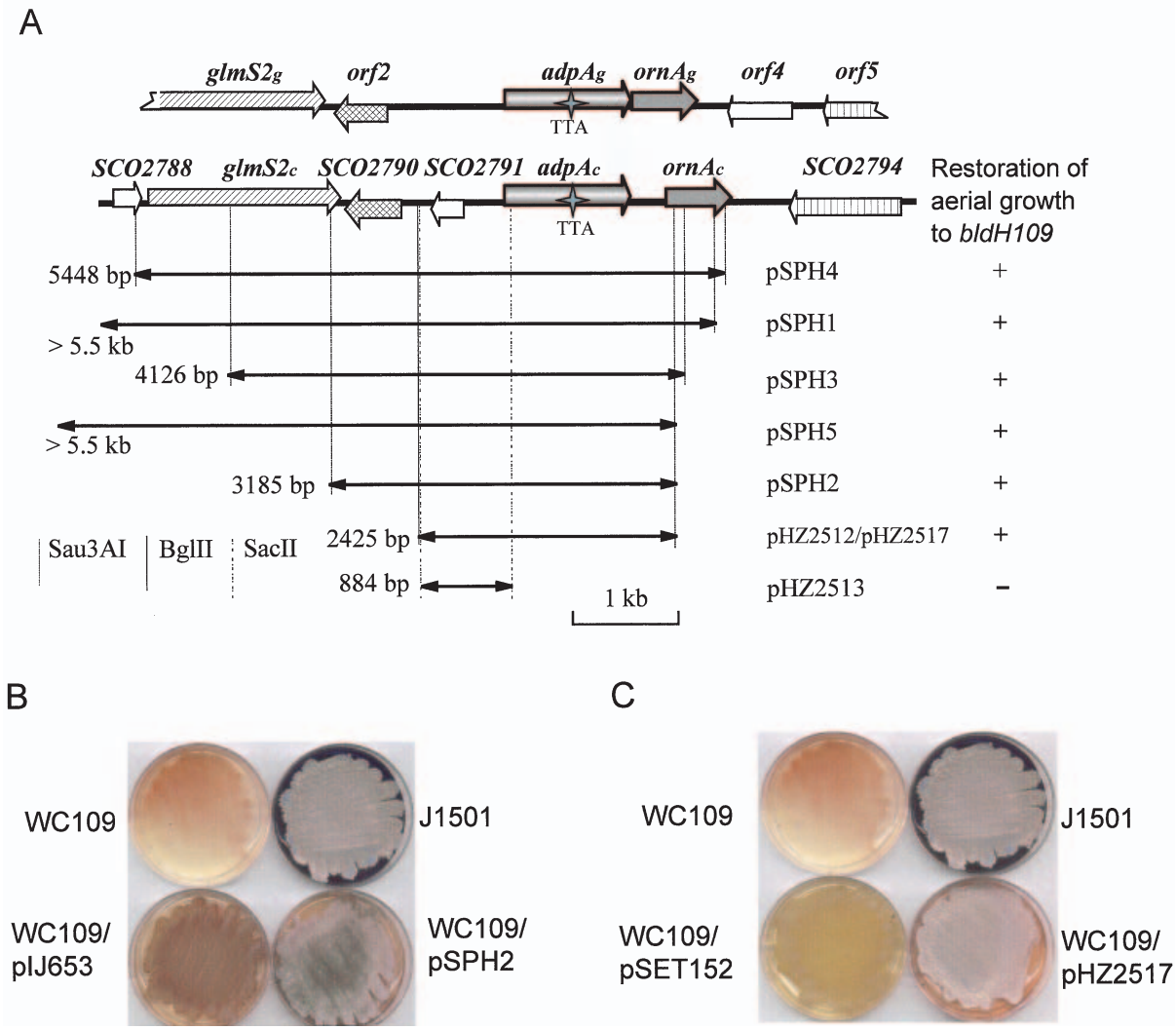

Fig. 1. Complementation of the *bldH* mutation of WC109 and localization of the complementing DNA to *adpA*$_c$.
A. Inserts of the *bldH*-complementing clones and subclones, and comparison with the equivalent region of *Streptomyces griseus* (Ohnishi *et al.*, 1999; 2000). The vector in pSPH1, 2, 3, 4, 5 and pHZ2512 was pIJ699. The vector in pHZ2513 and pHZ2517 was pSET152. The size of each insert based on the genome sequence is given on the left. The left insert plasmid boundaries of pSPH1 and pSPH5 were not sequence identified; thus, only the estimated size is given.
B. Plate showing complementation of *bldH109* in WC109 by pSPH2, containing *adpA*$_c$ in the multicopy vector pIJ699. The cultures were grown on R2 medium for 3 days.
C. Plate showing complementation of *bldH109* in WC109 by pHZ2517, containing *adpA*$_c$ in the integrating vector pSET152. The cultures were grown on R2 medium for 3 days.

adpA$_c$/AdpA$_c$ from J1501

ATG AGC [207 nt] ACG GGG GGC CTG [966 nt] CCG TGA (GGTGA) [1197 nt]
M S [69 aa] **T G G L** [322 aa] **P *** [397 aa]

adpA$_c$'/AdpA$_c$' from WC109

ATG AGC [207 nt] ACG GGG GG**G** CCT [967 nt]CC GTG AGG TGA [1203 nt]
M S [69 aa] **T G G P** [323 aa] **V R *** [399 aa]

Fig. 2. The sequence of *adpA*$_c$ from WC109 reveals a frameshift. An extra G (in bold) inserted after the G at nt 221 in *adpA*$_c$ shifted the reading frame a nucleotide forward, so the amino acid sequence after 74 amino acids was changed. The length of the new gene product is 399 amino acids, compared to 398 amino acids for the wild type AdpA$_c$. The coding sequence is in bold, and the underlined bold denotes the amino acid sequence that has been changed in the mutant.

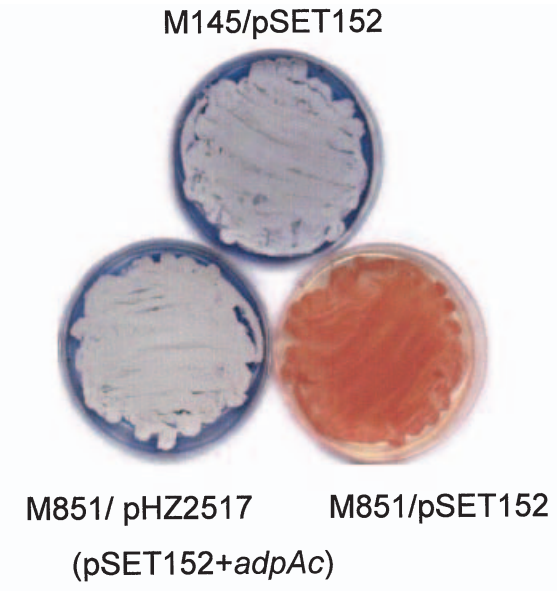

Fig. 3. Deletion of *adpA*$_c$ causes a Bld phenotype that is complemented by *adpA*$_c$. M851 was an *adpA*$_c$ in frame-deleted derivative of M145. The cultures shown here contained the integrating vector pSET152, which in one case carried a copy of *adpA*$_c$ (pHZ2517; Fig. 1A). Cultures were grown on R2 medium for 3 days.

resulted in a yellow colour characteristic of undecylprodiginines ('Red' antibiotic), and not of actinorhodin which would turn blue (data not shown). The constructed mutation was responsible for the phenotype, as a wild-type copy of *adpA*$_c$ introduced on the pSET152 integrating vector fully restored the wild-type phenotype.

If *adpA*$_c$ is a major and direct target for *bldA*, then the extracellular complementation pattern of M851 should be like that of *bldA* mutants and WC109, which have been shown to be stimulated to form aerial hyphae by growth close to a *bldG* mutant, and to be able to stimulate aerial growth of an adjacent *bldK* culture (Nodwell *et al*., 1996). Figure 4 shows that this was indeed the case: M851 restored a fringe of aerial mycelium production to a *bldK* mutant, whereas a *bldG* mutant restored a fringe of aerial mycelium production to M851.

Transcription of adpA$_c$ *does not depend on a γ-butyrolactone signalling molecule*

In *S. griseus*, *adpA*$_g$ occupies a crucial position in the signal transduction system by which the γ-butyrolactone signalling molecule A factor activates morphological differentiation and secondary metabolism (Horinouchi, 2002). Expression of *adpA*$_g$ is directly repressed by ArpA, a γ-butyrolactone binding protein, and is induced at transition phase by the production of A factor. *S. coelicolor* produces a different spectrum of γ-butyrolactones. The *S. coelicolor* genome contains only one homologue of *afsA*, the *S. griseus* gene believed to be responsible for A factor biosynthesis (Horinouchi *et al*., 1989; Bentley *et al*., 2002), and disruption of this gene (*scbA*) eliminated γ-butyrolactone production and had the unexpected effect of increasing pigmented antibiotic production, while having no obvious effects on morphological differentiation (Takano *et al*., 2001). In addition, a mutant disrupted in *scbR* (the gene next to *scbA*), a homologue of *arpA*, which in *S. griseus* encodes the A factor-binding protein, produced no γ-butyrolactones, yet showed delayed production of Red (Takano *et al*., 2001). We analysed *adpA*$_c$ expression by S1 nuclease mapping using RNA isolated from liquid cultures of a wild type and its *scbA*, *scbR* disruption derivatives. Three apparent mRNA 5′ ends were detected (Fig. 5A) presumably corresponding to three promoters, and their expression was more or less constant during growth in the wild type (Fig. 5B); the same three mRNA 5′ ends were detected in samples from surface cultures of M145 and M600 (Fig. 5C). There was little difference in the profile of these three mRNAs in any of the strains used, except that, in surface cultures, p1 appeared to be used more abundantly, and the signals were diminished in the latest time samples, possibly approximately coinciding with the onset of sporulation in the aerial mycelium. Gel retardation experiments conducted using ScbR did not show any ScbR binding to the *adpA*$_c$ promoter region (data not shown). These results suggest that, unlike in the case of *S. griseus*, *adpA*$_c$ is not part of a γ-butyrolactone signal transduction cascade. Consistent with this, there is considerable sequence diver-

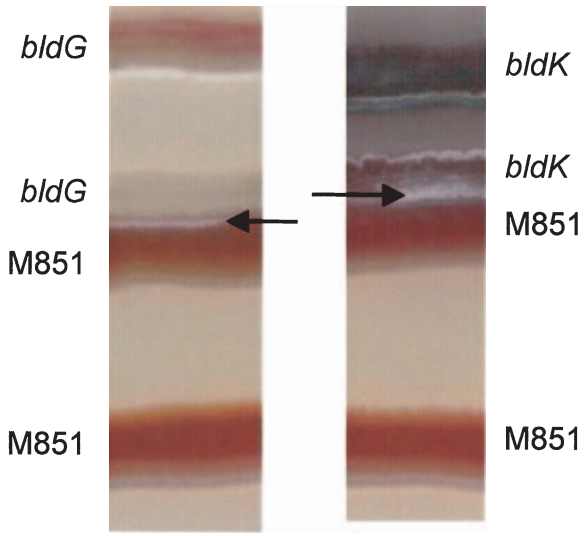

Fig. 4. The pattern of extracellular complementation exhibited by an *adpA*$_c$ mutant. M851 (Δ*adpA*$_c$) restored a fringe of aerial mycelium production in *bldK* (arrow), while *bldG* restored a fringe of aerial mycelium production in M851 (arrow). The strains were grown on R5 for 3 days.

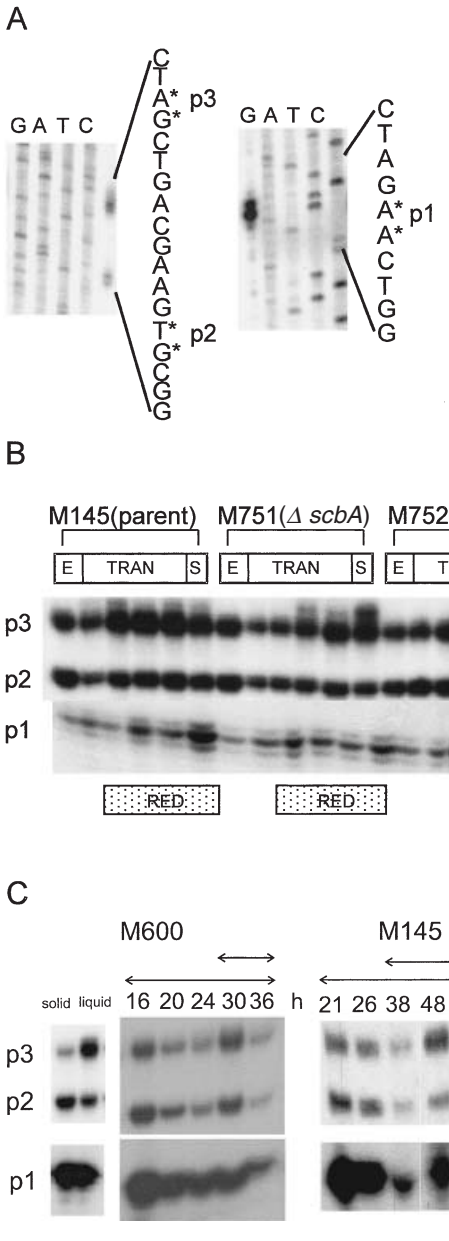

Fig. 5. Transcription of *adpA*$_c$ is unaffected by mutations eliminating γ-butyrolactone production in liquid cultures.
A. S1 nuclease mapping of the transcriptional start sites of *adpA*$_c$ was conducted using a 463 bp PCR product uniquely labelled at the 5′ end. The asterisks indicate the probable transcriptional start points; the sequences shown are those of the template strand. Lanes G, A, T and C are sequence ladders derived from the same labelled primer that was used to generate the PCR product.
B. Transcription analysis of *adpA*$_c$ using RNA isolated from liquid SMMS-grown cultures of *S. coelicolor* M145, M751 (Δ*scbA*) and M752 (Δ*scbR*). p1, p2 and p3 indicate the three promoters of *adpA*$_c$. E, TRAN and S indicate the exponential, transition and stationary phases of growth respectively. The shaded boxes labelled RED denote the presence of undecylprodigiosin, and the box labelled A denotes actinorhodin in the mycelium.
C. Transcription analysis of *adpA*$_c$ using RNA isolated from solid R5-grown cultures of *S. coelicolor* M145 and M600. p1, p2 and p3 indicate the three promoters of *adpA*$_c$. The numbers denote the time (h) after which the RNA was harvested, and arrows indicate the presence of vegetative mycelium or aerial mycelium in the cultures. A control shows that the signals from solid and liquid cultures of M600 co-migrate.

gence between the *adpA* upstream regions of the two species in the region previously shown to bind ArpA in *S. griseus*, which corresponds to the major promoter of *adpA*$_g$ (Fig. 6; Ohnishi *et al*., 1999). Interestingly, although none of the three *adpA*$_c$ promoters corresponds in position to the published promoter of *adpA*$_g$, they all have quite extensive similarity to equivalent regions in the non-coding region upstream of *adpA*$_g$.

Sigma BldN is present in an adpA$_c$ *mutant*

In *S. griseus*, AdpA regulates a large number of genes (Horinouchi, 2002). One of these (*adsA*) encodes a sigma factor needed for development (Yamazaki *et al*., 2000). In *S. coelicolor*, the homologous protein, σ^{BldN}, is also necessary for aerial growth (Bibb *et al*., 2000). Levels of σ^{BldN} are affected by several different *bld* mutations. Notably, WC109 (*bldH109*) lacks σ^{BldN}, as detected by Western blotting, and contains no *bldN* mRNA (Bibb *et al*., 2000; Bibb and Buttner, 2003). On the simple assumption that *bldH* and *adpA*$_c$ are the same gene, we expected that the *adpA*$_c$ disruption mutant should also lack *bldN* expression. We analysed extracts of the mutant by Western blotting with antiσ^{BldN} antibodies and unexpectedly found an undiminished signal in the mutant compared with the *bldH*$^+$ parent (Fig. 7). This result may indicate that either WC109 contains another mutation as well as its *adpA*$_c$ frameshift or the frameshift in *adpA*$_c$ in WC109 affects σ^{BldN} levels indirectly through polarity on the downstream oligoribonuclease gene *ornA.* However, in the *adpA*$_c$ insertion mutant that was constructed first to obtain the in frame mutant, σ^{BldN} was also present; so, in the polarity model, we would have to assume that the insertion has a less severe polarity effect than the *bldH109* frameshift. Whatever the explanation for the discrepancy between the *bldH109* and *adpA*$_c$ phenotypes with respect to *bldN*, the undiminished expression of *bldN* in the *adpA*$_c$ mutant again showed that *adpA* plays a somewhat different role in *S. coelicolor* from that previously found in *S. griseus*.

The TTA codon in adpA$_c$ *contributes markedly to the* bldA *dependence of normal development*

Our discovery that a TTA-containing gene, *adpA*$_c$, is needed for normal aerial mycelium development, and is involved in the same step in the extracellular signalling cascade for sporulation as *bldA*, led to the possibility that it might be the sole *bldA* target gene essential for differentiation. To test this, we generated a mutant copy of

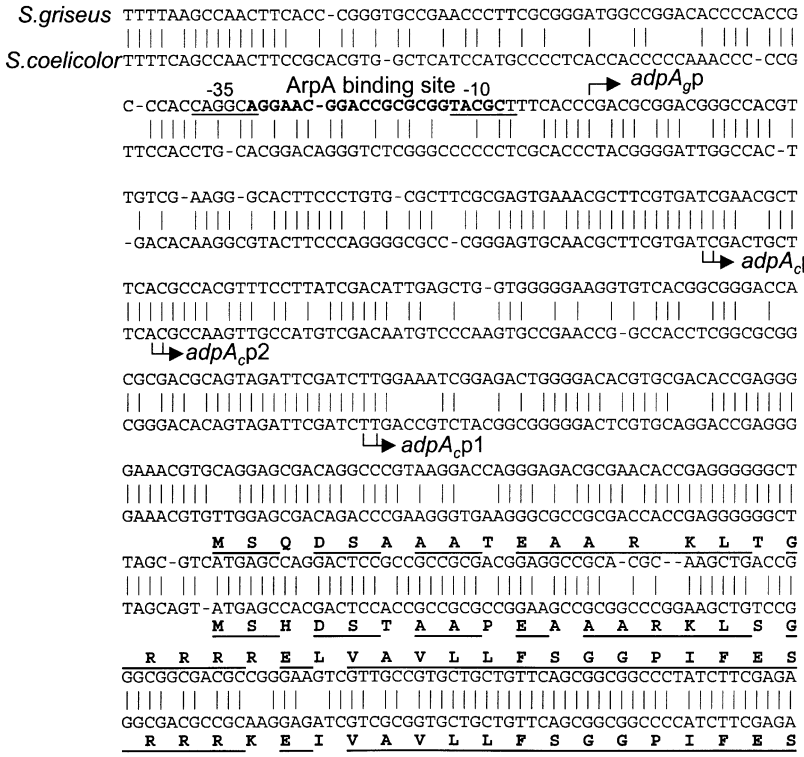

Fig. 6. Sequence comparison of the $adpA_c$ and $adpA_g$ promoter regions. The upper line denotes the DNA sequence from *S. griseus* and the lower that from *S. coelicolor*. Transcriptional start sites are indicated by arrows. The coding sequences of $adpA_c$ and $adpA_g$ are indicated in bold. The underlined coding sequences represent homologous sequences. Presumptive −10 and −35 regions of $adpA_g$ are underlined, and the ArpA binding site is in bold (Ohnishi *et al.*, 1999).

$adpA_c$ in which the TTA codon had been changed to the alternative leucine codon TTG. This mutant $adpA_c$ was inserted into pSET152, both with and without the downstream *ornA* gene, to give pHL72 and pHZ2528 respectively. The constructs were then introduced into a *bldA* mutant (J1700). The TTA-free $adpA_c$ restored aerial mycelium formation, but only when coupled *in cis* to *ornA* (Fig. 8), suggesting that *bldA* affects the translation of both $adpA_c$ and the downstream TTA-free *ornA*, and showing that the TTA codon in $adpA_c$ is a major contributor to the morphological deficiency of *bldA* mutants. Consistent with the idea of a *cis*-acting effect of the TTA codon in $adpA_c$ on *ornA*, some of the J1700/pHZ2528 $adpA_c$(TTA)/$adpA_c$(TTG) heterogenote colonies displayed sporulating sectors (which remained unpigmented), suggesting that recombinational interaction of the two $adpA_c$ copies had inserted the TTG codon into the $adpA_c$ copy located in its natural chromosomal position, where *bldA*-independent expression could bypass possible polarity on *ornA*.

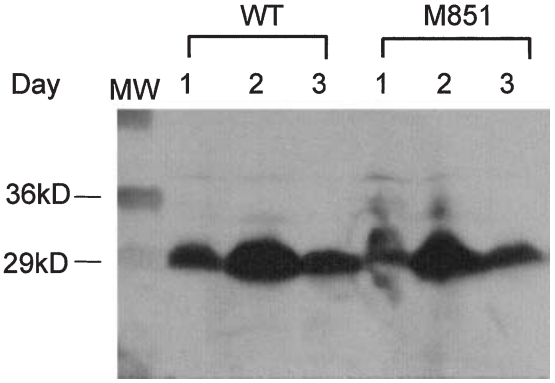

Fig. 7. Western immunoblot analysis of σ^{BldN} expression during growth on R2YE solid medium of *S. coelicolor* M145 and the $adpA_c$ mutant M851. Molecular weight markers (MW) are shown in the leftmost lane in kDa. Days indicate the times for which the cultures were incubated.

Discussion

Several pieces of evidence indicate that the principal cause of the *bldH109* phenotype is a mutation in $adpA_c$. Thus, $adpA_c$ at least partially complements *bldH109*; *bldH109* contains a frameshift mutation in $adpA_c$; an $adpA_c$ knockout mutant is defective in aerial growth but retains pigmented antibiotic production; and the $adpA_c$ mutant has the same extracellular complementation phenotype as the *bldH109* mutant. The only other previously known candidate *bldH* mutant (WC181) proved not to be allelic with WC109. *bldH* (= $adpA_c$) is the first *S. coelicolor bld* gene shown to contain a TTA codon, raising the possibility that it could provide (part of) the explanation for the morphological deficiency of *bldA* mutants. This hypothesis was strongly supported by the finding that the morphological defect of a *bldA* deletion mutant was largely sup-

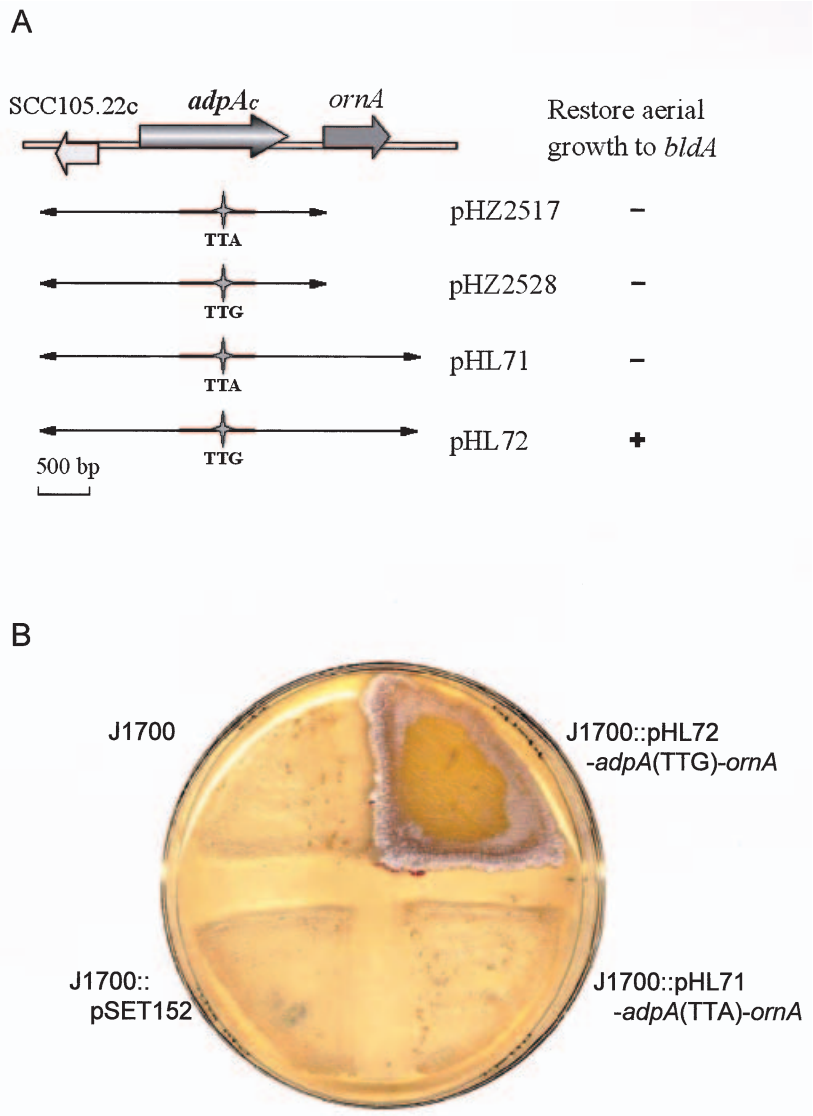

Fig. 8. Restoration of aerial hyphal formation to a *bldA* mutant when the TTA codon of $adpA_c$ is changed to a TTG codon.
A. The insert of each construct introduced into the chromosomal ϕC31 *attB* site of the *bldA* mutant J1700. The vector in these constructs was pSET152.
B. Restoration of aerial hyphal formation to J1700 by the TTA-free $adpA_c$–*ornA* cassette. Cultures were grown on R2YE for 6 days.

pressed by a TTA-free copy of $adpA_c$ when the mutation was *in cis* with a copy of the downstream gene *ornA*. Further work will be needed to establish whether this requirement for *ornA* is indeed a reflection of co-transcription of the two genes, especially in view of the 341 bp interval between them – in *S. griseus* the equivalent interval is 9 bp (Ohnishi *et al.*, 2000).

We can now account for most of the main aspects of the *bldA* phenotype: failure to make actinorhodin is because of the presence of a TTA codon in *actIIorf4*, the essential pathway-specific activator gene for actinorhodin biosynthesis (Fernandez-Moreno *et al.*, 1991; Passantino *et al.*, 1991); failure to make undecylprodigiosin pigments is because of a TTA-containing gene, *redZ*, that is the first of two pathway-specific activators in the mini-cascade leading to undecylprodigiosin biosynthesis (White and Bibb, 1997; Guthrie *et al.*, 1998); failure to make methylenomycin results from TTA codons in regulatory elements in the gene cluster for methylenomycin biosynthesis (S. O'Rourke, C. J. Bruton, N. Hartley and K. F. Chater, manuscript in preparation); and failure to make aerial hyphae is largely attributable to a TTA codon in a regulatory gene, $adpA_c$. It was interesting to find that $adpA_c$ is not needed for undecylprodigiosin synthesis, in view of the fact that its apparent orthologue in *S. griseus* is needed for streptomycin biosynthesis, aerial growth and yellow pigment formation. In addition, the regulation of $adpA_c$ and $adpA_g$ seems to differ, notably in their dependence on γ-butyrolactones; and the promoter regions of *adpA* in the two species have only punctuated similarity to each other and show different transcriptional start points (Fig. 6). The *S. coelicolor* genome contains some seven *adpA*-like genes, of which $adpA_c$ is the most similar and the only one to show local synteny with *adpAg*. While it is clear that $adpA_c$

is not important for the production of at least one of the pigmented antibiotics of *S. coelicolor* (undecylprodigiosin), we cannot rule out the possibility of such a role for other *adpA*-like genes – indeed, if any of those genes do carry out such a role, then it is possible that $adpA_c$ does interact with secondary metabolism pathways, with this activity being masked by the presence of one or more other genes with overlapping function. A similar explanation is possible for the observation that, in *S. coelicolor*, $adpA_c$ is not needed for the expression of *bldN*, whereas in *S. griseus*, the equivalent sigma factor gene (*adsA*) is $adpA_g$ dependent (Yamazaki *et al*., 2000). We did not observe any obvious actinorhodin production in the constructed $adpA_c$ mutant, for reasons that remain to be determined. However, we note that actinorhodin production can be activated in a *bldA* mutant by changing the TTA codon in the *actII-orf4* pathway-specific regulatory gene (Fernandez-Moreno *et al*., 1991). As the *bldA* mutation would have effectively inactivated $adpA_c$ expression in this strain, actinorhodin production is evidently not strongly dependent on $adpA_c$. AdpA proteins are members of the AraC family of regulators, and $AdpA_g$ has been shown to bind DNA (Ohnishi *et al*., 1999), reinforcing the growing evidence that most *bld* genes are regulatory. Thus, the subsequent steps in the *bld* gene-mediated extracellular signalling cascade are defined by mutations in *bldG* (encoding a homologue of such antiantisigma proteins as SpoIIAA in *Bacillus subtilis*; Bignell *et al*., 2000), *bldC* and *bldD* (encoding DNA-binding proteins; A. Hunt, personal communication; Elliot *et al*., 1998; 2001). It follows that the *bld* genes that define the extracellular complementation cascade are not likely to be directly involved in the synthesis of a diffusible extracellular factor. We therefore assume that each of these regulatory genes directly or indirectly activates a pathway for production of the relevant factor. In addition, the pattern of extracellular complementation among the *bld* mutants used to define the cascade implies, for any one mutant, that none of the *bld* genes defining other steps is inactive in that mutant by any mechanism other than signal deficiency. In other words, we suggest that none of these *bld* genes is dependent for expression in a gene-to-gene manner on any of the *bld* genes governing other steps in the cascade. This in turn implies that the *bld* genes represent at least four distinct intracellular regulatory systems that must be activated to permit morphological differentiation on the rich R2YE medium, and which intercommunicate via the extracellular signals. In the simplest case, the extracellular signals would bind directly to the product of the *bld* gene involved in the next step in the cascade to activate production of the next signal. Signal production is presumed to be associated with different aspects of physiology, also under *bld* gene control, that are characteristic of the region of the colony from which aerial hyphae emerge. These aspects might include starvation, secondary metabolism, storage metabolism, cell–cell contact or controlled cell death. Only when all the *bld* gene-dependent processes have been activated, we suggest, will it be appropriate for aerial hyphae to emerge. This model has also been discussed in some detail by Chater and Horinouchi (2003).

Experimental procedures

Bacterial strains, plasmids, growth conditions

Streptomyces coelicolor A3(2) strains (Table 1) were manipulated as described previously (Kieser *et al*., 2000). For routine subcloning, *Escherichia coli* K-12 strains JM101, DH5α (Sambrook *et al*., 1989) and ET12567 (*dam dcm hsdS*; MacNeil *et al*., 1992) were grown and transformed according to Sambrook *et al*. (1989). ET12567 was used to propagate unmethylated cosmid DNA for introduction of DNA into *S. coelicolor* by transformation or conjugation. pIJ699, a high-copy-number positive selection vector (Kieser and Melton, 1988), was used for shotgun cloning in *Streptomyces*. pIJ2925 was a high-copy-number pUC-type plasmid used for routine subcloning (Kieser *et al*., 2000). pSET152 (Bierman *et al*., 1992), which integrates into the *S. coelicolor* chromosome by site-specific recombination at the bacteriophage φC31 attachment site, *attB* (Kuhstoss and Rao, 1991), was used to introduce single copies of genes into the *S. coelicolor* chromosome. SCC13 and SCC105 (Redenbach *et al*., 1996) were two cosmids carrying $adpA_c$. *E. coli* BW25113/pIJ790 was the host for λRED-mediated PCR-targeted mutagenesis (Gust *et al*., 2003), and *E. coli* DH5α/BT340 was the host for FLP recombinase-mediated deletion of disruption cassettes to leave non-polar, phenotypically unmarked deletion mutations (Datsenko and Wanner, 2000). pIJ773 (Gust *et al*., 2003) was used as the template for amplification of a disruption cassette containing *aac(3)IV* (conferring apramycin resistance) and the RK2 origin of transfer (*oriT*), flanked by recognition sites for FLP recombinase.

Several different *Streptomyces* media were used. SMMS medium was as reported by Takano *et al*. (2001). MS agar (Kieser *et al*., 2000) was used to make spore suspensions and for plating out conjugations with *E. coli* ET12567 con-

Table 1. *S. coelicolor* strains used in this study.

Strain	Relevant genotype	Source or reference
M145	Prototroph	Kieser *et al*. (2000)
M600	Prototroph	Chakraburtty and Bibb (1997)
NS17	*bldK::aadA*	Nodwell *et al*. (1996)
WC103	*hisA1 uraA1 strA1 bldG103*	Champness (1988)
WC109	*hisA1 uraA1 strA1 bldH109*	Champness (1988)
WC181	*hisA1 uraA1 strA1′ bldH181′*	Champness (1988)
J1700	*hisA1 uraA1 strA1 bldA39*	Lawlor *et al*. (1987)
M751	M145 Δ*scbA*	Takano *et al*. (2001)
M752	M145 Δ*scbR*	Takano *et al*. (2001)
M757	M145 $adpA_c$::*aadC(3)IV*	This study
M851	M145 Δ$adpA_c$	This study
J1501	*hisA1 uraA1strA1*	Kieser *et al*. (2000)

taining the RP4 derivative pUZ8002 (Flett *et al*., 1997). R2, R2YE or R5 media and minimal medium (MM mannitol) (Kieser *et al*., 2000) were used for scoring sporulation. All *Streptomyces* cultivation was at 30°C.

Shotgun cloning of bldH

pIJ699 was digested with *Bam*HI and *Bgl*II, then the 4.8 kb *Bgl*II fragment was gel purified. Self-religation of this vector fragment forms a long uninterrupted perfect palindrome, rendering the plasmid non-viable in host bacteria (Kieser and Melton, 1988). After partial digestion of M145 genomic DNA with *Sau*3AI, 4–8 kb fragments were agarose gel purified and ligated with the purified 4.8 kb pIJ699 *Bgl*II fragment. The ligation mixture was introduced into WC109 (*bldH109*) protoplasts, and transformants were selected by flooding the regeneration plates with thiostrepton (25 μg ml^{-1}). Five individual colonies were found to form aerial mycelium at day 5 among ≈ 9000 transformants. The plasmids isolated from these colonies were named pSPH1, pSPH2, pSPH3, pSPH4 and pSPH5. For subcloning to localize the complementing DNA (Fig. 1), first the insert of pSPH2 was excised as a 3.2 kb *Hin*dIII fragment and inserted into pIJ2925 to give pHZ2511. The 2.4 kb *Bgl*II fragment of pHZ2511 was then ligated with *Bgl*II-digested pIJ699 to give pHZ2512, and the 0.9 kb *Sac*I fragment was ligated with *Sac*I-digested pSET152 to give pHZ2513. The 2.4 kb *Bgl*II fragment was also cloned in pSET152 to give pHZ2517.

Identification of the insert boundaries of the bldH *complementing clones*

The pSPH1 to pSPH5 plasmid insert boundaries were amplified by ligation-mediated PCR (LM-PCR) and sequenced. The oligonucleotides used were: UNIV5-AD1 (5′-GACTCGC GAATTCCGACAGTTGA), AGA1-AD2 (5′-GGCCTCAACT GTCG) and UNI699 (5′-CTAACGTCTGGAAAGACGAC). UNIV5-AD1 and AGA1-AD2 were annealed to give an adaptor with a 5′-GGCC overhang, which is cohesive with *Eag*I digestion ends. UNI699 was complementary to a sequence near the cloning site of pIJ699. The plasmids to be sequenced were digested with *Eag*I and then ligated with the adaptor. With the ligation mixture as template, UNIV5-AD1 and UNI699 were used to prime amplification of the two vector-insert junctions of each clone. The PCR product was gel purified and sequenced with UNI699 as primer. The sequences of the plasmid-insert boundaries were compared with the *S. coelicolor* genome sequence (http://www.sanger.ac.uk/Projects/S_coelicolor/) to reveal the inserts.

PCR sequencing of bldH

The *adpA$_c$* alleles of WC109 and WC181 were sequenced as follows. First, the entire *adpA$_c$* segments were PCR amplified from both strains using *Pfu*, a high-fidelity DNA polymerase. The primers were adp1 (5′-CCGAATTCCACCTGCACGGA CAGG) and adp2 (5′-AGGGATCCGTCTGCTCACCTCACG), which were complementary to sequences upstream and downstream of the *adpA$_c$* ORF but with an *Eco*RI and a *Bam*HI site respectively. The amplification included the region from 303 bp upstream of the translation start codon GTG to 14 bp downstream of the stop codon. The purified 1525 bp PCR products of the two strains were digested with *Bam*HI and *Eco*RI and cloned into pIJ2925 to give pHZ2516 and pHZ2519 respectively. pHZ2516 and pHZ2519 were then sent to TaKaRa Biotechnology (Dalian) for sequencing. The resulting sequences were compared with the EMBL database to identify any mutations. The DNA region around the mutated point found in WC109 was further confirmed by sequencing the PCR products using genomic DNA of both strains as templates. The primers for PCR and sequencing were adp19 (5′-CCATGTCGACAATGTCCCAAG) and adp4 (5′-GGGTCGACGTGCACCGACGG). The promoter regions of both strains, from 500 bp upstream to 111 bp downstream of the GTG start codon, extending 125 bp into the upstream divergent gene, were PCR amplified and sequenced. The primers used were adp2 (5′-GTCTGCTGCTGCGGCGT TCCG) and adp11 (5′-GAACTCTCGAAGATGGGGCCG).

S1 nuclease mapping

RNA was isolated as described by Strauch *et al*. (1991). For each S1 nuclease reaction, 30 or 40 μg of RNA was hybridized in NaTCA buffer [Murray, 1986; solid NaTCA (Aldrich) dissolved to 3 M in 50 mM PIPES, 5 mM EDTA, pH 7.0] to about 0.002 pmol (≈ 10^4 Cerenkov counts min^{-1}) of the following probes. For *adpA$_c$*, the oligonucleotide 5′-CACCCT TCGGGTCTGTCGCTC, which anneals within the *adpA$_c$* coding region, was uniquely labelled at its 5′ end with [^{32}P]-ATP using T4 polynucleotide kinase and used in the PCR with the unlabelled oligonucleotide 5′-GTCTGCTGCTGCGGCG TTCCG, which anneals upstream of the *adpA$_c$* promoter, to generate a 463 bp probe. The cosmid SCC13 (Redenbach *et al*., 1996) was used as the PCR template. For *hrdB*, the probe was made as described previously (Aigle *et al*., 2000). Subsequent steps were as described by Strauch *et al*. (1991). The sequence ladder was generated using a Sequenase 7-deaza-GTP sequencing kit (USB) with the same labelled primer as was used for the probe.

Construction and complementation of in frame deletion mutants of adpA$_c$

A mutant *adpA$_c$* allele, in which most of the *adpA$_c$* coding region (amino acids 57–378 out of 398) was deleted, was constructed by PCR targeting using oligonucleotide primers (5′-CATCTTCGAGAGTTCCATACCGCTGCGGTGTTCGGG ATCATTCCGGGGATCCGTCGACC and 5′-GCGGCGG GTCTGG A ACGGGACCGCGTTCTCCGGGGCGAGGTGT AGGCTGGAGCTGCTT) with 5′ ends overlapping the 5′ and 3′ ends of the *adpA* coding sequence, and 3′ (priming) ends designed to amplify the apramycin resistance disruption cassette of pIJ773. The PCR product was introduced into *E. coli* BW25113/pIJ790 containing cosmid SCC13, preinduced for λRED functions by the addition of arabinose, to obtain an *adpA$_c$*-disrupted version of SCC13. The disrupted cosmid was isolated and transferred via *E. coli* strain ET12567/pUZ8002 to *S. coelicolor* M145 by conjugation. Single crossover exconjugants were selected on MS agar containing

apramycin. Three such colonies were taken through two rounds of non-selective growth on MS agar, and spores were then plated for single colonies, which were scored for kanamycin sensitivity. Deletions within *adpA$_c$* were confirmed by Southern hybridization (Kieser *et al*., 2000) using a PCR-generated probe (labelled with ^{32}P by random oligopriming; Pharmacia). One such strain was designated M757. To construct a phenotypically unmarked in frame deletion, the *adpA$_c$*-disrupted cosmid SCC13 *adpA$_c$::aac(3)IV* was introduced into *E. coli* DH5α/BT340 to excise the disruption cassette from the cosmid by FLP recombinase. The resulting cosmid SCC13 Δ*adpA$_c$* was then transferred to *S. coelicolor* M757 by conjugation via ET12567. Single cross-over exconjugants were selected on MS agar containing kanamycin and apramycin. Three such colonies were then taken through one round of non-selective growth on MS agar, and spores were plated for single colonies, which were scored for kanamycin and apramycin sensitivity. All the colonies tested were kanamycin and apramycin sensitive and were deleted for *adpA$_c$*. Deletion within *adpA$_c$* was confirmed by Southern hybridization using the same probe described above, and one such strain was designated M851. To complement the mutants, pHZ2517 (see above), which was a pSET152 derivative containing *adpA$_c$* and its promoter, was transferred into *S. coelicolor* by conjugation via ET12567. Exconjugants were purified by single-colony isolation on medium containing apramycin, and plasmid integration was confirmed by Southern hybridization.

Site-directed mutation of the TTA codon of adpA$_c$ *and construction of relevant integrative plasmids*

There is a TTA codon 674–676 nucleotides downstream of the start codon of *adpA$_c$*. In order to change the TTA leucine codon to a TTG leucine codon, the insert of pSPH2 was cloned into pIJ2925 at the *Hin*dIII site to give pHZ2511. Then, pHZ2511 was digested, and the 2.4 kb *Bgl*II fragment, which carries the entire *adpA$_c$*, was cloned into the *Bam*HI site of pIJ2925 to give pHZ2514, which was used as the PCR template for site-directed mutagenesis. Two synthetic oligonucleotides were designed: ADPA-TTGL (5′-CAGGTCTTT **G**CCGGAGGAGATCGG) and ADPA-TTGR (5′-CTCCTCCG G**C**AAAGACCTGTCGAGG). ADPA-TTGL and ADPA-TTGR were complementary to opposite strands of the TTA codon region of *adpA$_c$* and contained the desired mutation as shown in bold/underline. The site-directed mutation procedure was modified from the Stratagene Quickchange kit instructions. The PCR was carried out with *Pfu* DNA polymerase. DMSO was added to the reaction mixture to a final concentration of 5%. The cycling conditions were 95°C for 30 s, 55°C for 1 min, 68°C for 10 min and 50 s (12 cycles). After temperature cycling, the PCR product was treated with *Dpn*I to digest the methylated parental DNA template and to select for mutation-containing synthesized DNA. The mixture was then used to transform *E. coli* DH5α. Plasmid DNA from two transformants was sequenced to show that the inserted DNA fragment of both plasmids contained the designed TTA→TTG mutation and there was no other mutation. Then the 2.4 kb *Bgl*II fragment was excised from the mutated plasmid, gel purified and inserted into the *Bam*HI site of pSET152 to give pHZ2528. Thus, pHZ2528 carried the intact *adpA$_c$* with a TTA→TTG mutation and the phage φC31 attachment function for integrating into the chromosomal *attB* site. In order to get an integrative construct carrying the TTA-free *adpA$_c$* together with the downstream gene *ornA*, pHL72 was constructed by replacing the 0.7 kb *Bcl*I–*Eco*RI fragment of pHZ2528 with a 1547 bp *Bcl*I–*Eco*RI fragment from a PCR amplification primed with adpa3 (5′-TGTCGGTGTTCGGGA TCG-3′) and ornA-C-EI (5′-CCGGAATTCAAGTGTACAGGG TCCGAAGG-3′), with M145 DNA as template. adpa3 was complementary to the *adpA$_c$* sequence. ornA-C-EI was complementary to a sequence downstream of *ornA*, but contained an engineered *Eco*RI site. pHL71 was the same as pHL72 but still carried the TTA codon.

Immunoblot analysis

Western blotting was done exactly as described by Bibb and Buttner (2003).

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