Proteomic Analysis of the *Streptomyces griseus* Ribosomal Fraction

Genki Akanuma,1 Hideaki Nanamiya,2 Yoshihiro Mouri,2 Morio Ishizuka,1 and Yasuo Ohnishiy

1Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo 112-8551, Japan
2Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Received July 17, 2012; Accepted September 14, 2012; Online Publication, December 7, 2012 [doi:10.1271/bbb.120556]

The *Streptomyces griseus* 70S ribosome fraction was analyzed by radical-free and highly reducing two-dimensional (RFHR 2D) gel electrophoresis and mass spectrometry. Among the 60 putative ribosomal proteins that are encoded by the *S. griseus* genome, 48 were identified in the 70S ribosome fraction prepared from mycelia grown in liquid culture for 12, 36, and 48 h. Ribosomal protein S3 was detected at two different positions on the 2D gel, and the distribution changed completely in the course of the growth, suggesting that it was modified or processed. The SGR3624 protein was also identified in the 70S ribosome fraction, but detailed cellular fractionation analysis indicated that it localizes mainly at the membrane rather than the ribosome. An SGR3624-deleted mutant showed slow growth on solid media, indicating that SGR3624 has an important role in the growth of the substrate mycelium in solid culture.

Key words: *Streptomyces*; ribosome; proteomics

The bacterial 70S ribosome consists of large and small subunits, designated 50S and 30S, respectively. The 50S subunit is composed of the 23S and 5S rRNAs and more than 30 ribosomal proteins, whereas the 30S subunit is composed of the 16S rRNA and more than 20 ribosomal proteins.1–4) Protein synthesis is mediated by the 70S ribosome and various GTPases, which are required not only for the initiation and elongation of polypeptide synthesis but also for the release of tRNA from the ribosome.4–7) Several ribosome-associated proteins provide additional functions to the ribosome. For example, the trigger factor, which localizes at the exit site of the ribosome by binding to ribosomal proteins L23 and L29, prevents newly synthesized proteins from misfolding and aggregating.38) The signal recognition particle (SRP), which consists of the Fh1 protein and 4.5S RNA,9,10) also localizes near the exit site of the ribosome by binding to L23.11) SRP recognizes the hydrophobic signal sequence in the nascent polypeptide on the ribosome, and then mediates the transport of secreted proteins and membrane proteins to the appropriate locations.12–14) Ribosome modulation factor (RMF), which binds to 70S ribosomes, stimulates their dimerization to make particles of 90S, and then hibernation promoting factor (HPF) converts the 90S particles to 100S particles, which have no translational activity.15–17) Thus ribosome-associated proteins have various important functions.

The Gram-positive, soil-dwelling, filamentous bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites and by its complex morphological differentiation. In the streptomycin producer *Streptomyces griseus*, aerial mycelium formation and secondary metabolite production are triggered by the low-molecular-weight signaling molecule, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrrolactone).18,19) A-factor switches on the transcription of *adpA*, which encodes a transcriptional activator, by binding to ArpA, the A-factor receptor protein that binds the *adpA* promoter, and dissociating DNA-bound ArpA from the DNA.20) AdpA activates the transcription of a number of genes that are required for morphological development and secondary metabolite formation.21) Hence, regulation at the transcriptional level is undoubtedly important for morphological development and secondary metabolism in *Streptomyces*. In addition, several studies have indicated that ribosome functions are closely associated with the production of secondary metabolites.22–24) *Streptomyces* appears to have a variety of regulatory mechanisms that control the activity as well as the intracellular concentration of ribosomes. We assume that some ribosome-associated proteins are involved in this regulation, but no comprehensive analysis of ribosome-associated proteins in *Streptomyces* has been reported. Determination of the complete genome sequence of *S. griseus* has revealed the existence of six rRNA operons and 60 genes that encode putative ribosomal proteins (Table 1).25) *Streptomyces* species have several paralogous genes for some ribosomal proteins.25–28) For example, in the *S. griseus* genome, genes encoding ribosomal proteins L28 (SGR545 and SGR1916), L31 (SGR547, SGR2177, and SGR6846), L33 (SGR546, SGR2884, and SGR6845), S14 (SGR544 and SGR2821), and S18 (SGR549 and SGR3672) are duplicated or triplicated,25) but to date the details of the composition of ribosomal proteins in the *Streptomyces* ribosome have not been determined.

Radical-free and highly reducing two-dimensional (RFHR 2D) gel electrophoresis is a powerful tool for...
separating ribosomal proteins which are usually small and basic and cannot be separated well by standard 2D electrophoresis.\textsuperscript{20} In the first electrophoresis of RFHR 2D analysis, basic proteins migrate to the cathode and acidic proteins to the anode, according to the net charge at pH 8.2.\textsuperscript{20} In the second electrophoresis, proteins migrate according to the net charge at pH 3.6 and are separated by the gel filtration effect with 18% acrylamide-6M urea gel.\textsuperscript{20} Here we analyzed the high-salt-washed 70S ribosome fraction by RFHR 2D gel electrophoresis and mass spectrometry to identify the ribosome proteins in \textit{S. griseus}, preceding the identification of proteins that might be loosely associated with the ribosome. We identified 48 ribosomal proteins among the 60 putative ribosomal proteins encoded by the \textit{S. griseus} genome. In addition to these canonical ribosomal proteins, three proteins (SGR3624, SGR4527, and SGR5341) were identified in the high-salt-washed 70S ribosome fraction. SGR4527 is a putative HPF that appears to be required for the formation of the 70S dimer. SGR5341 is a multimer-forming phage shock protein homolog that does not appear to be associated with the ribosome. SGR3624 is a protein of unknown function. Its homologs are widely distributed among bacteria. We examined the function of SGR3624 by gene disruption, and an SGR3624-deleted mutant showed slow growth on solid media, but detailed cellular fractionation analysis indicated that SGR3624 is a membrane protein rather than a ribosome-associated protein. Thus SGR3624 has an important role in the growth of the substrate mycelium of \textit{S. griseus}, although its molecular function remains to be elucidated.

\textbf{Materials and Methods}

\textbf{Bacterial strains and growth conditions.} \textit{S. griseus} IFO13350 (\textsuperscript{-}NBRC102592) was obtained from the Institute of Fermentation, Osaka (IFO) (Osaka, Japan). Cells were grown in YMPD medium (0.2\% yeast extract, 0.22\% meat extract, 0.4\% Bacto peptone, 0.5\% NaCl, 0.2\% MgSO\textsubscript{4}-7H\textsubscript{2}O, and 1\% glucose, pH 7.2). The YMPD agar contained 2.2\% agar. The following antibiotics were added as necessary: neomycin (20\,\mu g/mL) and thiostrepton (50\,\mu g/mL).

\textbf{Preparation of 70S ribosomes.} \textit{S. griseus} was precultured at 30 \textdegree C for 2 d in 100 mL of YMPD medium in a 500-\text{ml} shaking (Sakaguchi) flask with reciprocal shaking (120\,rpm). Then 25\,\text{ml} of the culture was centrifuged at 8,000 \times g and the mycelia were harvested. The mycelial pellet was washed twice with YMPD and resuspended in 5\,\text{ml} of YMPD medium. Two \text{ml} of the resulting mycelial suspension were inoculated into 1L of YMPD. The culture was incubated at 30 \textdegree C with reciprocal shaking (120\,rpm) in a 5-L baffle flask. Mycelia were collected after 12, 36, and 48 h of incubation, resuspended in buffer I (10\,\text{mm} Tris–HCl pH 7.6, 15\,\text{mm} (CH\textsubscript{3})\textsubscript{2}COOH, 100\,\text{mm} CH\textsubscript{3}COONH\textsubscript{4}, 6\,\text{mm} \beta\text{-mercaptoethanol}, and 2\,\text{mm} phenylmethylsulphonyl fluoride) and disrupted by passage through an Amino French pressure cell (8,000 psi, Thermo Fisher Scientific Inc., Waltham, MA). After removal of cell debris, the supernatant was centrifuged at 30,000 \times g for 30\,min at 4 \textdegree C in a Hitachi P55SST2 rotor (Hitachi, Tokyo). The supernatant was centrifuged at 190,000 \times g for 100\,min at 4 \textdegree C in a Hitachi P55SST2 rotor. The precipitate was dissolved in buffer I and applied to 10–40\% sucrose density gradient centrifugation (Hitachi P5282 rotor, 500 A\text{260 units} per tube) in buffer I at 43,000 \times g for 18h. Forty fractions were collected, and those containing 70S ribosomes were identified by a peak in the absorbance at 260\,nm. The 70S peak fraction was centrifuged in buffer II (20\,\text{mm} Tris–HCl pH 7.6, 15\,\text{mm} (CH\textsubscript{3})\textsubscript{2}COOH, 1\,\text{mm} CH\textsubscript{3}COONH\textsubscript{4}, 6\,\text{mm} \beta\text{-mercaptoethanol}, and 2\,\text{mm} phenylmethylsulphonyl fluoride) at 190,000 \times g for 100\,min at 4 \textdegree C in a Hitachi P55SST2 rotor. The precipitate was dissolved in buffer III (20\,\text{mm} Tris–HCl pH 8.0, 0.2\,\text{mm} dithiothreitol, and 2\,\text{mm} phenylmethylsulphonyl fluoride). Ribosomal proteins were prepared by the acetic acid method\textsuperscript{60} and, after dialysis against 1\% acetic acid, were lyophilized and stored at -80 \textdegree C until use.

\textbf{RFHR 2D gel electrophoresis and protein identification.} RFHR (radical-free and highly reducing) 2D gel electrophoresis\textsuperscript{20} was performed essentially according to published procedures.\textsuperscript{31} Each protein spot on the 2D gel was identified by mass spectrometry, as described previously.\textsuperscript{32} MALDI-TOF peptide mass fingerprint data were compared with the \textit{S. griseus} sequence database (DDBI, accession no. AP009493) using the Mascot search engine (Matrix Science, London, UK), and the proteins were identified using the Probability-Based MOWSE Score algorithm. The search parameters were as follows: mass accuracy, 0.5 Da; missed cleavage, none; fixed modification, carboxamidomethylation of Cys residues; variable modification, oxidation of Met residues. A MOWSE score > 52 guarantees identification of the protein with > 95\% probability. All proteins identified in this study gave MOWSE scores > 52.

\textbf{DNA manipulation.} DNA was manipulated in \textit{Streptomyces} spp.\textsuperscript{33} and \textit{E. coli},\textsuperscript{34,35} as previously described. The primers used are listed in Table S1 (see Biosci. Biotechnol. Biochem. Web site).

\textbf{RT-PCR.} Total RNA was isolated from wild-type cells grown for 24h in YMPD using the Ambion RNQueuos-Midi kit (Life Technologies, Carlsbad, CA). Complementary DNA was then synthesized by the Invitrogen ThermoScript RT-PCR system (Life Technologies, Carlsbad, USA) and random hexamers following the manufacturer’s instructions, and was PCR-amplified using the primers listed in Table S1 (10 pmol each) under the following thermal conditions: 96 \textdegree C for 45 s, 60 \textdegree C for 1 min, and 72 \textdegree C for 30 s (30 cycles).

\textbf{Construction of the SGR3624 mutant.} A plasmid that contained upstream (8.5\,kb) and downstream (3.5\,kb) regions of SGR3624 (which excludes the entire 744-bp SGR3624-coding sequence except for the start and stop codons) was constructed as described previously.\textsuperscript{36} Briefly, from the genomic shotgun library for sequencing of the \textit{S. griseus} IFO13350 genome,\textsuperscript{25} we selected a plasmid that contained a 12-kb region including SGR3624. This was introduced into \textit{E. coli} BW25141 carrying pKD78. The apramycin-resistance gene (aac(3)\,IV) was PCR-amplified using primers 3624FRF and 3624FRT. The amplified DNA fragment was composed of a 39-bp DNA fragment, the aac(3)IV gene, and another 39-bp DNA fragment. The 39-bp DNA fragments attached to the ‘S’ and ‘3’ ends of the aac(3)IV gene encoding the ‘S’ and ‘3’ flanking regions of SGR3624, respectively. Using this DNA fragment, the coding sequence of SGR3264 on the plasmid was replaced with the aac(3)IV gene via \textit{Al}RE mediated recombination in \textit{E. coli} DH5\textalpha. Next, the apramycin-resistance gene in the recombinant plasmid was eliminated via FLIP recombine-induced excision, leaving a \textit{Scar} sequence. Then the kanamycin/neomycin-resistance gene was inserted into the plasmid, and the resulting plasmid was introduced into \textit{S. griseus} IFO13350 by protoplast transformation. A transformant in which the recombinant plasmid was integrated into the chromosome as a result of a single crossover event was selected from the neomycin-resistance colonies. This strain was repeatedly cultured in YMPD medium without neomycin. Then protoplasts were prepared and spread on a regeneration medium (R5)\textsuperscript{33,34} without neomycin. From these protoplasts, two neomycin-sensitive colonies were obtained, a SGR3624 mutant from which the wild-type SGR3624 gene was deleted, and a regenerated wild-type strain. Deletion was confirmed by Southern hybridization using an appropriate probe (data not shown).

\textbf{Construction of pTYM3624-FLAG for use in complementation and localization tests.} pTYM3624-FLAG containing the SGR3624 with a 3\texttimes\text{-FLAG} tag sequence was constructed as follows: the promoter region of \textit{hrdB}, which encodes housekeeping-sigma factor, and the SGR3624-coding sequence were PCR-amplified using primer sets PhrdBF plus PhrdBR and 3624HisF plus 3624HisR, respectively. The two resulting fragments were then mixed and amplified with primers PhrdBF (containing a BamHI site) and 3624HisR2 (containing an EcoRI site). The fragment was cloned into the BamHI-EcoRI sites of
Proteomic Analysis of *S. griseus* Ribosomal Fraction

were resuspended in 40 mL of buffer V (20 mM Tris–HCl pH 8, 100 mM CH$_3$COONa, and 6 mM β-mercaptoethanol) supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland; one tablet per 15 mL of buffer V). The extract obtained was centrifuged at 12,000 × g for 30 min at 4°C to remove cell debris and aluminum oxide. The supernatant was subjected to centrifugation at 100,000 × g for 30 min at 4°C in a Hitachi P55ST2 rotor. The precipitate obtained was resuspended in 4 mL of buffer VI (20 mM Tris–HCl pH 8.0, 100 mM CH$_3$COONa, and 6 mM β-mercaptoethanol), and centrifuged at 5,200 × g for 10 min at 4°C to remove insoluble materials. Next, 1.9-mL aliquots of the supernatant were layered onto 10–40% sucrose density gradients in buffer VI, and extract aliquots were subjected to further ultracentrifugation, at 190,000 × g, for 100 min at 4°C in a Hitachi P55ST2 rotor. The precipitate obtained was resuspended in 0.2 mL of buffer IV, and extract aliquots were layered onto 10–40% sucrose density gradients in buffer V (20 mM Tris–HCl pH 8.0, 100 mM CH$_3$COONa, and 6 mM β-mercaptoethanol), and centrifuged at 4,000 × g for 10 min at 4°C to remove tailing from the major L4 spot (5.2 kDa). Enlargements in panels B, C, and D are indicated by dotted rectangles. For the 12-h sample, spots on the 2D gel were identified as L4 by peptide mass fingerprinting. This spot was identified as L4 by peptide mass fingerprinting. This spot was identified as L4 by peptide mass fingerprinting.

Sucrose density gradient sedimentation analysis. The *S. griseus* ΔSGR3624 strain harboring pTYM3624FLAG (ΔSGR3624 SGR3787::pTYM3624FLAG) was grown in 100 mL of YM medium at 30°C for 24 h. After the addition of KCl at a final concentration of 1 M, mycelia were collected by centrifugation at 13,000 × g for 10 min at 4°C, and were washed with 25 mL of wash buffer (50 mM Tris–HCl pH 7.4, 10% v/v glycerol, and 1 mM DTT). The mycelial pellet was frozen in liquid N$_2$ and then disrupted by grinding it in a mortar with aluminum oxide (1.5 g per 1 g wet weight mycelia). The cell material was extracted from the paste with 5 mL of buffer IV (10 mM Tris–HCl pH 8, 10 mM (CH$_3$COO)$_2$Mg, 100 mM CH$_3$COONa, and β-mercaptoethanol) supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland; one tablet per 15 mL of buffer IV). The extract obtained was centrifuged at 12,000 × g for 30 min at 4°C to remove cell debris and aluminum oxide. The supernatant was subjected to centrifugation at 30,000 × g for 30 min at 4°C, and then it was subjected to further ultracentrifugation, at 190,000 × g, for 100 min at 4°C in a Hitachi P55ST2 rotor. The precipitate obtained was resuspended in 4 mL of buffer V (20 mM Tris–HCl pH 8.0, 15 mM (CH$_3$COO)$_2$Mg, 1 mM CH$_3$COONa, and 6 mM β-mercaptoethanol), and centrifuged at 5,200 × g for 10 min at 4°C to remove insoluble materials. Next, 1.9-mL aliquots of the supernatant were layered onto 1.9 mL of buffer V containing a 30% (w/v) sucrose bed and centrifuged at 190,000 × g for 3 h at 4°C in a Hitachi P55ST2 rotor. The precipitate obtained was resuspended in 0.2 mL of buffer IV, and extract aliquots were layered onto 10–40% sucrose density gradients in buffer VI (20 mM Tris–HCl pH 8.0, 100 mM CH$_3$COONa, and 6 mM β-mercaptoethanol) that contained either 1 mM or 15 mM of (CH$_3$COO)$_2$Mg, and this was centrifuged at 4°C for 18 h at 65,000 × g (Hitachi P40ST rotor, 50 A$_{260}$ units per tube). Twenty fractions (0.5 mL in each) were collected from the top of the tube, and those containing 30S, 50S, and 70S ribosomes were identified by peaks in the absorbance at 260 nm. Then 2-µL aliquots of each fraction (from fractions 6 to 20) were subjected to agarose gel (1% agarose) electrophoresis in 1× TBE to examine the presence of each tRNA in each fraction. Also, 0.4-µL aliquots of each fraction were subjected to trichloroacetic precipitation, and the precipitates obtained were resuspended in 40 µL of loading buffer (50 mM Tris–HCl pH 6.8, 0.1 M dithiothreitol, 2% wt/v SDS, 0.1% wt/v bromophenol blue, and 10% v/v glycerol). Fifteen µL of each fraction was loaded onto a 15% SDS–PAGE gel to detect the proteins in each fraction by Coomassie Brilliant Blue staining. To detect the SGR3624-3×FLAG protein, 15 µL of each fraction was electrophoresed and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA). Immunodetection procedures, in which anti-FLAG (Sigma–Aldrich), Pierce SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific Inc, Waltham, MA) was used to amplify the signal as the substrate of horseradish peroxidase, and substrate (Thermo Fisher Scientific Inc, Waltham, MA) was used to amplify the signal as the substrate of horseradish peroxidase, and detection was done using the LAS 1000 lumino-image analyzer (Fujifilm, Tokyo).

Results

Identification of the ribosomal proteins in *S. griseus*

To identify the constituent proteins of the *S. griseus* ribosome, we prepared a 70S ribosome fraction from mycelia grown in YMPD liquid medium for 12 h (early exponential phase), 36 h (early stationary phase), and 48 h (stationary phase), and separated them by RFHR 2D gel electrophoresis (Fig. 1A). Because we prepared the 70S ribosome fractions by sucrose density-gradient sedimentation, followed by centrifugation in high-salt buffer, most of the proteins detected in the 2D gels were considered to be the ribosome components (ribosomal proteins) and ribosome-related proteins tightly associated with the ribosome. More than 50 spots were almost identically visualized on the 2D gels of all samples, and the proteins of all the spots of the 12-h sample were analyzed by peptide mass fingerprinting, as

Fig. 1. Two-Dimensional Gel Electrophoresis of Ribosomal Proteins.

A, Ribosomal proteins (750 µg) prepared from mycelia grown in YMPD liquid for the indicated durations were analyzed by RFHR 2D gel electrophoresis. The hollow arrowhead for each gel image indicates the candidate spot for L7/L12. L34 was not detected on the 2D gel of the 48-h sample, due to its low molecular weight (5.2 kDa). Enlargements in panels B, C, and D are indicated by dotted rectangles. For the 12-h sample, spots on the 2D gel were identified. Gray circles indicate proteins in the small subunit of the ribosome, and hollow circles show the proteins in the large subunit of the ribosome. B, Differential appearance of the spot corresponding to S3 at different growth phases. The areas of the 2D gels containing the spots of the S3 protein are extracted from the gel images. S3 protein spots were identified by peptide mass fingerprinting. C, The areas of the 2D gels containing the spots of the L9 protein are extracted from the gel images. D, The areas of the 2D gels containing the spots of SGR3541, SGR4527, and SGR3624 are extracted from the gel images. The spot indicated by a hollow arrow was identified as L4 by peptide mass fingerprinting. This spot appears to have been a result of tailing from the major L4 spot during first electrophoresis.
the stationary phase (48 h) was detected at a more basic, on the 2D gel of the ribosomes prepared from mycelia at just above the protein spot of S4. In contrast, the S3 spot localization in YMPD), the protein spot of S3 was detected mycelia at early exponential growth (12 h after inocu-
lation in YMPD), the protein spot of S3 was detected following proteins: L7/L12, L21, L36, S1, and S5 (see ‘Discussion’ below).

Differential appearances of the spots corresponding to S3 on the 2D gels were observed in the ribosome fractions prepared from mycelia during different growth phases. On the 2D gel of the ribosomes prepared from mycelia at early exponential growth (12 h after inoculation in YMPD), the protein spot of S3 was detected just above the protein spot of S4. In contrast, the S3 spot on the 2D gel of the ribosomes prepared from mycelia at the stationary phase (48 h) was detected at a more basic, lower molecular weight region (designated S3′) (Fig. 1B). This indicates that the ribosomal protein S3 in S. griseus was modified or processed during growth. The other ribosomal proteins were detected at similar positions on the 2D gels of all the samples (Fig. 1A). Furthermore, the amounts of all the ribosomal proteins, except for L9, remained almost constant during growth. The amount of L9 was greatly decreased after the culture entered the stationary phase (Fig. 1C).

Identification of three proteins that are not canonical ribosomal proteins

Our 2D gel analysis also showed three proteins, SGR3541, SGR4527, and SGR3624, that were not annotated as ribosomal proteins, and have not been characterized to date in S. griseus (Fig. 1D). SGR5341 shows homology with PspA (22% identity), a phage shock protein A in E. coli. Given that PspA forms a huge multimer in the cell, we assumed that SGR5341 was not associated with the ribosome, but co-fractionated with it due to multimerization. In fact, in B. subtilis, LiaH, which also shows homology with SGR5341 (24% identity), forms large oligomeric rings with a high molecular weight, of at least 1,250 kDa. At present, however, we cannot completely eliminate the possibility that SGR5341 is associated with the ribosome. SGR4527 was detected in the 70S ribosome fraction prepared from mycelia at the stationary phase rather than the exponential phase (Fig. 1D). SGR4527 shows 29% identity with E. coli HPF (hibernation promoting factor), which is required for the formation of the 70S dimer, so-called 100S ribosome. A possible function of SGR4527 is described below under “Discussion.” SGR3624 was detected in the ribosome fractions prepared from mycelia at the early- to mid-stationary growth phases (Fig. 1D). The amount of SGR3624 was larger in the 70S ribosome fraction prepared from mycelia at the mid-stationary phase than at the early stationary phase (Fig. 1D). SGR3624 is a protein of function unknown and its homologs are widely distributed among bacteria (Fig. S1). The function of no SGR3624 homolog has been characterized so far.

SGR3624 had a function in the growth of the substrate mycelium

We identified the transcription unit including SGR3624 by RT-PCR. As shown in Fig. 2A, SGR3624 was co-transcribed with SGR3623 and SGR3625 to SGR3628, indicating that SGR3623 to SGR3628 were transcribed as a polycistronic messenger. This is in agreement with the prediction of a previous report that SGR3629 (pheA), whose gene product is involved in phenylalanine biosynthesis, has its own promoter and that the orthologs of SGR3623-3628 in Streptomyces coelicolor A3(2) and Streptomyces avermitilis are co-transcribed from the promoter of scoA, an ortholog of SGR3623. To eliminate possible polar effects on downstream genes within the same operon, we constructed an SGR3624-deleted mutant in which the coding sequence of SGR3624 (from +4 to +747, where +1 denotes the translation start site) was deleted without insertion of any DNA fragments. This deletion mutant showed a reduced growth rate on YMPD solid medium (Fig. 2B) and on minimal medium (data not shown). In contrast, deletion of SGR3624 did not significantly affect the growth rate in YMPD liquid culture (data not shown). When pTYM19-3624FLAG was integrated into the chromosome of the ΔSGR3624 strain, the growth rate was restored (Fig. 2B). These results suggest that SGR3624 has an important role in the growth of substrate mycelium in solid culture.

SGR3624 was localized mainly to the membrane

As described above, we found that SGR3624 has a function in the growth of substrate mycelium in solid culture. We hypothesized that SGR3624 affects translation by interacting with the ribosome, but the localization of SGR3624 to the ribosome was questionable, as the SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and SOSUI 1.11 (http://bp.nuap.nagoya-u.ac.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SGR no.</th>
<th>Spot on 2D</th>
<th>Protein</th>
<th>SGR no.</th>
<th>Spot on 2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>2873</td>
<td>L31</td>
<td>6846</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>2831</td>
<td>L32</td>
<td>1909</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>2834</td>
<td>L33</td>
<td>546</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>2833</td>
<td>L33</td>
<td>2884</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>2822</td>
<td>L33</td>
<td>6845</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L6</td>
<td>2819</td>
<td>L34</td>
<td>3699</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L7/L12</td>
<td>2870</td>
<td>ND</td>
<td>L35</td>
<td>5906</td>
<td>ND</td>
</tr>
<tr>
<td>L9</td>
<td>3671</td>
<td>L36</td>
<td>2810</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L10</td>
<td>2871</td>
<td>S1</td>
<td>5534</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L11</td>
<td>2874</td>
<td>S2</td>
<td>1863</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L13</td>
<td>2802</td>
<td>S3</td>
<td>2828</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L14</td>
<td>2824</td>
<td>S4</td>
<td>6030</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L15</td>
<td>2815</td>
<td>S5</td>
<td>2817</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L16</td>
<td>2827</td>
<td>S6</td>
<td>3674</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L17</td>
<td>2806</td>
<td>S7</td>
<td>2845</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L18</td>
<td>2818</td>
<td>S8</td>
<td>2820</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L19</td>
<td>1885</td>
<td>S9</td>
<td>2801</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L20</td>
<td>5907</td>
<td>S10</td>
<td>2835</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L21</td>
<td>4593</td>
<td>ND</td>
<td>S11</td>
<td>2808</td>
<td>ND</td>
</tr>
<tr>
<td>L22</td>
<td>2829</td>
<td>S12</td>
<td>2846</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L23</td>
<td>2832</td>
<td>S13</td>
<td>2809</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L24</td>
<td>2823</td>
<td>S14</td>
<td>544</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L25</td>
<td>4382</td>
<td>S14</td>
<td>2821</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L27</td>
<td>4954</td>
<td>S15</td>
<td>1784</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L28</td>
<td>545</td>
<td>ND</td>
<td>S16</td>
<td>1889</td>
<td>ND</td>
</tr>
<tr>
<td>L28</td>
<td>1916</td>
<td>S17</td>
<td>2825</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L29</td>
<td>2826</td>
<td>S18</td>
<td>549</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L30</td>
<td>2816</td>
<td>S18</td>
<td>3672</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>L31</td>
<td>547</td>
<td>ND</td>
<td>S19</td>
<td>2830</td>
<td>ND</td>
</tr>
<tr>
<td>L31</td>
<td>2177</td>
<td>S20</td>
<td>4983</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

○, detected
ND, not detected
In the present study, we successfully identified 48 ribosomal proteins in the 70S ribosome fraction out of 60 putative ribosomal proteins that are encoded by the <i>S. griseus</i> genome (Fig. 1B and Table 1). To our knowledge, this is the first study to determine the composition of ribosomal proteins in the <i>Streptomyces</i> 70S ribosome. In addition to these canonical ribosomal proteins, we identified three proteins, SGR5341, SGR4527, and SGR3624. Because the high-salt-washed 70S ribosome fraction was subjected to 2D gel analysis, most ribosome-associated proteins might have dissociated from the ribosome during the purification procedures. Hence this study clarifies the fundamental composition of the <i>S. griseus</i> ribosome, and can serve as a basis for further investigation to identify novel ribosome-associated proteins by other experimental approaches, such as monolith chromatography-based purification of the ribosome.42)

We did not identify the following proteins: L7/L12, L21, L36, S1, and S5. S1 is dispensable for growth in Gram-positive bacteria.43,44) In fact, it was not detected in the <i>B. subtilis</i> ribosome, either.31) L36 was not detected, probably due to its small size (4.4 kDa) among ribosomal proteins, as described in a previous report.31)

The gene encoding L21 (rplU) is expected to be co-transcribed with the gene encoding L27 (rpmA), and the gene encoding S5 (rpsE) is located in a large cluster of 21 genes encoding ribosomal proteins, the so-called S10-spc cluster, which is highly conserved among eubacteria.45) Because L27 and the other ribosomal proteins encoded by the S10-spc cluster were detected by our 2D analysis, rplU and rpsE should have been expressed under the growth conditions imposed in this study. Hence the absence of spots corresponding to L21 and S5 may have been due to dissociation of these proteins from the ribosome during the purification procedures. It is also possible that the spots of L21 and S5 cannot be separated from the other protein spots. Furthermore, we failed to identify some spots on the 2D gels by MALDI-TOF peptide mass fingerprinting analysis, probably due to the low MOWSE score. For
example, we could not identify a spot that probably corresponded to L7/L12. Although many ribosomal proteins are considered to be basic proteins, the isoelectric point of L7/L12 is expected to be 4.4. Together with the molecular size of L7/L12 (13 kDa), it is most likely that the spot detected under the L25 spot was L7/L12 (Fig. 1A). Indeed, in the profiles of the RFRHR 2D gels of the *B. subtilis* ribosome, the spot corresponding to L7/L12 was detected in a similar position. \(^{31}\)

The spot corresponding to ribosomal protein S3 was found at different positions on the 2D gels of the ribosomes prepared at different growth phases. The S3 spot moved to a more basic, lower molecular weight region with growth (Fig. 1C). In *S. coelicolor* A3(2), the phosphorylation of several ribosomal proteins, including S3, which influences subunit association and the translation of poly (U), has been reported. \(^{46}\) One possible explanation is that S3 is modified by phosphorylation (or dephosphorylation). Other modifications are also possible. Another strong possibility is that S3 is partially degraded by post-translational protein processing. Ribosomal proteins S3 and S4 exhibit helicase activity by interacting with the mRNA in the entrance tunnel of the ribosome. This activity is required to constrain the mRNA in a clamp-like fashion. \(^{47}\) Thus it is likely that modification or processing of S3 can control the translation activity at the stationary phase. Identification of the detailed molecular composition of S3 by high-resolution mass spectrometry analysis, such as LC-MS/MS, should help to clarify the biological significance of S3 at different growth phases.

*S. griseus* has five paralogous sets of ribosomal protein-encoding genes, but only one of each gene product, SGR1916 (L28), SGR2177 (L31), SGR2884 (L33), SGR2821 (S14), and SGR3672 (S18), was detected for these ribosomal proteins. The alternative ribosomal protein-encoding genes did not appear to be expressed under the conditions tested in this study. In *S. coelicolor* A3(2), the genes encoding alternative ribosomal proteins L28 (*rpmB2*), L31 (*rpmE2*), L32 (*rpmF2*), L33 (*rpmG2*), and S14 (*rpsN2*) are regulated by Zur, a zinc-specific transcription repressor controlling zinc transport operons. \(^{48,49}\) In addition, transcription of *rpmG3*, which encodes the alternative ribosomal protein L33, is controlled by σ^H*, a sigma factor essential for the oxidative stress response. \(^{49}\) Under the growth conditions tested in this study, zinc starvation, which accompanies derepression of Zur and oxidative stress, did not appear to occur, and thus these paralogous genes did not appear to be expressed. In *B. subtilis*, YtA, a
homolog of L31 (RpmE), is produced only under zinc-deficient growth conditions by derepression of Zur, and is incorporated into the ribosome in place of RpmE. Because the L31 homolog gene in *S. coelicolor* A3(2) is also regulated by Zur,[48,49] it is most likely that a zinc-dependent alternation of L31 proteins also occurs in *Streptomyces* species.

The 100S ribosome is found in *E. coli* cells only during the stationary phase.15,16) Because the translation activity of the 100S ribosome was not detected, it is believed that 100S ribosome formation is one of the strategies for acclimation during the stationary phases, in which efficient protein synthesis may not be needed anymore. Recently, it has been found for *Staphylococcus aureus*, as well as *B. subtilis*, that the homologs of HPF are also responsible for the dimerization of 70S.50,51) Hence it is likely that SGR4527 also promotes the formation of 70S dimers in *S. griseus*, though the details remain unclear.

Among the three uncharacterized proteins identified in our 2D gel analysis, we attempted to determine the function of SGR3624 in this study. Our phylogenetic analysis indicated that orthologs of SGR3624 are distributed among Actinobacteria, as well as a few species belonging to Firmicutes, Proteobacteria, and Cyanobacteria (Fig. S1). According to the Pfam database (http://pfam.sanger.ac.uk/), all of these orthologs contain a common domain of unknown function, DUF1775, but to the best of our knowledge there has been no report on the function of this family. According to the InterPro database (http://www.ebi.ac.uk/interpro/), SGR3624 contains the N-terminal domain of G1e, which overlaps with the DUF1775 region. G1e is an essential mRNA export factor in both yeast and human cells.52,53) It is also required for efficient translation.54) Taking these facts together with the fact that SGR3624 was found in the ribosome fraction, we first assumed that SGR3624 is involved in the function of the ribosome, but we found that SGR3624 is localized mainly to the membrane rather than the ribosome. In addition, an in vitro translation assay using EGFP as reporter[44] showed no apparent effect of the absence of SGR3624 on translation activity (data not shown). These results suggest that SGR3624 is a membrane protein, and hence does not directly affect the translation activity of the ribosome. However, we cannot rule out the possibility that the function of SGR3624 is correlated with the function of the ribosome in vivo. For example, SGR3624 may be required to anchor the ribosome to the membrane.

We found that a null mutation of SGR3624 exhibited a delay in growth on solid medium, indicating that SGR3624 has an important role in the growth of substrate mycelium. Very recently, it was reported that SGR3625 (scoC), which is co-transcribed with SGR3624, is involved in the utilization of copper under low-copper conditions.41) Morphological development and antibiotic production in *Streptomyces* are stimulated by supplying copper to the culture medium.56) A delay in the development of the scoC mutant was observed in both *S. coelicolor* A3(2) and *S. griseus*, probably due to a decrease in the activity of copper-containing proteins, such as the cytochrome oxidase (cox) complex.41) Because SGR3624 (scoB) is transcribed with SGR3625 (scoC) and its expression is dependent on the copper concentration, SGR3624 may also be involved in the utilization of copper, but it has been reported that disruption of scoB did not significantly affect cox activity in *S. coelicolor* A3(2).43) Further analysis is required to elucidate the molecular function of SGR3624.

**Acknowledgments**

G.A. was supported by the Japan Society for the Promotion of Science (JSPS). This work was supported in part by a Funding Program for Next Generation World-Leading Researchers from the Bureau of Science, Technology, and Innovation Policy, Cabinet Office, Government of Japan (grant no. GS006).

**References**


