Sequence Analysis of the Ribosomal L11 Protein Gene (*rplK*=*relC*) in *Streptomyces lavendulae* Using a Deletion Allele

SHINICHI KAWAMOTO, DEYU ZHANG and KOZO OCHI*

National Food Research Institute, 2-1-2 Kannondai,Tsukuba, Ibaraki 305-8642, Japan

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Mutants with mutations in the *relA* (coding for ppGpp synthetase I) or *rplK* (coding for the ribosomal protein L11; formerly *relC*) gene fail to synthesize ppGpp (guanosine 5'-diphosphate 3'-diphosphate) (reviewed by CASHEL *et al.*[1]). On the basis of isolation and analysis of relaxed (Rel) mutants in several *Streptomyces* species, OCHI[2] has proposed that ppGpp plays a central role in triggering the onset of antibiotic production. During the last two years, with the aid of gene engineering techniques more informative results that help to clarify the possible role of ppGpp in initiating antibiotic production were provided by three independent laboratories. CHAKRABURTTY *et al.*[3] and MARTINEZ-COSTA *et al.*[4] reported that disruption of *relA* in *Streptomyces coelicolor* results in an actinorhodin-nonproducing phenotype in addition to the failure to synthesize ppGpp, while propagation of the *relA* gene completely abolishes these defective phenotypes. By comparing the *rplK* gene sequences of the wild-type strain to presumed *relC* mutant strains, we found a deletion within the mutant *rplK* gene[5,6]. When the wild-type *rplK* gene was propagated using a low copy-number vector in a *relC* mutant of *Streptomyces griseus* or *S. coelicolor*, the ability to produce ppGpp, streptomycin (in *S. griseus*) and actinorhodin (in *S. coelicolor*), all of which had been lost in the *relC* mutants, was completely restored[5,6]. These results all support the hypothesis that ppGpp is a pivotal signal molecule essential for initiating the onset of antibiotic production.

Putative *relC* mutant strains of *Streptomyces lavendulae* (No. 32 and 123) have been isolated and characterized[7,8]. These mutants are resistant to thiopeptin and thiostrepton and defective both in formycin production and in the ability to accumulate ppGpp during nutritional shift-down. When ribosomal proteins were analyzed by two-dimensional PAGE, the pattern from mutant 32 was significantly different from that of the parent strain, although mutant 123 shows no observable difference[8]. These results are summarized in Table 1. In this paper we report our attempts to clone and compare the *S. lavendulae relC* (=*rplK*) gene present in the wild-type and mutant strains.

The 1.0 kb fragments containing the *rplK* gene taken from the parent strain MA406 and from mutants No. 32 and 123 were cloned into the vector pGEM-5Zf(+). PCR was performed using the genomic DNA as templates and primers designed from *nusG* and *rplA* sequences in *S. griseus* as previously described[10]. The complete nucleotide sequences were also determined (accession No. D87847). Sequence comparisons between the wild-type and mutant 32 revealed that the mutant contained a large 90 bp deletion within the ORF of the *rplK* gene, which results in the loss of 30 amino acids from the L11 protein. In contrast, mutant 123 revealed a small 12 bp deletion, which results in the loss of only a tetrapeptide. These results are summarized in Fig. 1 and noted together with previously known results for *relC* mutants of *S. griseus* and *S. coelicolor*. Accordingly, mutants 32 and 123 are both assigned as *relC* mutants. It should be noted that the deletions found are all located in a specified region.

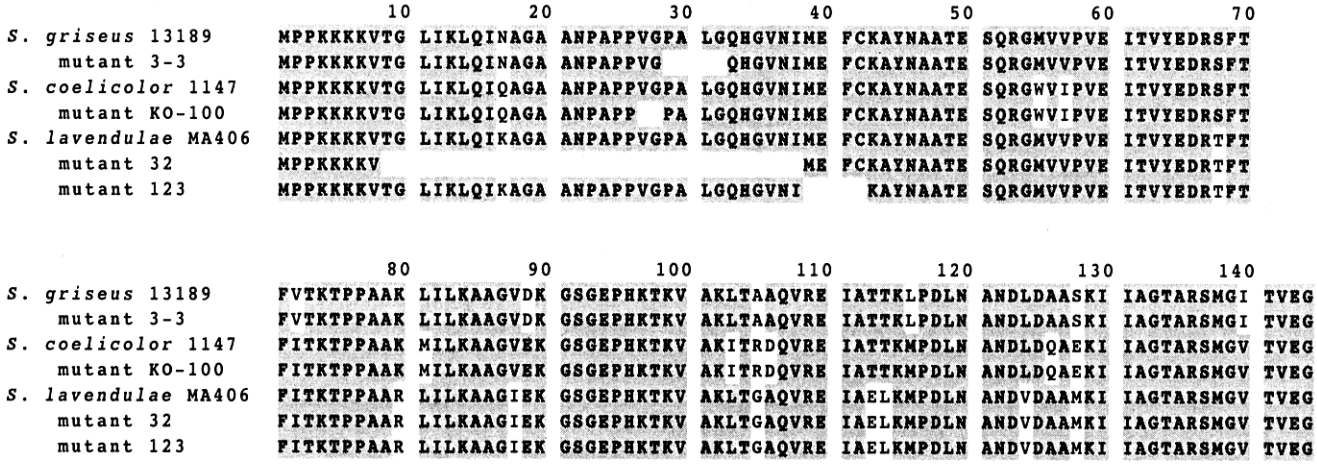
In order to clarify whether or not the mutant L11 proteins are integrated normally into the ribosomes, Western analysis was conducted (Fig. 2A). Care was taken to avoid different (if any) reactivity of anti-L11 antiserum to wild-type and mutant L11 proteins. Thus, we prepared an anti-L11 antiserum specific for the *C*-terminal region (see Experimental), since the mutant L11 proteins have no lesion in that region (Fig. 1). Also, to avoid the possibility that certain ribosomal proteins may be released from ribosomal particles during the washing steps for purification, crude ribosomal proteins were prepared without any washing and then were subjected directly to PAGE, followed by immunoblotting with anti-L11 (and anti-L6) antiserum. As shown in Fig. 2, incorporation of the mutant L11 proteins in the ribosomes in mutants 32 and 123 were both approximately half of that shown for the wild-type protein located in the parental ribosome, as determined by comparison with diluted ribosome samples prepared from the wild-type cells . The mutant L11 protein from mutant 32 migrated faster than the parental strain or mutant 123, reflecting its smaller molecular size which is due to the large deletion. Ribosomes from the *S. coelicolor relC* mutant KO-100, examined as a control,

Table 1. Summary of strain characteristics (from references 4, 8~12).

Organism	Resistance to thiopeptin (μg/ml)	ppGpp accumulated[a] (pmol/mg dry wt)	Two-dimentional PAGE pattern of ribosomal proteins	Growth
S. lavendulae MA406	0.5	275	(wild-type pattern)	good
mutant 32	6	44	missing a protein spot from the original spot	grossly impaired
mutant 123	6	53	no observable difference	grossly impaired
S. griseus 13189	0.5	815	(wild-type pattern)	good
mutant 3-3 (*relC*)	7	96	missing two protein spots from the original spots	grossly impaired
S. coelicolor 1147	1	243	(wild-type pattern)	good
mutant KO-100 (*relC*)	200	35	severely (but not completely) missing a protein spot	good

[a] Maximal ppGpp accumulated during Casamino acid deprivation.

Fig. 1. Amino acid sequence comparisons of the ribosomal L11 proteins in the wild-type and mutant strains of *S. griseus*, *S. coelicolor*, and *S. lavendulae*.

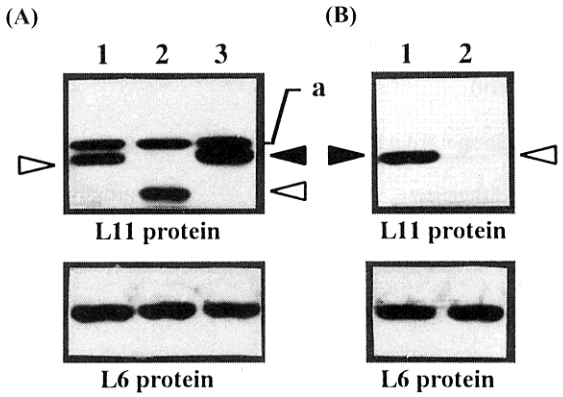


The open boxes represent the location of the deleted sequence in each mutant. L11 protein sequences for *S. griseus* 13189, *S. coelicolor* A3(2) 1147, and their mutants were taken from references 5 and 6.

contain only one-eighth the amount of L11 protein found in the ribosomes of the parent strain (Fig. 2B). These results correlate well with previous results using anti-L11 antiserum for the whole L11 protein[6]. No difference in the incorporation level of the L6 protein, which was used as an internal standard, was detected between the ribosomes of the parental and mutant strains. Interestingly, in *S. lavendulae* there exists another protein (designated band a in Fig. 2) that reacts with anti-L11 antiserum. This protein was not further investigated. In a similar analysis of the supernatants obtained following pelleting of the ribosomes (and therefore representing cytoplasmic proteins) neither the L11 nor the L6 was detectable (data not shown).

In the present study we have demonstrated by gene cloning and sequence analysis that the putative *relC* mutants of *S. lavendulae* (strains 32 and 123) are indeed *relC* mutants, as proven by the existence of a deletion mutation within the *rplK* gene coding for ribosomal protein L11. When the ribosomal proteins from mutant 123 and the parental strain were examined by two-dimensional PAGE analysis, the patterns generated showed no observable difference between the two strains[8]. Apparently, this is due to the fact that mutant

Fig. 2. Western analysis of ribosomal L11 and L6 proteins.



Closed and open arrowheads show the wild-type and mutant L11 proteins, respectively. Band "a" designates an unidentified protein that crossreacted with the anti-L11 antiserum. (A) *S. lavendulae*; lane 1, mutant 123; lane 2, mutant 32; lane 3, wild-type MA406. (B) *S. coelicolor*; lane 1, wild-type 1147; lane 2, *relC* mutant KO-100.

123 produces a protein only 4 amino acids shorter than wild-type (Fig. 1), which was integrated into the ribosomes in a manner not dramatically reduced as compared to wild-type (Fig. 2). The *relC* mutants listed in Fig. 1 were all originally isolated as thiopeptin (an analogue of thiostrepton)-resistant isolates which were significantly impaired in the ability to accumulate ppGpp during nutritional shift-down[2,7,9]. It should be noted that the regions deleted within the *rplK* gene represent the most highly conserved region of protein L11 among Gram-positive and Gram-negative bacteria (see KAWAMOTO *et al.*[5]), implying that it may be important for ribosome function. Indeed, the deletion mutations resulted not only in resistance to thiopeptin but also in a deficiency in accumulating ppGpp, apparently due to a failure to activate the *relA* gene product, ppGpp synthetase I. In fact, L11-deficient ribosomes are completely inactive for binding a stringent factor (=ppGpp synthetase I) which is responsible for the synthesis of ppGpp during the stringent response[11,12]. It is also known that L11 forms part of the target site for members of the thiazole family of antibiotics such as thiostrepton and thiopeptin. These antibiotics bind the same rRNA domain as L11[13], inhibit ribosome binding of EF-G and EF-Tu[14], and bind cooperatively with L11[15]. A recent approach using a limited proteolysis technique has shown that native L11 consists of two functional domains; the *N*-terminal domain is responsible for the cooperative binding of L11 and thiostrepton to RNA, and the *C*-terminal domain is the RNA binding domain[16]. This RNA binding domain is highly homologous to the homeodomain class of eukaryotic DNA binding proteins[17]. It is striking that mutations in L11, conferring resistance to thiopeptin in *Streptomyces* species, were all found within a specific *N*-terminal region (Fig. 1). Our results therefore correlate well with data described in the current literature. Also noteworthy is the fact that the mutations found were all inframe deletion mutations. This implies that in *Streptomyces* the *C*-terminal region of L11 protein, together with the *N*-terminal region, plays a part in the acquisition of resistance to thiopeptin and thiostrepton (or if not, the truncated protein may still be taking place to assist in the normal assembly of other ribosomal proteins into core particles).

Experimental

Ribosomes from cells grown to mid-exponential phase in GYM medium were prepared and subjected SDS-PAGE as described previously[5]. Each lane contained 0.20 A_{254nm} units of ribosomes. Mouse polyclonal antiserum against L6 protein used was described previously[5]. In the present study we prepared a rabbit polyclonal anti-L11 antiserum specific for the *C*-terminal region of L11 protein as follows. The plasmid pL11C2, which was constructed by inserting the gene fragment for the *S. griseus* L11 protein *C*-terminal region downstream from the gultathion *S*-transferase (GST) gene to generate a fusion protein, was introduced into *E. coli* for expression. A *Bam*HI site was created within the coding region of the *rplK* gene by PCR using pL11W, which expresses the whole *S. griseus* L11 protein in *E. coli*[5], as a template. The DNA primers used were: 5′ GAGTTCTGCAAGGGATCCAACGCCGCG3′ (*Bam*HI site underlined) and 5′ ATGCCTCGAGGGTTCCTCTA3′ (corresponding to the vector sequence of pL11W; *Xho*I site underlined). The PCR-amplified fragment was digested with *Bam*HI (cutting at positions between codons 44 and 45 of the L11 protein; see Fig. 1) and *Xho*I, then ligated into the expression vector pGEX-4T-2 (Stratagene) previously digested with *Bam*HI and *Xho*I, generating a plasmid named pL11C2. The polyclonal antiserum was prepared in a rabbit using the recombinant GST-L11 fusion protein produced in *E. coli*, purified by affinity chromatography using a glutathion Sepharose 4B column (Pharmacia Biotech).

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