RESEARCH LETTER

Heterogeneous rRNAs are differentially expressed during the morphological development of *Streptomyces coelicolor*

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Abstract

It is generally assumed that all mature rRNA molecules assembled into ribosomes within a single cell are identical. However, sequence analysis of *Streptomyces coelicolor* genome revealed that it harbors six copies of divergent rRNA operons that may express and constitute three and five different kinds of small subunit (SSU) and large subunit (LSU) rRNA molecules, respectively, in a single cell. Phylogenetic analyses of the LSU rRNA genes and the internal transcribed spacer between SSU and LSU genes indicated that the LSU gene of *rrn*A and *rrn*E operons might be the result of interspecies recombination between rRNA genes in closely related streptomycetes. Profiling of rRNA species using primer extension analysis showed that heterogeneous rRNA transcripts are expressed and assembled into ribosomes in the cell. As the cells developed from germination to sporulation, the relative amount of LSU rRNA molecules derived from three rRNA operons (*rrn*A, D, and E) gradually decreased from ~85% to ~60%, whereas the distribution of LSU rRNA molecules from two other operons (*rrn*B and F) and *rrn*C operon gradually increased from ~10% to ~20% of the total LSU rRNA. These findings indicate that heterogeneous rRNA molecules are differentially expressed during the life cycle of this developmentally complex microorganism.

Introduction

Streptomycetes are Gram-positive bacteria that have a developmentally complex life cycle and the morphological differentiation of these bacteria normally coincides with physiological differentiation characterized by the production of antibiotics.

Like most other bacteria, *Streptomyces* species contain multiple copies of rRNA operons and rRNA genes that are closely linked and organized in the order of 16S–23S–5S. Six rRNA operons are present in most *Streptomyces* species including *Streptomyces coelicolor* (Baylis & Bibb, 1988a), *Streptomyces lividans* (Suzuki *et al.*, 1988), *Streptomyces rimosus* (Plohl & Gamulin, 1991), *Streptomyces griseus* (Kim *et al.*, 1993), and *Streptomyces nodosus* (Yap & Wang, 1999), while *Streptomyces ambofaciens* (Pernodet *et al.*, 1983) has four and *Streptomyces venezuelae* (Farina *et al.*, 1996) has seven operons. Up to four promoters have been identified, which regulate the expression of rRNA operons in *Streptomyces* and the transcriptional activity of each promoter seems to vary under different growth conditions (Baylis & Bibb, 1988b; van Wezel *et al.*, 1994; de Leon & Mellado, 1997). Sequence identities between rRNA genes of different *Streptomyces* species are more than 90%.

Each organism has evolved to possess a unique rRNA species that serves best its physiological needs, although much of the structure and function of rRNA is evolutionarily conserved. For this reason, rRNA sequences have been used for identification and classification of microorganisms for several decades. However, it has been reported recently that intragenomic heterogeneity exists among haloarchaeal rRNA genes (Bouncher *et al.*, 2004). This phenomenon seemed to result from intergenomic recombination within a distantly related bacterial species while it is not known whether different rRNA genes are all expressed within a cell.

In this study, an example was also found of an intragenomic heterogeneity of rRNA genes in the *S. coelicolor* genome, which harbors six copies of divergent rRNA

operons that differ at *c.* 0.6% of the nucleotide positions in the large subunit (LSU) rRNA gene and at *c.* 0.2% of the nucleotide positions in the small subunit (SSU) rRNA gene. These rRNA operons may express and constitute three different SSU and five different LSU rRNA molecules in a single cell. In the present study, it was investigated whether heterogeneous rRNA species are expressed and assembled into ribosomes in *S. coelicolor* and their possible role and origin was proposed.

Materials and methods

Culture conditions

Liquid media used to culture *S. coelicolor* M145 were chemically defined (CD) medium supplemented with 1% casamino acids or pregermination medium (Ryu *et al.*, 2007). The CD medium contained 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.52% K_2HPO_4, 0.05% NaCl, 0.005% KCl, 0.005% $MgSO_4 \cdot 7H_2O$, and 0.0001% $ZnSO_4 \cdot 7H_2O$. Pregermination medium contained 1% yeast extract, 1% casamino acids, and 10 mM $CaCl_2$.The solid medium used was R2YE (R5) (Kiser *et al.*, 2000).

Isolation of total RNA and ribosomes

To prepare *S. coelicolor* cells from liquid medium, the spores were first germinated. Spores (10^7–10^8) were incubated in 5 mL of 50 mM TES at 50 °C for 10 min, cooled to room temperature under cold tap water, and incubated further at 37 °C and 250 r.p.m. for 3 h. 4 mL of pregermination medium was added. Germinated cells were harvested, washed twice in 50 mL of CD medium, resuspended in 50 mL of CD medium containing 1% casamino acids, and grown at 30 °C and 250 r.p.m. until the $OD_{450\,nm}$ reached 0.7–0.8. Four milliliters of cultures were mixed with two volumes of RNAprotect™ bacteria reagent (Qiagen) for total RNA preparation using the RNeasy Kit (Qiagen) according to the manufacturer's recommendations. Crude ribosomes were prepared from 400 mL of cultures grown in the same way as described above and isolated by the method of Powers & Noller (1991). rRNA was purified from the crude ribosomes using phenol extraction and ethanol precipitation.

To isolate total RNA from the cells grown on the solid medium, freshly prepared spores ($\sim 10^5$) were spread on cellophane paper laid on an R5 agar plate and incubated at 30 °C. To collect cells, 5 mL of mixture of 1 × TE (pH 8.0) and RNA protect™ bacteria reagent (1 : 2 volume to volume, Qiagen) was added to the plate and the cells were transferred to an oakridge tube. The collected cells were harvested at 18 000 ***g*** for 1 min, the supernatant was discarded, and total RNA was isolated using the RNeasy Kit.

Modified primer extension

The isolated RNA was analyzed by primer extension, using a modified method described by Lee *et al.* (1997). Oligodeoxynucleotides were 5′ end-labeled with [γ-^{32}P]ATP (3000 Ci mmol^{-1}, 1 Ci = 3.7 × 10^{10} Bq) and phage T4 polynucleotide-kinase. The primers used to synthesize cDNA from SSU rRNA and LSU rRNA were Sc16S-PE1 (5′-CGCGGGCTCA TCCTTCACCG) and Sc23S-PE1 (5′-TTCAAAAGGCACG CAGTCAACGA), respectively. Ten picomoles of the primers were labeled in a 50 μL of reaction containing 15 pmol of [γ-^{32}P]ATP, and 10 U of PKN in 1X reaction buffer at 37 °C for 30 min. Labeled primers were purified using microspin G-25 columns (GE Healthcare). Total RNA samples (0.5–5.0 μg) were mixed with 0 · 5 pmol of 5′ end-labeled primer, three kinds of dNTPs, and one kind of ddNTP in 6.5 μL of H_2O, incubated at 65 °C for 5 min, and quenched on ice. The synthesis of cDNA was carried out using 4 U of AMV reverse transcriptase in 10 μL of 1X AMV cDNA synthesis buffer in the presence of 20 U of a recombinant RNase inhibitor (RNase OUT). The samples were sequentially incubated at 42 °C for 30 min and 45 °C for 30 min for cDNA synthesis. The reaction was stopped by incubating the samples at 75 °C for 5 min, and 10 μL of gel loading buffer II (Ambion) was added. Samples were loaded on a 10% acrylamide gel containing 8 M urea in 1 × TBE buffer and electrophoresed at a constant voltage (20 V cm^{-1}) for 7 h in 1 × TBE buffer. Extending primers annealed to the rRNA in the presence of three kinds of dNTPs and one kind of ddNTP, the relative proportions of rRNA in the samples were identified from the relative intensities of the differently terminated cDNA bands in a sequencing gel. The radioactivity of each band was detected using a phosphorimager and measured by OPTIQUANT™ image analysis software.

Results and discussion

Sequence analysis of rRNA genes in *S. coelicolor* genome

Sequence analysis of rRNA-coding regions of six rRNA operons found in the *S. coelicolor* genome revealed that, as shown in Table 1, three different SSU rRNA and five LSU rRNA sequences are present in the genome, indicating the presence of heterogeneity of rRNA molecules in *S. coelicolor.* The most variable region was present in the region between nucleotide position 631 and 652 in LSU rRNA that corresponds to the helix 533 in the *Escherichia coli* LSU rRNA. In *E. coli*, this variable region is a part of the stem composed of 2 base pairs (bp) between positions 543–550 and 544–549 adjacent to a tetra loop, whereas, in *S. coelicolor*, the corresponding stem is extended to 8–9 bp (Fig. 1). Analysis of nucleotide frequencies of this stem region at positions 543,

Table 1. Nucleotide distributions in the variable regions of *Streptomyces coelicolor* rRNA

	Nucleotide sequence																															
rRNA	SSU rRNA			LSU rRNA																												
Position*	180	197	207	631	632	633	635	636	637	639	644	645	646	647	648	650	651	652	695	717	756	1701	1704	1705	1706	1707	1722	1723	1724	1725	1727	1730	1732
*rrn*A and D	C	U	C	G	G	–	U	G	G	G	C	U	C	C	A	–	C	C	G	A	U	C	G	U	G	U	A	C	A	C	U	U	G
*rrn*B	U	U	G	G	A	C	U	C	A	G	C	U	U	G	G	G	U	C	A	G	–	C	G	U	G	U	A	C	A	C	U	U	G
*rrn*C	C	G	C	C	G	C	A	G	G	–	U	C	C	U	–	G	C	G	G	G	U	C	G	U	G	U	A	C	A	C	U	U	G
*rrn*E	C	U	C	G	G	–	U	G	G	G	C	U	C	C	A	–	C	C	G	G	–	A	U	C	A	G	C	U	G	A	A	A	U
*rrn*F	C	U	C	G	A	C	U	C	A	G	C	U	U	G	G	G	U	C	G	A	C	C	G	U	G	U	A	C	A	C	U	U	G

*Nucleotide positions correspond to rRNA sequences from *rrn*F operon.

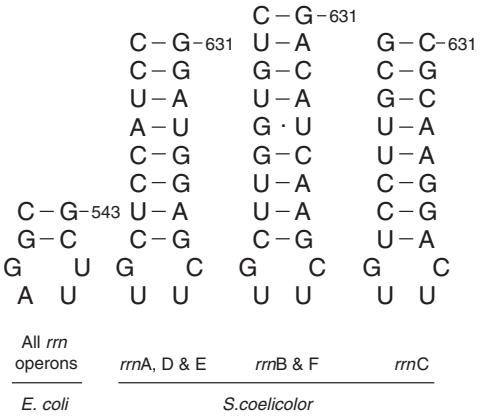

Fig. 1. Secondary structure of the variable region in *Streptomyces coelicolor* LSU rRNA. Secondary structures of stem loops containing the most variable region in *S. coelicolor* LSU rRNA are compared with that of the corresponding region in *Escherichia coli* LSU rRNA.

544, 549, and 550 in *E. coli* LSU rRNA revealed that the stem region is highly variable (Cannone *et al.*, 2002). Most substitutions found in these heterogeneous rRNA genes are either compensatory mutations that occur in stems or are located in loop regions, suggesting that they should have little or no effect on the structure of rRNA. Given the complexity of the ribosome and interactions of the rRNA molecules with the numerous ligands, the overall structure of rRNA seems to be maintained to carry out the general function of the ribosome.

Identification of the heterogeneous rRNA molecules in *S. coelicolor*

Because high levels of intragenomic variability of the LSU rRNA genes exist in *S. coelicolor* genome, experiments were conducted to test whether heterogeneous LSU rRNA molecules are expressed in an *S. coelicolor* cell and, if expressed, are assembled into ribosomes. For this, total RNA as well as ribosomes were isolated from the cells grown in CD medium and a modified primer extension method was used to detect divergent rRNA species in RNA samples. This method utilizes extension of the primer annealed to the 3′ region of the variable region of rRNA in the presence of three kinds of dNTPs and one kind of ddNTP (Fig. 2a and d). The relative proportions of each group of rRNA species in the sample can be identified by measuring the relative intensities of the differently terminated cDNA bands. Two groups of SSU and three groups of LSU rRNA species expressed in the cells can be detected using two primers as shown in Fig. 2. LSU rRNA molecules containing the same variable sequences (nucleotide positions between 631 and 652) that are derived from three operons (*rrn*A, D, and E) constituted *c.* 60% of the total LSU rRNA, while LSU rRNA molecules from *rrn*B and F were *c.* 25% of the total LSU rRNA and those from the

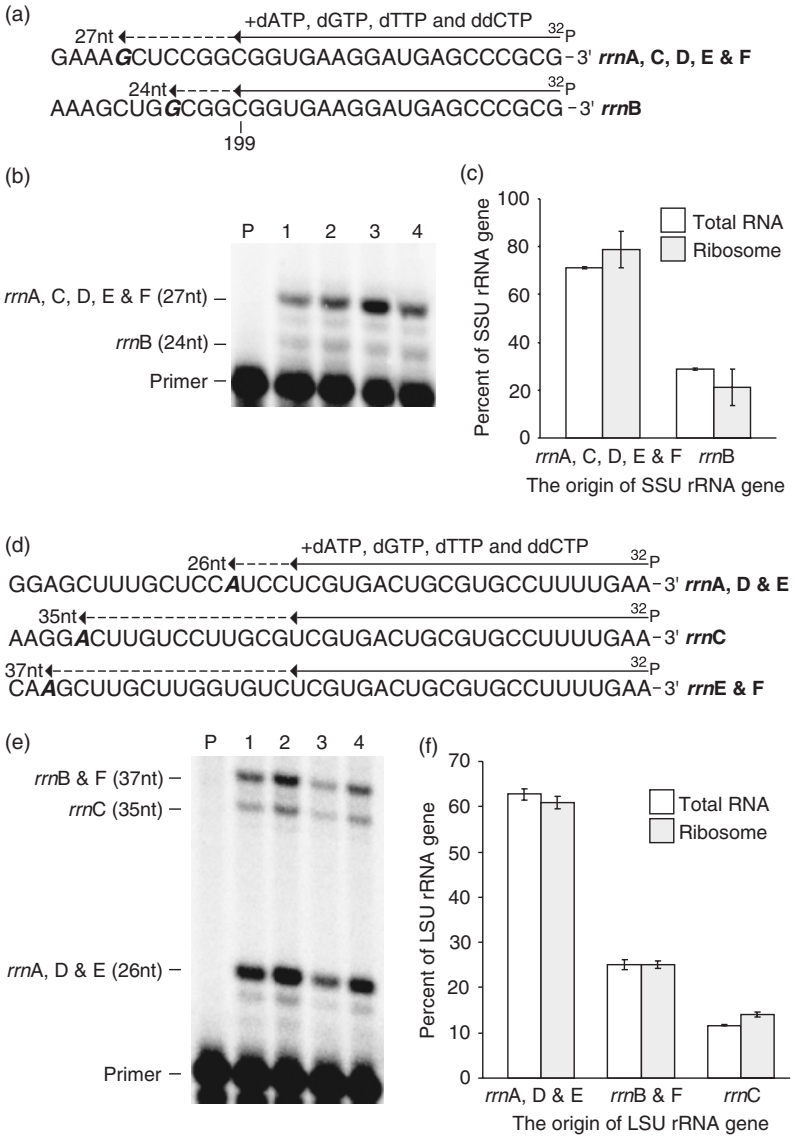

Fig. 2. Expression of heterogeneous rRNA species in *Streptomyces coelicolor*. Schematic representations of modified primer extension method are shown for SSU rRNA (a) and LSU rRNA (d). The cDNA products synthesized from specific SSU rRNA (b) or LSU rRNA (e) templates were resolved in a 10% sequencing gel. The relative abundances of each group of rRNA species in total RNA (lanes 1 and 2) or in ribosomes (lanes 3 and 4) are quantitated and shown for SSU rRNA (c) and LSU rRNA (f), respectively. A modified primer extension method was used to determine the relative abundance of LSU rRNA species by annealing the ^{32}P-end-labeled primer complementary to the sequence of the variable region and extending through the variable region using AMV reverse transcriptase. The extension mixture contained three kinds of dNTPs and one kind of ddNTP as indicated. The relative proportions of each group of rRNA species in the samples were identified from the relative intensities of the differently terminated cDNA bands in a sequencing gel. In lane P, samples from an extension reaction carried out without RNA were loaded.

*rrn*C operon occupied about 15%. SSU rRNA molecules from the *rrn*B operon occupied about 30% of the total SSU rRNA, while 70% of the SSU rRNAs were from the rest of the rRNA operons. The distributions of hetergeneous rRNA species in total RNA were similar to those in ribosomes, indicating that they were equally well assembled into ribosomes (Fig. 2c and e).

Differential expression of heterogeneous LSU rRNA molecules during the morphological development

Because heterogeneous LSU rRNA molecules were synthesized and assembled into ribosomes in a cell, it was speculated that different rRNA molecules may be differentially expressed during the morphological development. To characterize the expression profiles of rRNA species in relation to the developmental state of the cell, spores of *S. coelicolor* were seeded and grown on R5 agar medium, and total RNA was isolated from the cells at 12-h intervals up to 108 h after seeding the spores to measure the relative amount of each group of LSU rRNA species present in a cell. As shown in Fig. 3, LSU rRNA species containing the variable regions between positions 631 and 652 derived from three groups of *rrn* operons (*rrn*A, D, and E, *rrn*B and D, or *rrn*C) were expressed differentially during the development. As the cells developed from germination to sporulation, the relative amount of LSU rRNA molecules containing the same variable sequences derived from three operons (*rrn*A, D, and E) gradually decreased from ~85% to

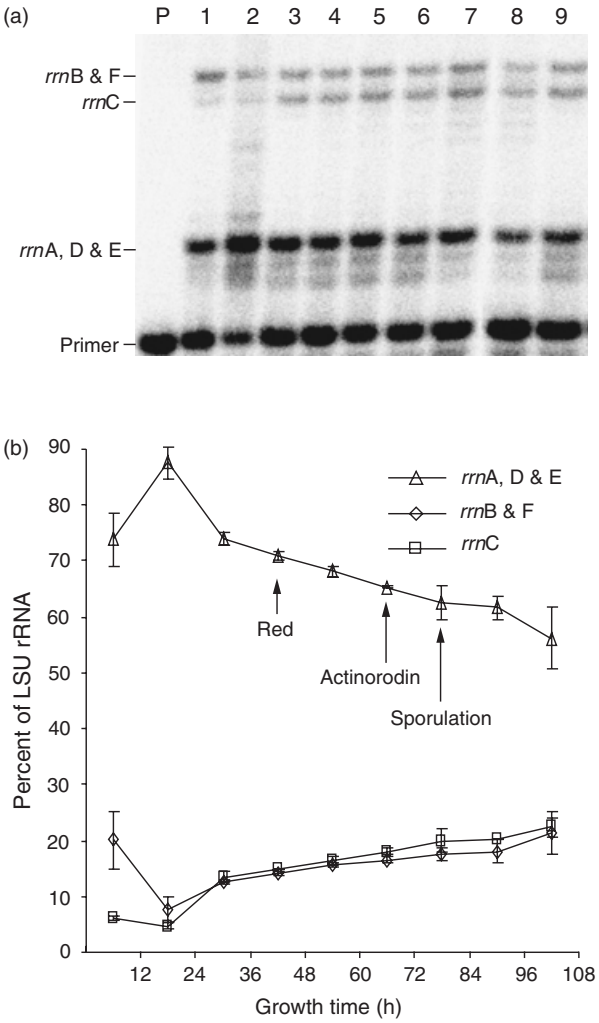

Fig. 3. Expression profiles of heterogeneous LSU rRNA species during morphological development. (a) Total RNA was isolated from the cells grown on R5 medium at 12-h intervals up to 108 h after seeding the spores (lanes 1–12) to measure the relative amount of each group of LSU rRNA species using the same method as described in Fig. 1d. In lane P, samples from an extension reaction carried out without RNA were loaded. (b) The experiments were repeated three times and averaged. SE of the mean (±numbers) is used to indicate the range of the assay results in the graph. The times at which the cells produced undecylprodigiosin (red) and actinorodin, and sporulated are indicated.

~60%, whereas the distribution of LSU rRNA molecules from two other operons (*rrn*B and F) and *rrn*C operon gradually increased from ~10% to ~20% of the total LSU rRNA. These results showed that different species of LSU rRNA molecules were differentially expressed during the morphological development. While it is difficult to assess the activity of multiple promoters of each rRNA operon and the relative stability of each rRNA species by analyzing the data presented in Fig. 3, assuming that rRNA synthesis is stopped when the cells enter the stationary phase, it seems that LSU rRNA molecules from *rrn*B, *rrn*C, and *rrn*F operons are more stable than those from other operons (*rrn*A, D, and E).

The differential expression of rRNA species and the maintenance of the intragenomic variability of rRNA operons by evolutionary pressure imply the existence of the functional divergence of heterogeneous rRNA species. Such a relationship between intragenomic rRNA divergence and functionality has been observed in the apicomplexan *Plasmodium berghei* where two types of SSU rRNA were differentially expressed depending on different stages of the life cycle of this organism (Gunderson *et al.*, 1987), raising a possibility that heterogeneous rRNA species may play a role in the control of gene expression depending upon certain developmental cues in *S. coelicolor*.

Phylogenetic analyses of rRNA operons

To explain the origin of different rRNA genes in *S. coelicolor*, phylogenetic analyses of the region encompassing the variable region of LSU rRNA genes as well as the internal transcribed spacer (ITS) between SSU and LSU rRNA genes were performed and the relationships of rRNA operons of *S. coelicolor* with those of other bacterial species were identified. A phylogeny of the ITS of *rrn* operons from *Streptomyces* is shown in Fig. 4. The ITS gene tree showed that *S. coelicolor rrn*A and *rrn*E genes clustered outside the clade formed by the other four rRNA genes of this organism and LSU genes from other *Streptomyces* species. Sequence alignment of ITS from *S. coelicolor* and other *Streptomyces* species identified in the ITS gene tree confirmed that the ITS of *rrn*A and *rrn*E from *S. coelicolor* is 98–99% identical to that of homologs from *S. lividans*. The ITS of the other four rRNA genes from *S. coelicolor* was $< 94\%$ identical to that of *S. coelicolor rrn*A and *rrn*E. When phylogenetic trees of the ITS and the variable region of the LSU genes of *Streptomyces* species were compared with each other by the same taxon sampling method, the overall structure was very similar, although the ITS gene tree seemed to be more resolved (data not shown). Because of the low resolution of LSU gene trees, sequences present in the variable region present in LSU genes from *S. coelicolor* (positions between 631 and 652) and homologs from *S. lividans* and *S. ambofaciens* were aligned. The results revealed that the sequence of *rrn*A, *rrn*D, and *rrn*E from *S. coelicolor* matches 95% with that of *S. lividans*, while the sequence of *rrn*B and *rrn*F from *S. coelicolor* was perfectly identical to that of *S. ambofaciens* (data not shown).

The similarities observed in the ITS between *rrn*A and *rrn*E from *S. coelicolor* and that of homologs from *S. lividans*, and in a 22-bp stretch between LSU genes of *rrn*A, D, and E from *S. coelicolor* and that of *S. lividans* as well as between LSU genes of *rrn*B and F from *S. coelicolor* and that of

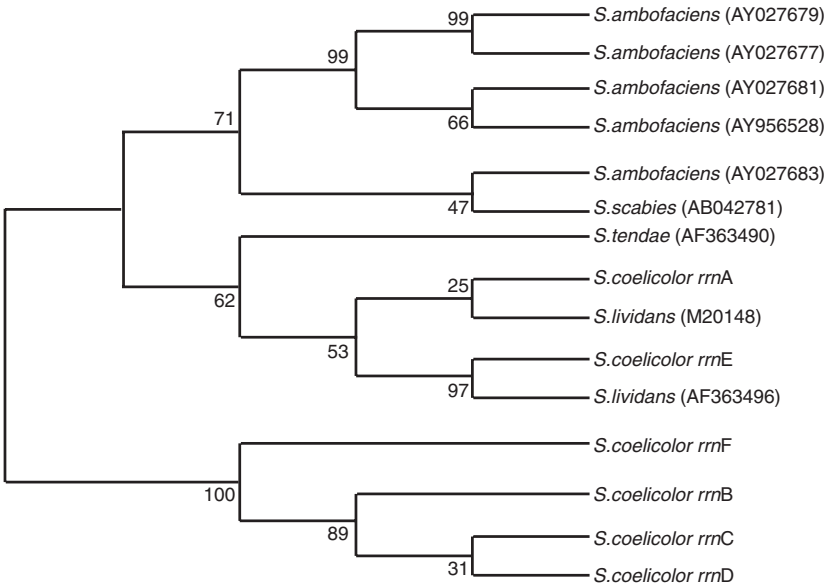

Fig. 4. Phylogenetic analysis of the ITS region between SSU and LSU rRNA. Sequences were aligned with their closest match (> 90%) found in the NCBI database as determined by the BLASTN algorithm at the NCBI website. Alignment was performed with CLUSTALX software. The phylogenetic trees were created with the MEGA 3·1 software using neighbor-joining analyses. Bootstrap values were from 1000 replicates.

S. ambofaciens indicate the existence of interspecies recombination events. It appears that LSU genes of *S. coelicolor* have not been congruently evolved with SSU genes because classifications of SSU and LSU rRNA genes do not correlate to each of the corresponding rRNA operons (Table 1). However, there seems to be coordinated evolution of the LSU genes and the ITS partners in these operons, considering that two of the ITS of *rrn*A and E from *S. coelicolor* share homology with that of an *S. lividans* homolog.

The existence of functional heterogeneous rRNA molecules and the maintenance of divergent rRNA genes through evolution, along with differential expression of heterogeneous rRNA species at different stages of development in *S. coelicolor* observed here, imply that this developmentally complex bacterium has an unidentified regulatory circuit that utilizes heterogeneous rRNA molecules to control gene expression at the post-transcriptional level by selectively translating specific mRNA species.

Acknowledgements

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