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Title
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Permalink
https://escholarship.org/uc/item/8vc0k593

Journal
Nucleic acids research, 36(3)

ISSN
1362-4962

Authors
Haiser, Henry J
Karginov, Fedor V
Hannon, Gregory J
et al.

Publication Date
2008-02-01

DOI
10.1093/nar/gkm1096

Peer reviewed
Developmentally regulated cleavage of tRNAs in the bacterium *Streptomyces coelicolor*

Henry J. Haiser¹, Fedor V. Karginov², Gregory J. Hannon² and Marie A. Elliot¹,*

¹Department of Biology, McMaster University, 1280 Main Street West, Hamilton, ON L8S 4K1, Canada and ²Cold Spring Harbor Laboratory, Watson School of Biological Sciences, Howard Hughes Medical Institute, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

Received October 5, 2007; Revised November 16, 2007; Accepted November 19, 2007

**ABSTRACT**

The ability to sense and respond to environmental and physiological signals is critical for the survival of the soil-dwelling Gram-positive bacterium *Streptomyces coelicolor*. Nutrient deprivation triggers the onset of a complex morphological differentiation process that involves the raising of aerial hyphae and formation of spore chains, and coincides with the production of a diverse array of clinically relevant antibiotics and other secondary metabolites. These processes are tightly regulated; however, the genes and signals involved have not been fully elucidated. Here, we report a novel tRNA cleavage event that follows the same temporal regulation as morphological and physiological differentiation, and is growth medium dependent. All tRNAs appear to be susceptible to cleavage; however, there appears to be a bias towards increased cleavage of those tRNAs that specify highly utilized codons. In contrast to what has been observed in eukaryotes, accumulation of tRNA halves in *S. coelicolor* is not significantly affected by amino acid starvation, and is also not affected by induction of the stringent response or inhibition of ribosome function. Mutants defective in aerial development and antibiotic production exhibit altered tRNA cleavage profiles relative to wild-type strains.

This morphological transition is associated with a shift from primary to secondary metabolism in the vegetative hyphae, and involves the production of pigmented antibiotics. The onset of both aerial hyphae formation and secondary metabolism is controlled in part by a class of genes termed the ‘bld’ (for ‘bald’) genes, while the sporulation process is controlled by the ‘whi’ (for ‘white’) genes. The majority of *bld* and *whi* genes encode regulatory proteins; however, neither their downstream targets nor the signal(s) that stimulate their expression/activity are well understood (1,2).

There are several disparate lines of evidence that connect the ability to sense nutritional conditions with the onset of differentiation. The inability of many *bld* mutants to raise an aerial mycelium is conditionally dependent upon the carbon-source available in their growth medium (3); these same *bld* mutants are also defective in their ability to regulate their carbon source utilization (4). More recently, aerial development has been shown to be inhibited by both extracellular N-acetylglucosamine (5) and high concentrations of external organic acids (6), while both aerial hyphae formation and antibiotic production are affected by nitrate limitation or amino acid starvation (7,8).

In many bacteria, the response to amino acid starvation is associated with the synthesis of the small molecule ppGpp by the ribosome-associated *relA* gene product. ppGpp accumulation is accompanied by global changes in gene expression and cellular physiology that are believed to enhance survival under suboptimal growth conditions; this phenomenon has been termed the ‘stringent response’ (9). In the streptomycetes, the stringent response is associated with a stimulation of antibiotic production and morphological differentiation, particularly under nitrogen-limiting conditions (8,10). Typically, the stringent response is initiated by an increase in the ratio of uncharged tRNAs to aminoacylated tRNAs. The resulting interaction of uncharged tRNAs with the A site of the 50S ribosome causes stalling of protein synthesis and a subsequent activation of RelA activity. Ultimately, this triggers both the downregulation of stable

*To whom correspondence should be addressed. Tel: (905) 525-9140 x24225; Fax: (905) 522-6066; Email: melliot@mcmaster.ca

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RNA (tRNA, tRNA) synthesis and the upregulation of amino acid biosynthetic gene expression (11), although in S. coelicolor, recent work has revealed that many amino acid biosynthetic clusters are actually repressed in response to ppGpp (10).

Starvation conditions are also associated with the degradation of stable RNAs. This primarily affects rRNAs, and it is presumed that tRNA degradation slows protein synthesis, and at the same time releases nutrient stores (12). In contrast, tRNAs have been shown to be stable under starvation conditions (13). A number of factors contribute to the overall stability of tRNA molecules: extensive secondary and tertiary structure of the mature tRNAs mean they are less accessible to nucleases than other RNAs in the cell; the 3' termini of tRNAs are protected from exonucleases by aminoacylation; and charged tRNAs are often associated with ribosomes, elongation factors and acyl-tRNA synthetases, thus rendering them inaccessible to most ribonucleases. Despite their apparent stability under starvation conditions, those tRNAs most susceptible to degradation would be uncharged tRNAs that are not associated with the translation machinery. This tRNA sub-population would be most prevalent during conditions of nutrient deprivation, particularly amino acid starvation, and thus it may be possible to correlate tRNA degradation with the nutritional status of a cell. Here, we provide evidence for the accumulation of tRNA cleavage products, particularly tRNA halves, in a medium-dependent manner during the development of S. coelicolor.

**MATERIALS AND METHODS**

**Streptomyces strains and culture conditions**

Wild type S. coelicolor strain M145 and developmental mutants bldA (14), bldB (15), bldC (16), bldH (17,18), whiG (19) and whiB (19) were grown on R2YE or MS (mannitol soy flour) agar media, or minimal agar medium supplemented with 0.5% w/v mannitol (20) for 12–72 h (as indicated), or in liquid minimal medium (MM) supplemented with 0.5% w/v mannitol. All Streptomyces cultures were grown at 30°C. Escherichia coli strains were grown at 37°C in Luria-Bertani (LB) liquid or on LB solid medium. *Streptomyces* strains used in this study are summarized in Table 1.

RNA isolation

RNA was isolated as described previously (21); however, plate grown cultures were harvested by directly scraping cells from agar, overlaid with cellophane discs, into modified Kirby’s mixture [1% w/v N-lauroylsarcosine sodium salt, 6% w/v sodium 4-amino salycilate, 6% v/v phenol mixture (pH 7.9) made in 50 mM Tris (pH 8.3)]. Total RNA samples were quantified by UV spectroscopy using the Ultrospec 3100 pro (Biochrom), and RNA quality was assessed using agarose gel electrophoresis.

RNA detection

Total RNA was detected using either SYBR Gold (Molecular Probes) staining or 3' pCp end-labeling with T4 RNA Ligase (Roche). Samples were run on 12% denaturing polyacrylamide gels, and were visualized using either UV light or autoradiography, respectively.

**Oligonucleotides**

The sequences of all DNA oligonucleotides used in this study, as well as the hybrid RNA/DNA adaptor used for RNA cloning, are summarized in Table 2.

**RNA cloning**

Total RNA (~100 µg) was separated on a denaturing 12% polyacrylamide gel and the region corresponding to the abundant 30-35 nt RNA species was excised. RNA was eluted from the gel slice overnight at 4°C in 20 mM Tris (pH 8.0), 0.5% SDS, 1 mM EDTA, and 0.4 M sodium acetate. Cloning of the RNA species was carried out using the method described by Lau *et al.* (22), with minor modifications. Briefly, RNA was recovered from the eluate by ethanol precipitation with 20 µg of glycogen, and samples were re-suspended in 30 µl H2O. Ligation of the 3'-adapter (Modban; Table 2) was carried out for 1 h at 37°C in a 20 µl reaction volume consisting of 13 µl purified RNA, 10 µM 3'-DNA adapter oligonucleotide (IDT DNA Technologies), 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 60 µg/ml BSA, 10% DMSO, 40 U T4 RNA Ligase (Amersham Biosciences). The ligation mixture was ethanol precipitated, and separated on a 12% denaturing polyacrylamide gel followed by purification of the ligated products as described above, except that the RNA was resuspended in 20 µl H2O. The 3'-adapter-ligated RNA was then ligated to the DNA/RNA 5'-adapter (Nelson’s linker; IDT DNA Technologies; Table 2) as described above, with the inclusion of 1 mM ATP. The ligation mixture was again ethanol precipitated and separated on a 12% denaturing polyacrylamide gel prior to purification. The RNA was then used as a template for reverse transcription (RT) using 200 U SuperScript II reverse transcriptase (Invitrogen) and the BanOne oligonucleotide (Table 2), whose sequence corresponded to the inverse complement of the 3'-adapter. The RT reaction was carried out at 42°C for 1 h. The resulting cDNA was used as template for PCR amplification with *Taq* DNA polymerase (Invitrogen) using oligonucleotides BanOne and BanTwo (Table 2). Products ~70 bp in size were recovered from a 2% agarose gel using a gel extraction kit (Qiagen) and

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### Table 1. *S. coelicolor* strains

| *S. coelicolor* strain | Genotype | Reference |
|------------------------|----------|-----------|
| M145                   | SCP1− SCP2− | 20        |
| J1501                  | hisA1 araA1 strA1 pg1 SCP1+ SCP2+ | 18 |
| C109                   | J1501 bldH | 17        |
| J660                   | bldC18 mthB2 cysD18 agaA1 SCP1+SCP2* | 15 |
| J669                   | bldB14 mthB2 cysD18 agaA1 SCP1+SCP2* | 15 |
| J1700                  | J1501 bldA39 | 14   |
| J2400                  | M145 whiG::hyg | 19 |
| J2402                  | M145 whiB::hyg | 19 |
Table 2. Linker and oligonucleotide sequences used in this study

| Linker/oligo name | Sequence (5' to 3') | Function |
|-------------------|---------------------|----------|
| BanOne            | ATGTAGGGTGCCTACAG   | Primer for reverse transcription and PCR amplification of cDNA |
| BanTwo            | ATCGTGGCACCTGAAA    | Primer for PCR amplification of cDNA |
| ModBan            | AMP-5-p-3'-CTGTAGGCCACCATCAATdi-deoxyC-3' | 3' Adaptor for RNA cloning |
| Nelson's linker   | 5'-ATCGtagcaccgaa-3' | 5' Adaptor for RNA cloning |
| M13 forward       | GAAAGACGGCAG        | Sequencing of cDNA clones |
| M13 reverse       | CAGGAAAAACGCTATGAC  | Sequencing of cDNA clones |
| 5' Ala            | GCTCTACAACATGAGCTATAGGCC  | Northern blot analysis |
| 3' Ala            | GAATTTGAACTCTGACCTTGCATGCAATGC  | Northern blot analysis |
| 5' Asn            | GCCGCGTCGTCTCTGGCAATTTGAAGCTACCG  | Northern blot analysis |
| 5' His            | AACCAGGTGCTACACGGCTAGCTACACCAC  | Northern blot analysis |
| 3' His            | GTGAGTGACGGGACTTGAACCCCGCGGCATCCTG  | Northern blot analysis |
| 5' Leu            | CGCCGCGTGCTCCATTCCACCATCGGG  | Northern blot analysis |
| 3' Leu            | GGACTTTGAACCCGACCGCCCTCGAAGGGCGAC  | Northern blot analysis |
| 5' Met            | CCCAGGAGCTACCGAGCTGCCACCCCCC  | Northern blot analysis |
| 3' Met            | CGGGGACAGGATTTGACATCGGAGCTTCTG  | Northern blot analysis |
| 5' Ser            | AGTGCGCGCGCATAGCGGGATACGGCGAGCCCTCC  | Northern blot analysis |
| 3' Ser            | GATTGTGACCCACCGGGACTTGGGCGCGAC  | Northern blot analysis |
| 3' Trp            | CTGAAACCACCGCGTTTTGG  | Northern blot analysis |

aThe sequence written in lower case represents RNA.

were directly cloned into the pCR®2.1–TOPO® vector (Invitrogen). Plasmid DNA was isolated from positive clones, and was sequenced using M13 forward and M13 reverse oligonucleotides (Table 2). The resulting sequences were then analyzed using BLAST (23).

Northern analysis
Total RNA samples were separated on 12% denaturing polyacrylamide gels and transferred to Zeta-Probe nylon membranes (BioRad) using a Trans-Blot semi-dry transfer cell (BioRad) (25 V for 30 min). Membranes were cross-linked using an XL-1000 UV crosslinker (Spectronics). 5′ end-labeled oligonucleotides, corresponding to either the 5′ or the 3′ half of the tRNA of interest, were hybridized with the membranes overnight at 42°C in ULTRAhyb-oligo hybridization buffer (Ambion). Membranes were washed twice with 2× SSC, 0.1% SDS for 30 min, followed by a single wash with 0.2× SSC, 0.1% SDS for 10 min. Detection and quantification of signals were achieved using a Storm 820 phosphorimagier (Molecular Dynamics) and ImageQuant v 5.2 (Molecular Dynamics) software. Where applicable, the change in the ratio of full-length tRNA:tRNA half was determined using intensity values obtained from the ‘Volume Report’ function of this software. To correct for background signals, we subtracted the intensities of equally sized areas on the blot, but adjacent to the bands, from all data points before using the resultant values for determining the ratio.

Translational inhibition and stringent response assay
Wild type S. coelicolor strain M145 was grown in 20 ml liquid MM supplemented with 0.5% w/v mannitol for ~40 h at 30°C. Cultures were then homogenized using a glass homogenizer before adding the indicated antibiotic or chemical (spectinomycin [200 μg/ml], hygromycin [50 μg/ml], thiostrepton [50 μg/ml], serine hydroxamate [SHX; 25 mM]). Cultures were then incubated for 30, 60 or 90 min, after which RNA was isolated as described above.

RESULTS
Detection of medium-dependent 30–35 nt RNA species
The initial goal of this work was to characterize small regulatory RNAs from S. coelicolor. To this end, we 3′ end-labeled total RNA isolated from cultures grown for 24 or 48 h on either solid R2YE (rich) or MS media, and size-fractionated this labeled RNA on a 12% denaturing polyacrylamide gel. R2YE is a standard rich medium for Streptomyces growth (containing both glucose and yeast extract), while MS was developed as a sporulation medium (24), and contains a poorly utilized carbon source (mannitol). We observed a distinct population of 30–35 nt RNAs in the RNA samples isolated exclusively from cultures grown on MS medium, but not from those grown on rich medium (Figure 1a). This small RNA sub-population was most abundant at 24 h, but was still detectable in the 48 h sample. To further explore the extent of this differential abundance, we examined RNA isolated from S. coelicolor cultures grown on MS medium over a 72 h developmental time course, which encompassed vegetative growth, aerial hyphae formation, and sporulation (Figure 1b). We discovered that these RNAs were not ubiquitously present in all RNA samples: they first appeared at times corresponding to the initiation of aerial hyphae formation (24 h) and then decreased in abundance as development proceeded through to late sporulation (Figure 1b). We also examined RNA isolated from cultures grown on rich R2YE medium over the same 72 h time course, and found that there were no 30–35 nt RNA species detectable at any time point, irrespective of the developmental stage (data not shown).

Cloning of 30–35 nt RNAs reveals an abundance of tRNA halves
To investigate the nature of this RNA population, we size-fractionated RNA isolated from cultures grown for 24 h on MS medium, and excised and purified the 30–35 nt
RNAs. 5' and 3' linkers were ligated onto the ends of the purified RNA molecules to facilitate RT and PCR amplification of the resulting cDNAs, which were then cloned and sequenced. The majority of cloned sequences corresponded to tRNA genes (72%) (Supplementary Table 1), while 25% of the cloned intergenic sequences matched rRNA or signal recognition particle (SRP) RNA degradation products. Intriguingly, the cloned tRNA gene sequences did not appear to result from the random degradation of tRNAs, but instead corresponded to ‘halves’ of tRNAs located either 5' or 3' of the anticodon sequence. Halves corresponding to 17 different tRNAs were identified in our cDNA library; however, the number of clones for each tRNA varied significantly (Table 3). When multiple clones were obtained for a particular tRNA half, we frequently observed heterogeneity at both ends of the cloned sequences (Supplementary Table 1). This suggested that either cleavage was not occurring at a specific sequence, but rather in the general vicinity of the anticodon loop, or that the products of the initial cleavage event were targeted by 5' and 3' exonucleases.

There seemed to be no bias towards the cloning of 5' halves (10 different tRNAs) or 3' halves (9 different tRNAs); however, both halves were cloned for only one tRNA (Table 3). There was also no correlation between the number of genes encoding a particular tRNA and the number of clones obtained. We did, however, detect a significant codon-usage bias, as more clones of frequently used tRNAs (146/170 – primarily those used most frequently) were obtained when compared with those used less frequently (24/170, predominantly those used least frequently), based upon the statistics of *S. coelicolor* codon usage provided by The Institute for Genomic Research (http://www.gem.re.kr/tigr-scripts/CMR2/codon_tables.spl?project=ntsc02).

We also found that for the majority of cloned 3' halves, there was evidence supporting the existence of a terminal CCA sequence, with either the entire CCA sequence being present or some portion of it (Supplementary Table 1). The terminal CCA sequence is added post-transcriptionally to the majority of tRNAs in *S. coelicolor* (25), in contrast to the situation in *E. coli*, where the terminal CCA is encoded within all tRNA genes (26). This suggested that the tRNA cleavage products were derived from mature tRNAs that were capable of being aminoacylated, and not from immature/precursor tRNA molecules.

Global cleavage of tRNAs within the anticodon loop generates 30–35 nt stable RNA species

Given that the majority of sequenced cDNA clones corresponded to tRNA halves, we wanted to determine whether these could be correlated with the 30–35 nt species observed in the RNA-labeling experiments, as it was possible that their abundance represented a cloning artefact stemming from preferential RT and/or amplification of tRNAs relative to other RNAs/cDNAs. We carried out northern blot hybridisation experiments using RNA separated on a denaturing polyacrylamide gel, and probed for an individual tRNA half using oligonucleotides specific for the 5' or 3' end of the tRNA^His^<sub>GUG</sub>. We found...
the appearance of the 30–35 nt RNA species could be directly correlated with the appearance of both 5′ and 3′ His tRNA halves (Figure 2). To investigate whether the appearance of tRNA halves was limited to particular tRNAs or whether it represented a more global phenomenon, we carried out northern blot analysis using a variety of probes for tRNA halves including: 5′ and 3′ Met (cloned many corresponding 5′ halves but no 3′ halves); 5′ Asn (cloned many corresponding 3′ halves); 3′ Ser (cloned many corresponding 5′ halves); and both 5′ and 3′ tRNA170UA, Atro which is the least used tRNA/codon in S. coelicolor, and is encoded by the bldA gene) (Figure 3). We detected tRNA halves corresponding to all of the 5′ tRNA halves examined (Figure 3), including the bldA tRNA, suggesting that cleavage within the anticodon loop is a general phenomenon in S. coelicolor. Interestingly, we did not observe any 3′ halves for Met, Leu or Ser tRNAs (Figure 3), despite having detected their corresponding 5′ halves through either cloning experiments or northern blot analysis. This observation could not be extended to all 3′ halves, however, as a number of sequences corresponding to 3′ halves had been cloned in our initial investigation and were also detected by northern blot analysis (Figure 2 and data not shown). This implied that the 3′ halves for particular tRNAs were significantly less stable than their 5′ half counterpart.

**tRNA cleavage profiles in developmental (bld) mutants are distinct from wild-type strains**

The timing of tRNA cleavage, and the accumulation of the 30–35 nt RNA species on MS medium, coincided with both the initiation of aerial hyphae formation and the production of antibiotics in S. coelicolor. As this morphological and physiological transition is controlled by the bld genes, we were curious to see whether tRNA cleavage would be affected by mutations in these genes, and chose to focus on two of the tRNA halves for which we had cloned numerous cDNA sequences (5′ His and 5′ Met; Table 3) and had examined previously in the wild-type strain (Figures 2 and 3). We isolated RNA from four different bld mutants (bldA, B, C and H) grown on MS medium. bldC and bldH both encode DNA-binding transcription factors (16,27); bldB encodes a small actinomycete-specific protein of unknown function (28,29), while bldA encodes the only tRNA specific for the rare LeuUA codon (30,31). We found that for all bld strains examined, the 5′ His tRNA halves accumulated with a slight delay relative to that observed for the wild-type strain (Figures 2 and 4): halves were detectable by 48 h in all bld mutants, and reached maximal levels at 72 h. This could be due to slower growth rates of the bld mutants, relative to the wild-type strain, in this experiment. Interestingly, the halves were predominantly degraded by 96 h in bldA, bldC and bldH, but were still readily detectable in a bldB mutant (Figure 4). In contrast, however, a very different result was obtained when probing with the 5′-specific Met oligonucleotide. In the wild-type strain, 5′ Met tRNA halves reached maximal abundance at 48 h, and were still readily detectable at 72 h (Figure 3). For bldB, a similar profile was observed (although this showed a delay relative to the wild type; Figure 4), but for bldA, bldC and bldH, Met-specific halves were not observed at any time point (Figure 4).

We also examined the appearance of tRNA halves in several mutants that were capable of raising aerial hyphae and producing antibiotics, but were blocked in their ability to form spore chains. These strains, referred to as whi mutants due to their white colony appearance, had tRNA cleavage profiles that closely resembled those of the wild-type strain for both 5′ Met and 5′ His tRNA halves (data not shown).

Taken together, these results demonstrate that bld (but not whi) mutants have patterns of tRNA cleavage and subsequent degradation that differ from the wild type, and from each other in the case of bldB. Given that bld mutants are defective in their ability to raise aerial hyphae (particularly bldB in this instance—see discussion below), these results would suggest that the appearance of tRNA

![Figure 2. Northern blot analysis for the 5′ and 3′ halves of histidine tRNA in a wild-type background. Total RNA samples harvested from MS medium were separated on 12% polyacrylamide gels and were subjected to northern blotting using probes complementary to the 5′ and 3′ halves of the histidine tRNA. Blots were exposed to a phosphorimagery.](image-url)

Table 3. Summary of cloned tRNA halves

| tRNA half | Amino Acid | Anticodon | # cloned | % of tRNAs cloned |
|-----------|------------|-----------|----------|-----------------|
| 3′        | Arg        | CCT       | 8        | 4.7%            |
| 3′        | Asn        | GTT       | 23       | 13.5%           |
| 5′        | Gln        | CTG       | 9        | 5.3%            |
| 3′        | Glu        | CTC       | 3        | 1.8%            |
| 3′        | Glu        | TTC       | 2        | 1.2%            |
| 5′        | Gly        | GCC       | 1        | 0.6%            |
| 5′        | His        | GTG       | 13       | 7.6%            |
| 5′        | His        | GTG       | 36       | 21.2%           |
| 5′        | Leu        | GAG       | 6        | 3.5%            |
| 5′        | Leu        | CAG       | 1        | 0.6%            |
| 5′        | Leu        | TAG       | 1        | 0.6%            |
| 3′        | Lys        | CTT       | 1        | 0.6%            |
| 5′        | Met        | CAT       | 34       | 20.0%           |
| 5′        | Phe        | GAA       | 1        | 0.6%            |
| 5′        | Pro        | CGG       | 6        | 3.5%            |
| 5′        | Ser        | TGA       | 21       | 12.4%           |
| 3′        | Thr        | GTG       | 2        | 1.2%            |
| 3′        | Val        | CAC       | 2        | 1.2%            |
|           |            |           | 170      |                 |

Figure 2. Northern blot analysis for the 5′ and 3′ halves of histidine tRNA in a wild-type background. Total RNA samples harvested from MS medium were separated on 12% polyacrylamide gels and were subjected to northern blotting using probes complementary to the 5′ and 3′ halves of the histidine tRNA. Blots were exposed to a phosphorimagery.
half is not dependent upon the initiation of aerial hyphae formation, antibiotic production or sporulation.

**tRNA cleavage is not dependent upon the stringent response or ribosome inhibition**

A similar tRNA cleavage phenomenon had recently been observed in *Tetrahymena* in response to amino acid deprivation (32), and we were curious about whether tRNA half accumulation in *S. coelicolor* could be correlated with amino acid limitation or deprivation of other nutrients. We had determined that tRNA halves could be detected in MS-grown strains (Figures 2–4); however, the composition of MS medium is not well defined (the major ingredient is soya flour), so we examined tRNA half accumulation in strains grown on a defined MM (20), where the only amino acid present was asparagine, which was provided as a nitrogen source. We observed an identical pattern of accumulation to that which we observed on MS medium (data not shown). We supplemented MM with yeast extract and proline, both individually and together, as these are the major source of amino acids added to the rich R2YE medium where tRNA halves were not detected. We did not observe a measurable decrease in tRNA halves, or even a delay in their appearance with any amino acid supplementation (data not shown), suggesting that amino acid starvation may not be the primary factor stimulating tRNA cleavage in *S. coelicolor*. To determine whether carbon source influenced the appearance of tRNA halves, we replaced the mannitol in MS medium (a poorly utilized carbon source) with glucose (which is the primary carbon source included in the rich R2YE medium). The growth and development of *S. coelicolor* on the mannitol and glucose containing media was comparable, and, as for the amino acid supplementation, we still observed an accumulation of tRNA halves in the RNA harvested from glucose-grown cultures (data not shown). We also examined the effect of high osmolarity on the accumulation of tRNA halves, by adding sucrose (to a final concentration of 10.3%, as is found in R2YE) to MS medium, and found that tRNA halves accumulated in the same manner as in a low-osmolarity medium. Finally, we investigated the effects of buffering the medium by adding TES buffer to MS medium. Studies have shown that vegetative growth is associated with increased acidification of unbuffered media, which the wild type, but not *bld* mutants, are capable of neutralizing during aerial hyphae formation (33). As for all other supplementation experiments, we saw no difference in tRNA half accumulation relative to that of the unbuffered control strain (data not shown).
Given our inability to prevent the cleavage of tRNAs through medium supplementation, we decided to assess whether we could stimulate tRNA cleavage. The initiation of aerial hyphae formation and antibiotic production has been linked to the stringent response in a number of Streptomyces species (34, 35, 8). The stringent response can be induced either by amino acid starvation or by exposure to SHX, where SHX is a serine analogue that prevents the charging of serine tRNAs through the competitive inhibition of seryl tRNA synthetases (36). We added SHX to liquid MM cultures that had been grown for 40 h, and continued growth for an additional 30, 60 or 90 min. While we saw a general trend towards increasing tRNA halves for 50 His (Figure 5), 50 Met and 50 Ser (data not shown) compared with an untreated control, this difference was not statistically significant. Similarly, we tested the effects of three antibiotics that specifically target the ribosome, and thus would arrest translation, to determine whether a decrease in translation (and hence decreased requirement for tRNAs) could stimulate tRNA cleavage. Spectinomycin targets the 16S rRNA within the 30S ribosomal subunit, preventing the translocation of the charged tRNA from the A-site to the P-site of the ribosome (37, 38); thiostrepton changes the conformation of ribosomal protein L11 and is thought to hinder the binding of elongation factors (39); while hygromycin primarily interacts with 16S rRNA and inhibits ribosomal translocation (40). As was observed for the SHX treatment, addition of spectinomycin and thiostrepton caused a small increase in detectable tRNA halves; however, again, this was not statistically significant after averaging the results of three independent biological trials (Figure 5). Hygromycin, on the other hand, caused a significant decrease in detectable tRNA halves (Figure 5), despite sharing ribosome-inhibitory characteristics with spectinomycin and thiostrepton.

DISCUSSION

We have discovered a unique tRNA cleavage event in the filamentous bacterium S. coelicolor, the timing of which coincides with both morphological differentiation and antibiotic production. Intriguingly, medium composition dictated whether tRNA halves were detected: tRNA halves were observed in RNA isolated from cultures grown on MM/MS medium (irrespective of carbon source), but not on rich medium. Previous work by Lee and Collins (32) showed that in Tetrahymena thermophila, tRNA cleavage within the anticodon loop (generating tRNA halves equivalent to those observed here) occurred in response to amino acid starvation. Propagation of T. thermophila requires a growth medium supplemented with 10 essential amino acids, and removal of even one of these from the growth medium was sufficient to stimulate a general tRNA cleavage response. In contrast, wild type S. coelicolor is capable of synthesizing all amino acids. We found that medium supplementation with additional amino acids had no effect on the appearance or accumulation of tRNA halves, suggesting that cleavage in S. coelicolor does not occur solely in response to amino acid starvation, and may instead occur in response to additional or alternative nutrient limitations. Intriguingly, tRNA cleavage has now also been observed in yeast during entry into stationary phase or during stress-induced apoptosis (D. Thompson and R. Parker, personal communication), suggesting that this phenomenon may be an evolutionarily conserved response to specific stresses.

bld mutants are defective in their ability to raise aerial hyphae, and in many cases, in their ability to produce antibiotics as well. These defects in aerial hyphae formation can, in some cases, be rescued by growth on alternative carbon sources such as mannitol (as is found in MS and minimal media), although carbon source has no effect on antibiotic production defects. Given the timing of tRNA
half appearance, we examined the tRNA profiles of several bld mutants to see whether defects in morphogenesis and physiology affected the pattern of tRNA half appearance. There appeared to be two distinct bld mutant tRNA profiles: the bldB mutant profile looked very similar to that of the wild type, while those of bldA, bldC and bldH were very similar to each other, but did not show the same global cleavage pattern seen in the wild type. Interestingly, bldB is the only mutant of these four whose developmental defects cannot be rescued by growth on alternative carbon sources. As the bldB tRNA cleavage profile was most like that of the wild type, this suggests that aerial hyphae formation is not a pre-requisite for tRNA cleavage (as bldB mutants fail to raise aerial hyphae on MS medium), and that tRNA cleavage is not a sufficient signal to stimulate aerial hyphae formation. Furthermore, as bldA, bldC and bldH mutants can form a modest aerial mycelium on MS medium, but have altered tRNA cleavage patterns, this suggests that normal tRNA cleavage is also not necessary for aerial hyphae formation. While it cannot be formally excluded that the different tRNA cleavage patterns in these four bld mutant strains are due to differing genetic backgrounds, it seems unlikely given that very different cleavage profiles were seen for two bld mutants having the same genetic background (bldB and bldC).

The generation of the tRNA halves appeared to result from a cleavage event occurring within the anticodon loop. Given the single-stranded nature of this loop region, it could serve as a possible substrate for the single stranded-specific endonuclease RNase E (41). Interestingly, in S. coelicolor, RNase E activity is upregulated during development (42), at a time that coincides with the appearance of tRNA halves, and thus could be responsible for the observed cleavage of tRNAs. This upregulation of RNase E activity was absent in a bldA mutant but was not affected by a mutation in bldC (42). As the tRNA cleavage profiles appeared to be virtually identical for bldA and bldC mutants, this suggests that the upregulation of RNase E activity does not play a major role in either the initial tRNA cleavage event or the ultimate degradation of the cleavage products. Unlike the global tRNA cleavage that we have observed here, cleavage of specific tRNAs has been observed previously in E. coli (43,44). E. coli produces several molecules having extremely specific tRNA cleavage capabilities: colicin D cleaves Arg tRNAs in their anticodon loop (43), while colicin E5 cleaves Tyr, His, Asn and Asp tRNAs in their anticodon loops (44). These colicins differ from each other both mechanistically, and at a sequence/structure level, and BLAST searches suggest that there are no similar proteins found in S. coelicolor (data not shown).

tRNAs undergo significant processing during their maturation into functional molecules. Mature 5’ ends are generated by a conserved RNase P cleavage event, whilst the generation of mature 3’ ends is far less conserved, even amongst bacteria; it may involve the concerted effort of several exonucleases and endonucleases such as RNase E, RNase III, and the recently discovered RNase Z (for which there is no obvious homologue in S. coelicolor) (45). In eukaryotes, the generation of full length tRNAs may also require the removal of introns through a splicing event that generates tRNA halves, which are then ligated together. There is no evidence for tRNA introns in S. coelicolor, and thus it seems unlikely that the tRNA halves observed are an intermediate in the synthesis process. Further supporting this is the observation that most of the 3’ halves cloned showed evidence of 3’ processing and addition of the terminal CCA sequence, which is the final step in tRNA processing and maturation.

The fate of the resulting 5’ and 3’ tRNA halves is clearly different in many instances. Strikingly, we were unable to detect 3’ halves for a number of tRNAs despite observing 5’ halves, in some cases in significant abundance (e.g. 5’ Met). This suggested that the 3’ halves might either be more susceptible to degradation, or are being preferentially degraded relative to the 5’ halves. One possible mechanism to explain this preferential degradation would be through the activity of a 5’ → 3’ exonuclease. Such activity has recently been demonstrated in Bacillus subtilis by an enzyme termed RNase J1 (46), which has a role in the maturation of 16S tRNA. RNase J1 has a demonstrated preference for single-stranded 5’ ends, particularly 5’ monophosphorylated substrates, (which often result from endonucleolytic cleavage events) or 5’ hydroxyl groups (which may be the product of spontaneous ‘in-line cleavage’ or cleavage by a single strand-specific endonuclease), but not primary transcripts having a 5’ triphosphorylated end (46). Cleavage within the anticodon loop would result in exposure of a single-stranded 5’ end of the 3’ half, which could then serve as a substrate for an RNase J1-like enzyme. In contrast, the 5’ end of the 3’ half would be protected from such nuclease activity through its base-pairing to the acceptor stem portion of the 3’ half, and would only become accessible when this base-pairing was disrupted through degradation of the 3’ half. There is an RNase J1 homologue in S. coelicolor (SCO5745), the gene for which is located immediately upstream of a ribosomal RNA gene cluster. This protein is predicted to have ~40% amino acid identity (60% similarity) to the B. subtilis RNase J1 protein, suggesting that it likely shares a similar function, although this has yet to be tested experimentally.
The biological function of tRNA cleavage in S. coelicolor, and other organisms, is not immediately obvious. It is conceivable that reducing the number of tRNAs available for translation would contribute to a slowing down of protein synthesis. Our results from the pCp-labeling experiments, shown in Figure 1b, reveal that at 24 h, the 30–35 nt RNA species (which likely represents all tRNA halves) appears to be as abundant as the full length tRNAs; this would be expected to have a considerable effect on the translational capability of the colony at this time point. This effect could be amplified by decreasing the availability of highly used tRNAs, as is suggested by the bias in cloning of sequences corresponding to tRNAs that specify highly used codons. Such a result would be in agreement with the bias in cloning of sequences corresponding to tRNAs that specify highly used codons.

This work was funded by the Natural Sciences and Engineering Council of Canada (NSERC) Discovery Grant (No. 312495). We would like to thank Debrah Thompson and Roy Parker for their willingness to share unpublished data. F.V.K. is supported by a post-doctoral fellowship (CF-07-058-01-GMC) from the Canadian Institute for Medical Research (CIRM) and by a doctoral fellowship from the German Academic Exchange Service (DAAD, Germany). T.H. was supported by a doctoral fellowship from the Natural Sciences and Engineering Council of Canada (NSERC) Discovery Grant (No. 312495). We would like to thank Mary Thompson, Christian Baron and Mark Buttner for helpful discussions and/or comments on the manuscript, and BldA, Chen, W., Ryding, J., Chang, S. and Bibb, M. (2007) The global role of ppGpp synthesis in morphological differentiation and antibiotic production in Streptomyces coelicolor A3(2). Genome Biol., 8, R161.

ACKNOWLEDGEMENTS

This work was funded by the Natural Sciences and Engineering Council of Canada (NSERC) Discovery Grant (No. 312495). We would like to thank Mary Yousef, Christian Baron and Mark Buttner for helpful discussions and/or comments on the manuscript, and Debrah Thompson and Roy Parker for their willingness to share unpublished data. F.V.K. is supported by a post-doctoral fellowship (# PF-07-058-01-GMC) from the American Cancer Society. Funding to pay the Open Access publication charges for this article was provided by McMaster University.

Conflict of interest statement. None declared.

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