

## RESEARCH LETTER

# The oligoribonuclease gene in *Streptomyces coelicolor* is not transcriptionally or translationally coupled to *adpA*, a key *bldA* target

Jason K. Sello &amp; Mark J. Buttner

Department of Molecular Microbiology, John Innes Centre, Norwich, UK

**Correspondence:** Jason K. Sello,  
Department of Chemistry, Brown University,  
324 Brook Street, Providence, RI 02912, USA.  
Tel.: +1 401 863 1194; fax: +1 401 863  
9046; e-mail: jason\_sello@brown.edu

Received 24 March 2008; accepted 28 May 2008.  
First published online 9 July 2008.

DOI:10.1111/j.1574-6968.2008.01260.x

Editor: Derek Jamieson

## Keywords

*Streptomyces*; differentiation;  
oligoribonuclease; translation; transcription;  
antibiotics.

## Introduction

Members of the *Streptomyces* genus of bacteria have a complex life cycle during which vegetative hyphae morphologically differentiate into sporogenous, aerial hyphae. A gene called *bldA* is required for both morphological differentiation and antibiotic production in *Streptomyces coelicolor*, the model streptomycete (Chater & Chandra, 2008). *bldA* encodes a tRNA that is necessary for the efficient translation of rare TTA codons (Lawlor *et al.*, 1987; Leskiw *et al.*, 1991). The genomes of *S. coelicolor* and *Streptomyces avermitilis* carry multiple TTA-containing genes, whose translation is likely to depend on *bldA* (Chater & Chandra, 2006, 2008). In *S. coelicolor*, *adpA* (also called *bldH*) encodes an AraC-like regulator that is required for aerial mycelium formation (Bentley *et al.*, 2002). *adpA* contains a TTA codon and translation of its mRNA is *bldA* dependent, at least partially explaining the inability of *bldA* mutants to differentiate (Nguyen *et al.*, 2003; Takano *et al.*, 2003). Thompson and colleagues reported that the morphological defect in a *bldA* mutant was partially suppressed by an *adpA* allele in which the TTA codon was changed to CTC (Nguyen *et al.*, 2003). In contrast, Takano *et al.* (2003) reported that the morphological defect in a *S. coelicolor bldA* mutant was

## Abstract

Transcriptional analysis of the gene in *Streptomyces coelicolor* encoding oligoribonuclease indicates that it is not transcriptionally or translationally coupled to *adpA*, a key *bldA* target for morphological differentiation. Interestingly, construction and phenotypic analysis of an *S. coelicolor* oligoribonuclease null mutant revealed a conditional defect in morphological differentiation that is reminiscent of *bldA* and other known developmental mutants.

partially suppressed only when a copy of *S. coelicolor adpA* with TTG in place of the TTA codon was introduced *in cis* with the downstream gene, *ornA*. *ornA* encodes an enzyme called oligoribonuclease, a 3'- to 5'-exoribonuclease that specifically hydrolyzes ribonucleic acid oligomers consisting of fewer than eight nucleotides (Zhang *et al.*, 1998). This member of the DEDDh family of exoribonucleases is found in all *Proteobacteria* (*Beta* and *Gamma* divisions), *Actinomycetes*, and all eukaryotes whose genomes have been sequenced (Zuo & Deutscher, 2001). Although it is essential in *Escherichia coli*, mutants of *Streptomyces griseus* and *S. coelicolor* lacking oligoribonuclease are viable but unable to form aerial hyphae (Ohnishi *et al.*, 2000).

*adpA* and *ornA* lie in the same orientation and order in *S. griseus*, *S. coelicolor*, and *S. avermitilis* (Ohnishi *et al.*, 2000; Omura *et al.*, 2001; Bentley *et al.*, 2002). In *S. griseus*, *ornA* is constitutively transcribed as a leaderless transcript, but is also inducibly transcribed as part of an operon with the upstream *adpA* gene from an A-factor-dependent promoter (Ohnishi *et al.*, 2000). The apparent requirement for TTA-free *adpA* *in cis* with *ornA* for suppression of the morphological defect of an *S. coelicolor bldA* mutant implied that *adpA* and *ornA* are cotranscribed and that poor translation of both genes contributes to the inability of *S. coelicolor*

*bldA* mutants to form aerial hyphae (Takano *et al.*, 2003). This hypothesis warranted further testing because *adpA* and *ornA* are separated by 341 bp in *S. coelicolor* (Bentley *et al.*, 2002).

Here, we report the results of S1 nuclease protection experiments designed to determine how *ornA* is transcribed in *S. coelicolor*. In addition, we describe replacement of the *ornA* gene in *S. coelicolor* with an antibiotic resistance cassette and phenotypic characterization and complementation of the null mutant. Our results indicate that the degradation of RNA oligomers by oligoribonuclease is critical for completion of the *S. coelicolor* life cycle, but expression of *ornA* is not likely to be contiguous on *bldA*-dependent translation of *adpA*.

## Materials and methods

### Strains

*Escherichia coli* strain DH5 $\alpha$  was used as the general cloning host. *Escherichia coli* ET12567 [pUZ8002] was used as the donor in the intergenic conjugations. *Escherichia coli* ET12567 is a methylation-defective strain (*dam-13::Tn9*, *dcm-6*, *hsdM*) (Gust *et al.*, 2003). pUZ8002 is a nontransmissible *oriT*-mobilizing plasmid. *Escherichia coli* BW25113 [pIJ790] was used as the host for  $\lambda$  RED recombination (Gust *et al.*, 2003). *Streptomyces coelicolor* M600 (SCP1<sup>-</sup>, SCP2<sup>-</sup>) (Kieser *et al.*, 2000) or *S. coelicolor* J3411 (SCP1<sup>-</sup>, SCP2<sup>-</sup>  $\Delta$ *ornA::apr*) were the recipients. *Streptomyces coelicolor* J3411 (SCP1<sup>-</sup>, SCP2<sup>-</sup>  $\Delta$ *ornA::apr*) and *S. coelicolor* J3411 harboring pJS93, pJS94, pHL71\*, or pHL72\* were constructed in this work.

*Escherichia coli* strains were grown in Luria–Bertani (LB) broth and agar supplemented with antibiotics. Apramycin (50  $\mu$ g mL<sup>-1</sup>), chloramphenicol (25  $\mu$ g mL<sup>-1</sup>), carbenicillin (100  $\mu$ g mL<sup>-1</sup>), kanamycin (50  $\mu$ g mL<sup>-1</sup>), and hygromycin (80  $\mu$ g mL<sup>-1</sup>) were added to the growth media as required.

Unless otherwise noted, *S. coelicolor* was grown on mannitol soya (MS) flour medium (20 g agar, 20 g mannitol, 20 g soya flour per 1000 mL water) or R2YE (103 g sucrose, 0.25 g K<sub>2</sub>SO<sub>4</sub>, 10.12 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 g glucose, 0.1 g DIFCO casaminoacids, 2 mL trace element solution, 5 g DIFCO yeast extract, 5.73 g Tris-[hydroxymethyl] methyl-2-aminoethane sulfonic acid per 1000 mL of water) (Kieser *et al.*, 2000). Apramycin (50  $\mu$ g mL<sup>-1</sup>) and hygromycin (40  $\mu$ g mL<sup>-1</sup>) were added to the growth media as required.

### Disruption of the *ornA* gene in *S. coelicolor*

PCR-targeted mutagenesis was used to replace *S. coelicolor* *ornA* with an apramycin resistance marker, *apr* (Gust *et al.*, 2003). The requisite PCR product was amplified from pIJ773 using primers: SCO2793 KO FOR – GCCCGATAGA CAGGCGCCACCGTAAAGTGGCCCGCATGATTCGGG

GATCCGTCGACC and SCO2793 REV – GCTTCCGGAC GCGGCCCGCCGCGGGCCCGCGCCCCTATGTAGGCTG GAGCTGCTTC. The PCR product was introduced into *E. coli* BW25113 [pIJ790] harboring cosmid 2StC13 and expressing  $\lambda$  RED recombinase. The resultant recombinant cosmid, 2StC13 *ornA::apr*, was introduced via *E. coli* strain ET12567 [pUZ8002] into *S. coelicolor* M600 by conjugation. M600 is a plasmid-free derivative of the wild-type strain (Kieser *et al.*, 2000). Ex-conjugants lacking the oligoribonuclease gene were identified by selection for apramycin resistance and kanamycin sensitivity. Gene replacement was confirmed by PCR analysis of both the recombinant cosmid and the genomic DNA from the null mutant, *S. coelicolor* J3411 ( $\Delta$ *ornA::apr*).

### Other genetic procedures

Standard genetic techniques with *E. coli* and *in vitro* DNA manipulations were used (Sambrook & Russell, 2001). *ornA* was amplified by PCR from cosmid 2StC13 (<http://streptomyces.org.uk>) using primers SCO2793 NdeI FOR (AACATATGAACGATCGCATGGTGTG; the engineered NdeI site is underlined) and SCO2793 BamHI REV (AAGGATCC AAGCTTCTTCCGGACGCGGCC; the engineered BamHI site is underlined) and cloned into the EcoRV site of pBlue-script KS+ (Stratagene). For use as a control in complementation experiments, site-directed mutagenesis was applied to change aspartate-130, a consensus residue in the DEDDh exonuclease family (Zuo & Deutscher, 2001), of the *S. coelicolor* oligoribonuclease to an alanine. A modification of the Stratagene Quickchange kit procedure was used to introduce the point mutation into *ornA* using the following primers: (GGATCGTCGCGCGTCTCCTCGATCA and GAG GAGACGGCGACGATCCGGTAG; the point mutation is underlined and in bold). Wild-type *ornA* and the *ornA* (D130A) point mutant were placed under the control of the strong, constitutive *ermE* promoter in the hygromycin-resistant, *attP*-integrative vector pIJ10275 (H.-J. Hong, unpublished data). The resultant plasmids (pJS93 and pJS94) were introduced into *S. coelicolor* J3411 ( $\Delta$ *ornA::apr*) by conjugation with *E. coli* strain ET12567 [pUZ8002] (Gust *et al.*, 2003) to yield *S. coelicolor* J3411 [pJS93] and *S. coelicolor* J3411 [pJS94].

XbaI–EcoRV fragments (3.5 kb) from plasmids pHL71 and pHL72 (Takano *et al.*, 2003) (Fig. 3) containing *adpA* and *ornA* (with and without the TTA codon in *adpA*, respectively) were cloned into the EcoRV site of pMS81, a hygromycin-resistant vector that integrates site specifically into the chromosome at the  $\Phi$ BT1 *attP* site (Gregory, 2003). These constructs (pHL71\* and pHL72\*) were introduced into the *S. coelicolor* J3411 ( $\Delta$ *ornA::apr*) strain by conjugation with *E. coli* strain ET12567 [pUZ8002] (Gust *et al.*, 2003), yielding *S. coelicolor* J3411 [pHL71\*] and *S. coelicolor* J3411 [pHL72\*].

## S1 nuclease mapping

RNA was isolated at various time points by published methods (Strauch *et al.*, 1991; Kieser *et al.*, 2000). In each experiment, 30 µg of RNA was hybridized with 1 fmol ( $10^3$  Cerenkov counts  $\text{min}^{-1}$ ) of the probe in NaTCA buffer (3 M sodium trichloroacetic acid in 50 mM PIPES (piperazine-1,4-bis(2-ethanesulfonic acid), 5 mM EDTA, pH 7.0) (Kieser *et al.*, 2000). The probe was amplified from the cosmid 2StC13 (<http://streptomyces.org.uk>) template using primer SCO2793 S1 Probe FOR (AGAGAACGCGGTCCC GTTCC) and primer SCO2793 S1 Probe REV (GGGCGGAT CACGATGTCG). The PCR product was cloned into pBluescript KS+ (Stratagene) to enable preparation of an S1 nuclease probe with a 75-bp, nonhomologous 3' end to be used as an internal control. Thus, the probe was amplified by PCR using the cognate reverse primer that was  $^{32}\text{P}$ -labeled at the 5' end with T4 polynucleotide kinase and  $^{32}\text{P}$ - $\gamma$ -ATP and the pBluescript T3-specific primer (GCGCAATTAACCCTCA CTAAAGGG). The probes were annealed with total mRNA and digested with the S1 nuclease. The digested probes were separated by polyacrylamide gel electrophoresis.

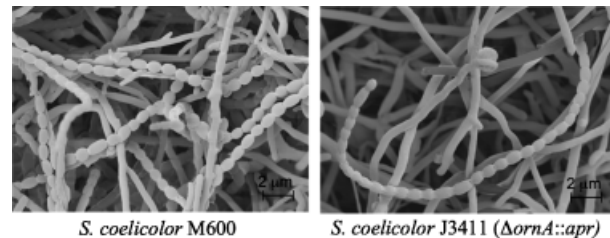
## Results

### Phenotypic analysis and complementation of an *S. coelicolor ornA* null mutant

The constructed *S. coelicolor ornA* null mutant, similar to that reported by Ohnishi *et al.* (2000), did not form aerial hyphae when grown on rich solid media like R2 or R2YE (Kieser *et al.*, 2000) (data not shown). Because the phenotypes of many developmental mutants of *S. coelicolor*, including *bldA* and *adpA* (*bldH*) mutants, are known to be conditionally dependent on growth media, the phenotype of the constructed *ornA* mutant was assessed on minimal medium (Pope *et al.*, 1996). The mutant grew quite differently on solid MS minimal medium (Kieser *et al.*, 2000) than on rich media. Specifically, the *S. coelicolor ornA* null mutant underwent morphological differentiation and sporulation in a density-dependent fashion when grown on MS medium. While single colonies of the null mutant formed aerial hyphae but did not sporulate, sporogenous aerial hyphae were readily apparent on confluent lawns of the *ornA* null mutant (Fig. 1). Scanning electron microscopic analysis of the surfaces of the null mutant grown on MS media indicated that morphological differentiation and sporulation did indeed occur, albeit more poorly than in wild-type *S. coelicolor* (Fig. 2). Accordingly, a confluent lawn of the *ornA* null mutant yielded < 5% of the number of spores harvested from lawns of the parent strain. This conditional defect in morphological differentiation is consistent with that of many other developmental mutants of *S. coelicolor*. While the *bldA* and *adpA* mutants of *S. coelicolor* do not



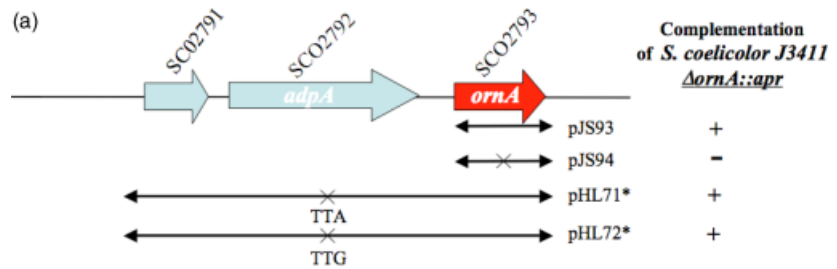
**Fig. 1.** Disruption of *ornA* has a dramatic effect on *Streptomyces coelicolor* physiology. Wild-type *S. coelicolor* M600 (left) and *S. coelicolor* J3411  $\Delta ornA::apr$  (right) grown on minimal MS medium for 5 days at 30 °C (Kieser *et al.*, 2000). The surfaces of the null mutant colonies lacked the gray-colored spores that cover the surfaces of wild-type colonies. The null mutant also dramatically over-produced the blue-pigmented antibiotic, actinorhodin, relative to the wild-type strain.



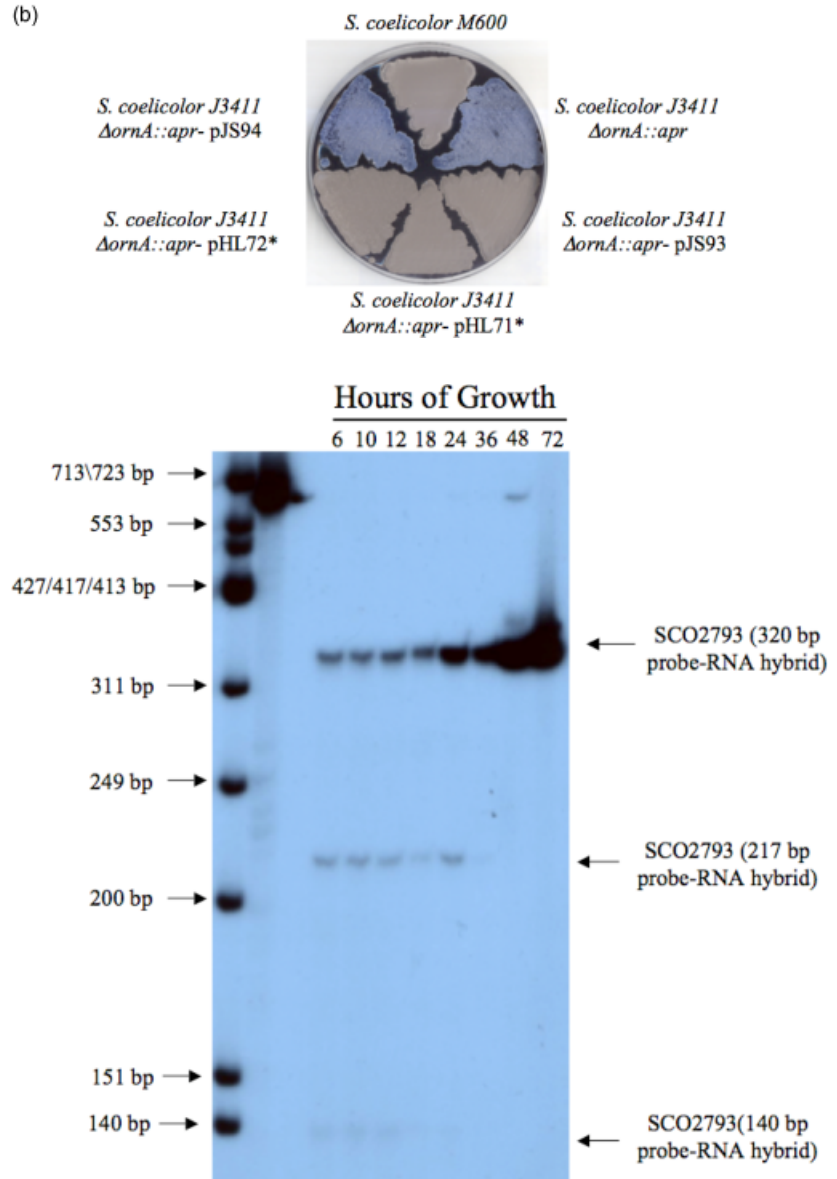
**Fig. 2.** The *ornA* null mutant is capable of sporulation on minimal media. Representative scanning electron micrographs of the wild-type strain (left) and the *ornA* null mutant (right) grown for 5 days on MS medium at 30 °C (Kieser *et al.*, 2000). While the wild-type strain had many chains of spores, very few sporogenous, aerial hyphae were apparent on the surfaces of the null mutant.

produce antibiotics, the *ornA* null mutant over-produces the blue-pigmented antibiotic, actinorhodin (Fig. 1). Unfortunately, exploitation of this antibiotic-overproduction phenotype is probably impossible because shaken liquid cultures of the *ornA* null mutant accumulated very little biomass in SMM (supplemented minimal medium) or YEME (yeast extract maltose extract medium) (Kieser *et al.*, 2000) (data not shown).

Complementation experiments were performed to verify that *ornA* was sufficient to suppress the null mutant phenotype. pJS93, containing *ornA* under the control of a constitutive promoter, completely suppressed the phenotype of the *S. coelicolor* J3411 ( $\Delta ornA::apr$ ), but pJS94 (encoding catalytically inactive OrnA D130A) did not (Fig. 3). Furthermore, pJS94 did not suppress the morphological defect of the *S. coelicolor* J1700, a *bldA* mutant (data not shown) (Lawlor *et al.*, 1987). Additional complementation experiments were performed with 3.5-kb XbaI–EcoRV fragments from plasmids pHL71 and pHL72 (Takano *et al.*, 2003) containing *adpA* and *ornA* (with and without the TTA codon in *adpA*, respectively). Both constructs suppressed the *ornA* null mutant phenotype (Fig. 3).



**Fig. 3.** Complementation of the *Streptomyces coelicolor ornA* null mutant. (a) *S. coelicolor* J3411  $\Delta ornA::apr$  complementing clones, and their comparison with the equivalent regions of the *S. coelicolor* chromosome (Bentley *et al.*, 2002). (b) Top view of a plate showing complementation of *S. coelicolor* J3411  $\Delta ornA::apr$  transformed by pJ93, pHL71\* and pHL72\*, and pJS94 (containing *ornA*, *adpA*, and *ornA*, TTA-free *adpA* and *ornA*, and *ornA* D130A, respectively). The cultures were grown on MS medium for 7 days at 30 °C. The *ornA* null strain complemented with the *ornA* gene *in trans* had the characteristic gray surface and amount of actinorhodin (blue pigment) production of the wild-type strain (M600).



**Fig. 4.** Transcriptional analysis of the *ornA* gene (SCO2793). S1 nuclease mapping of the SCO2793 mRNA transcript isolated from *Streptomyces coelicolor* M600 grown on R2YE solid medium for 3 days at 30 °C. Lane 1 shows the  $\phi$ X174/*Hinf*I ladder. Lane 2 shows the undigested probe including a 75-bp nonhomologous end derived from pBluescript-SCO2793 (705 nucleotides). Lane 3 shows the digested probe mixed with tRNA as a control. Lanes 4–11 show the S1 nuclease-protected probe-RNA hybrids.

**Transcriptional analysis of *S. coelicolor ornA***

In *S. griseus*, there are very clear data indicating that *adpA* and *ornA* are conditionally cotranscribed (Ohnishi *et al.*, 2000). In contrast, the results of complementation experi-

ments by Chater and colleagues suggested that *adpA* and *ornA* form an operon in *S. coelicolor* (Takano *et al.*, 2003). Furthermore, Horinouchi and colleagues showed that an *S. coelicolor ornA* mutant could be complemented by *ornA* alone, but it was not clear in these experiments whether

*ornA* transcription was driven from its own promoter or a vector promoter (Ohnishi *et al.*, 2000). To address these issues definitively, *ornA* transcription in *S. coelicolor* was analyzed by S1 nuclease protection using RNA isolated from growth on R2YE solid medium. When annealed to total mRNA, the 705-nucleotide probe (spanning from 140 bp upstream of the *adpA* stop codon to 140 bp downstream of the *ornA* start codon) was significantly digested by S1 nuclease, yielding three protected DNA fragments (330, 250, and 140 nucleotides in size) (Fig. 4). The number of protected fragments is consistent with transcription of the gene encoding oligoribonuclease from three distinct transcription start sites. The 140-bp fragment is indicative of a leaderless transcript. The 250- and 330-bp fragments are consistent with transcription from two distinct promoters whose activities appear to be growth phase dependent (Fig. 4). In any case, the sizes of the protected fragments indicate that *ornA* is not cotranscribed with *adpA* in *S. coelicolor*.

### Concluding remarks

The transcriptional analysis of the *ornA* gene in *S. coelicolor* reported here shows that *ornA* is not transcriptionally coupled to *adpA* and that it is transcribed in a growth phase-dependent fashion. Therefore, *bldA*-dependent expression of *adpA* cannot directly influence expression of *ornA* through transcriptional and translational coupling in *S. coelicolor*. Because *ornA* is not under *bldA* control, other explanations are needed to explain fully the morphological defect of the *bldA* mutant (Nguyen *et al.*, 2003; Li *et al.*, 2007).

Interestingly, disruption of the *S. coelicolor ornA* gene and phenotypic analysis of the null mutant uncovered a conditional defect in morphological differentiation, similar to that of other *S. coelicolor bld* mutants (Pope *et al.*, 1996). There is no clear explanation for the pleiotropic effects of disruption of the *ornA* gene or for the conditionality of the null mutant phenotype. The pleiotropy renews questions about how the buildup of RNA oligomers and/or a lack of 5'-ribonucleotide monophosphates in the oligoribonuclease null mutant could have such a dramatic effect on bacterial physiology (Zhang *et al.*, 1998; Ohnishi *et al.*, 2000).

### Acknowledgements

We thank Kim Findlay for performing scanning electron microscopic analyses and are grateful to Prof. Z.X. Deng for providing plasmids pHL71 and pHL72. Hee-Jeon Hong, Maureen Bibb, Sean O'Rourke, and Nicholas Bird are gratefully acknowledged for advice and reagents. Profs. Keith Chater and David Hopwood are acknowledged for helpful discussions. This work was supported by a Career

Award at the Scientific Interface from the Burroughs Wellcome Fund to J.K.S. and by a grant-in-aid to the John Innes Centre from the BBSRC.

### References

- Bentley SD, Chater KF, Cerdeno-Tarraga AM *et al.* (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor*. *Nature* **417**: 141–147.
- Chater KF & Chandra G (2006) The evolution of development in *Streptomyces* analysed by genome comparisons. *FEMS Microbiol Rev* **30**: 651–672.
- Chater KF & Chandra G (2008) The use of the rare UUA codon to define “expression space” for genes involved in secondary metabolism, development and environmental adaptation in *Streptomyces*. *J Microbiol* **46**: 1–11.
- Gregory MA, Till R & Smith MCM (2003) Integration site for *Streptomyces* phage  $\phi$ BT1 and development of site-specific integrating vectors. *J Bacteriol* **185**: 5320–5323.
- Gust B, Challis GL, Fowler K, Kieser T & Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* **100**: 1541–1546.
- Hesketh A, Bucca G, Laing E, Flett F, Hotchkiss G, Smith CP & Chater KF (2007) New pleiotropic effects of eliminating a rare tRNA from *Streptomyces coelicolor*, revealed by combined proteomic and transcriptomic analysis of liquid cultures. *BMC Genomics* **8**: 216.
- Kieser T, Bibb MJ, Buttner MJ, Chater KF & Hopwood DA (2000) *Practical Streptomyces Genetics*. John Innes Foundation, Norwich.
- Lawlor EJ, Baylis HA & Chater KF (1987) Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). *Gene Dev* **1**: 1305–1310.
- Leski BK, Lawlor EF, Fernandez-Abalos JM & Chater KF (1991) TTA codons in some genes prevent their expression in a class of developmental, antibiotic negative, *Streptomyces* mutants. *Proc Natl Acad Sci USA* **88**: 2461–2465.
- Li W, Wu J, Tao W *et al.* (2007) A genetic and bioinformatics analysis of *Streptomyces coelicolor* genes containing TTA codons, possible targets for regulation by a developmentally significant tRNA. *FEMS Microbiol Lett* **266**: 20–28.
- Nguyen KT, Tenor J, Stettler H, Nguyen LT, Nguyen LD & Thompson CJ (2003) Colonial differentiation in *Streptomyces coelicolor* depends on translation of a specific codon within the *adpA* gene. *J Bacteriol* **185**: 7291–7296.
- Ohnishi Y, Nishiyama Y, Sato R, Kameyama S & Horinouchi S (2000) An oligoribonuclease gene in *Streptomyces griseus*. *J Bacteriol* **182**: 4647–4653.
- Omura S, Ikeda H, Ishikawa J *et al.* (2001) Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci USA* **98**: 12215–12220.

- Pope MK, Green BD & Westpheling J (1996) The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis, and cell–cell signaling. *Mol Microbiol* **19**: 747–756.
- Sambrook J & Russell DW (2001) *Molecular Cloning: A laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Strauch E, Takano E, Baylis HA & Bibb MJ (1991) The stringent response in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **5**: 289–298.
- Takano E, Tao M, Long F, Bibb MJ, Wang L, Li W, Buttner MJ, Bibb MJ, Deng ZX & Chater KF (2003) A rare leucine codon in *adpA* is implicated in the morphological defect of *bldA* mutants of *Streptomyces coelicolor*. *Mol Microbiol* **50**: 475–486.
- Zhang X, Zhu L & Deutscher MP (1998) Oligoribonuclease is encoded by a highly conserved gene in the 3′–5′ exonuclease superfamily. *J Bacteriol* **180**: 2779–2781.
- Zuo Y & Deutscher MP (2001) Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* **29**: 1017–1026.