Purification, Cloning, and Preliminary Characterization of a Spiroplasma citri Ribosomal Protein with DNA Binding Capacity*

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The rpsB-tsf-x operon of Spiroplasma citri encodes ribosomal protein S2 and elongation factor Ts, two components of the translational apparatus, and an unidentified X protein. A potential DNA-binding site (a 20-base pair (bp) inverted repeat sequence) is located at the 3’ end of rpsB. Southwestern analysis of S. citri proteins, with a 30-bp double-stranded oligonucleotide probe (IRS), containing the 20-bp inverted repeat sequence and the genomic flanking sequences, detected an IRS-binding protein of 46 kDa (P46). P46 protein, which displays preferential affinity for the IRS, was purified from S. citri by a combination of affinity and gel filtration chromatographies. The native form of P46 seems to be homomultimeric as estimated by SDS-polyacrylamide gel electrophoresis analysis and gel filtration. A 2.5-kilobase pair S. citri DNA fragment comprising the P46 gene and flanking sequences was cloned and sequenced. Sequence analysis of this DNA fragment indicated that the P46 gene is located within the S10-spc operon of S. citri at the position of the gene coding for ribosomal protein L29 in the known S10-spc operons. The similarity between the N-terminal domain of P46 and the L29 ribosomal protein family and the presence of a 46-kDa IRS-binding protein in S. citri ribosomes indicated that P46 is the L29 ribosomal protein of S. citri. We suggest that P46 is a bifunctional protein with an L29 N-terminal domain and a C-terminal domain involved in IRS binding.

Spiroplasmas are wall-free bacteria belonging to the class Mollicutes, a group of microorganisms phylogenetically related to Gram-positive bacteria with low guanine + cytosine contents (1). Sequence analysis (70) of a 6.8-kbp DNA fragment (GenBank accession number AF012877) of the phytopathogenic mollicute Spiroplasma citri (2) made it possible to identify eight putative ORFs that encode ribosomal protein S2, elongation factor Ts, spiralin, 6-phosphofructokinase, pyruvate kinase, and three unidentified proteins (A, B, and X) (Fig. 1). Ribosomal protein S2 and the translational elongation factor Ts (EF-Ts), respectively, encoded by rpsB and tsf genes, are both components of the translational apparatus in prokaryotes. These genes are adjacent in Escherichia coli (3, 4) and Bacillus subtilis (5), whereas in the genome of the two mollicutes Mycoplasma genitalium (6) and Mycoplasma pneumoniae (7) they reside at different locations, and thus each of them may constitute monocistronic transcriptional units or be part of two different polycistronic operons.

The organization and relative orientation of rpsB and tsf of S. citri (Fig. 1) are analogous to those reported for E. coli (3, 4) and B. subtilis (5). In E. coli, rpsB and tsf form a single transcriptional unit, and an attenuation mechanism was proposed to explain the 1 to 3 ratio of EF-Ts to S2 (3). In B. subtilis, the absence of a rho-independent termination signal in the spacer region between rpsB and tsf, and between tsf and X, indicates that rpsB, tsf, and X might represent a single transcriptional unit. Transcriptional analyses of rpsB, tsf, and X genes of S. citri have recently revealed two different transcripts (70), one corresponding to the rpsB-tsf-x operon and the second to rpsB alone. These results suggested that a regulatory mechanism may act at the transcriptional level at the spacer region between rpsB and tsf. The only “regulatory” like sequence found in the rpsB-tsf region is an inverted repeat sequence at the 3’ end of rpsB (Fig. 1). This inverted repeat sequence is 20 bp long and represents two turns of helical DNA. It could be a binding site for a regulatory DNA-binding protein (8, 9).

In the study reported here, we have purified, by a combination of affinity and gel filtration chromatographies, a 46-kDa protein (P46) that displays preferential binding to a 30-bp double-stranded oligonucleotide containing the inverted repeat sequence. The gene coding for P46 has been cloned and sequenced. Sequence analysis has revealed significant similarities between the N-terminal part of P46 and the L29 eubacterial ribosomal protein family. Surprisingly, the S. citri protein is much larger than its eubacterial homologs, and the C-terminal domain of P46 shows significant similarities with the DNA-binding histone H1-like proteins found in some bacterial species. These results suggest a bifunctional role for P46. The protein could act as a ribosomal protein but also as a DNA-binding protein with a potential regulatory function.

EXPERIMENTAL PROCEDURES

Preparation of Crude Extracts—Crude extracts were prepared from S. citri strain R5A2 (ATCC 27556) cultivated in 5 to 10 liters of SP4 medium (10) at 32 °C. Cells from exponential growth phase were harvested by centrifugation at 17,000 × g for 40 min, washed with phosphate-buffered saline containing 70 g/liter sorbitol, and frozen at −20 °C overnight. Cells were then thawed on ice, resuspended in 10 to 30 ml of binding buffer BB (10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM

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‡ The abbreviations used are: kbp, kilobase pair(s); bp, base pair(s); FPLC, fast protein liquid chromatography; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; EF-Ts, elongation factor Ts.
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EDTA, 1 mM dithiothreitol, 12.5% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, and disrupted by sonication (5/10-s pulses, 50 watts, 0 °C, 1 min sonication, and 1 min on ice alternatively, 8 to 10 times with a VibraCell sonicator). Mixture was cleared by centrifugation at 12,000 × g for 20 min; the pellet was discarded, and the supernatant was used as source of isolated ribosomal proteins. Effect of preincubation with heparin column was assessed by SDS-PAGE separation of the proteins.

-90 °C for storage. The protein concentration of the supernatant, assayed by the Bradford procedure (11) using the Bio-Rad Protein Assay Kit with bovine serum albumin as a standard, was in the range of 1–10 mg/ml.

Southwestern Blot Analysis of Protein-DNA Interactions—The method for Southwestern analysis is a modification of the published "protein blotting" method (12). Crude or purified proteins were resolved by SDS-PAGE on 10% polyacrylamide gel using standard procedures (13). After electrophoresis, proteins were electroblotteded (14) to nitrocellulose membranes (C extra, Amersham Pharmacia Biotech) for 45 min at 8 watts in transfer buffer (25 mM Tris base, 190 mM glycine, and 15% methanol) using a semi-dry transfer apparatus (FastBlot Biometra). Membranes were dried 15 min at 37 °C and blocked for 60 min at 26 °C in buffer S (BB buffer containing 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, and 0.02% bovine serum albumin). Membranes were incubated in 10 ml of S buffer containing 10° cmap/ml end-labeled specific IRS probe. This probe is the synthetic double-stranded oligonucleotide (5′-GGTTTTGCTTTACCAAAGCAAAAAAGCTG-3′ and 3′-CGCTTTCTTTTCTTTTCTTTTCTCTTGCT-5′, containing the inverted repeat sequence [Fig. 1]). It was end-labeled with [γ-32P]ATP (ICN Biochemicals), purified on a Sephadex G-50 column (15), and used as a probe in Southern blot experiments (16) with S. citri restricted genomic DNA. A 3.5-kbp EcoRI DNA fragment was detected. The probe was then used to screen an S. citri genomic library, constructed in E. coli XL1 Blue by cloning EcoRI-restricted DNA fragments into plasmid pBS+ (Stragtenge Cloning Systems). One positive clone containing the 3.5-kbp EcoRI insert was selected. Insert was sequenced using the T7 sequencing kit (Amersham Pharmacia Biotech) together with pTAG SEQ 5′ and pTAG SEQ 3′ primers (R & D Systems). The insert was excised from the vector, separated on agarose gel, purified by the GeneClean Kit (Bio 101, La Jolla, CA), random primer-labeled with [α-32P]ATP (ICN Biochemicals), purified on a Sephadex G-50 column (15), and used as a probe in Southern blot experiments (16) with S. citri restricted genomic DNA. A 3.5-kbp EcoRI DNA fragment was detected. The probe was then used to screen an S. citri genomic library, constructed in E. coli XL1 Blue by cloning EcoRI-restricted DNA fragments into plasmid pBS+ (Stragtenge Cloning Systems). One positive clone containing the 3.5-kbp EcoRI insert was selected. Insert was sequenced using the T7 sequencing kit (Amersham Pharmacia Biotech) or the Thermo Sequence Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Sequence Analysis—Sequences were analyzed using the Wisconsin Package (version 9.0) of software programs from Genetics Computer Group (GCG, Madison, WI) (19). Potential ORFs were examined by codon bias analysis (20) from Sequid II software with the codon frequency table of S. citri based on previously sequenced genes (17). The proteins deduced from the ORFs were submitted for BLASTP (21) analysis at the National Library of Medicine, National Institutes of Health, Bethesda). Potentially homologous proteins were compared using GAP from GCG Package. The ProDom protein domain families data base (22, 23) was used for analysis of domain arrangement of predicted proteins computed with ProDom (24). Results

RESULTS

We describe here the purification, preliminary characterization, and identification of an S. citri protein that displays preferential binding affinity for a 30-bp oligonucleotide containing the 20-bp inverted repeat sequence present at the 3′-OH end of rpsB and preceding tuf (Fig. 1). The 30-bp sequence will be designated IRS.

Heparin-Agarose Affinity Chromatography—Crude extract of S. citri cells obtained by sonication in binding buffer BB, as described under "Experimental Procedures," was applied onto
a heparin-agarose affinity column. Bound proteins were eluted with a linear gradient of 0.05–1 M KCl. Protein concentration was monitored by absorbance at 280 nm (Fig. 2A). Aliquots of the eluted fractions were first analyzed by SDS-PAGE electrophoresis and Coomassie Blue staining (Fig. 2B). Detection of IRS-binding proteins was carried out by Southwestern blots (12, 27, 28). In short, proteins were resolved on SDS-PAGE, transferred on a nitrocellulose sheet, and incubated with radiolabeled IRS. After washing, IRS-binding proteins were detected by autoradiography. Southwestern analysis of eluted fractions detected a predominant IRS-binding protein of 46 kDa (P46) (Fig. 2C) eluted at approximately 0.37–0.42 M KCl as indicated in Fig. 2A. Two other minor IRS-binding protein species of approximately 25 and 38 kDa were detected in fractions 31–32 and 36, respectively. Part of the binding activity was lost after freezing/thawing cycles; therefore active fractions were used for further experiments immediately after preparation.

**Preferential Affinity of P46 for IRS**—To determine if the P46 protein displays some preferential affinity for the IRS sequence, we have used Southwestern competition assay. Aliquots of fraction F29 containing P46 protein eluted from the heparin column (Fig. 2) were resolved on three separate tracks (Fig. 3) by 10% SDS-PAGE and subjected to Southwestern analysis. Membranes were incubated with radiolabeled IRS (Fig. 3 lane A) or with radiolabeled IRS in the presence of cold competitors (Fig. 3, lanes B and C). Binding of radiolabeled IRS to P46 was not significantly reduced by a 200-fold molar excess of a non-labeled double-stranded oligonucleotide (Fig. 3, lane B), which does not contain the inverted repeat sequence, as compared with the IRS binding in the absence of competitor (Fig. 3, lane A). Competition by a 200-fold molar excess of non-labeled IRS reduced dramatically, or made undetectable, the binding of radiolabeled IRS (Fig. 3, lane C). These results strongly suggested that P46 exhibits preferential binding affinity for IRS.

**Gel Filtration Chromatography**—In a second step toward P46 purification, 200-μl aliquot of fraction F29 containing P46 protein retrieved from the heparin-agarose affinity column (Fig. 2) was loaded onto an FPLC Superose 12 column. Proteins were eluted at 0.2 ml/min with running buffer. Protein concentration was monitored by absorbance at 280 nm (Fig. 4A). Aliquots of eluted fractions were analyzed by SDS-PAGE, and proteins were stained with Coomassie Blue (Fig. 4B). IRS binding proteins were analyzed by Southwestern blots (Fig. 4C). Almost all of the P46 IRS binding activity was present in fractions 26–28, and, as judged by SDS-PAGE and Coomassie Blue staining, P46 was practically the only protein seen in these fractions. Comparison of the elution time of P46 from the Superose column with that of molecular mass markers including catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa), and trypsinogen (24 kDa) indicated that the P46 migration was consistent with an apparent molecular mass of 46 kDa.
within a ribosomal protein operon corresponding to the S10 and spc operons of E. coli (29–31), B. subtilis (32–34), Mycoplasma capricolum (35), M. genitalium (6), and M. pneumoniae (7, 36) (see Fig. 6).

No transcriptional signal sequences were found between the genes for ribosomal proteins S17 and L14, and therefore it appears that in S. citri, as in M. capricolum (35), M. genitalium (6), M. pneumoniae (36), and B. subtilis (34), operons S10 and spc are fused into one operon (Fig. 6), whereas in E. coli they are not (29, 37).

Two of the intergenic regions, between the genes for S3 and L16, P46 and S17, have overlapping translational stop/start codons, in which the third nucleotide of the stop codon TAA is the first nucleotide of the start codon ATG of the gene following. This kind of overlapping was also reported in E. coli (30, 31) and in M. capricolum (35) between the genes for L4 and L23, L16 and L29, and L29 and S17.

The gene coding for P46 is located within the S10-spc operon

Fig. 3. Effect of competitors on P46 affinity for IRS. 10 µl (4 µg) (tracks A–C) of P46 containing fraction 29 eluted from the heparin-agarose column (see Fig. 2) were resolved by 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. Tracks A–C were cut and treated separately. Control track A was directly incubated, for 1 h, with radiolabeled IRS. Tracks B and C were preincubated for 15 min before the addition of the radiolabeled IRS probe, with a 200-fold molar excess of an unlabeled double-stranded oligonucleotide competitor that does not contain the IRS (track B) or with a 200-fold molar excess of unlabeled IRS (track C) (see “Experimental Procedures”). After addition of the radiolabeled IRS to the hybridization mixtures containing the competitors, incubation was conducted as for the control track A. After washing, the membranes were subjected to autoradiography.

kDa), and ribonuclease A (13.7 kDa) indicated that P46 eluted at a position consistent with a molecular mass of approximately 180 kDa. This suggest that the native form of P46 is a homomultimer.

Identification of the Gene Coding Protein P46—To characterize further P46 we cloned its gene. First, P46 was partially sequenced. To that purpose an aliquot of fraction F27 containing P46 from the gel filtration purification step (Fig. 4) was resolved by 10% SDS-PAGE, and proteins were stained with amido black (16). The P46 band was excised from the gel and submitted to endoproteolysis with Endo Lys-C. The proteolytic products were separated by reversed phase high performance liquid chromatography and three peptides (P1, P2, and P3) were sequenced. None of these sequences exhibited homology to any known protein sequence available in the databases. Amino acid sequences of peptides P1 (KEYTYGTNWK) and P2 (KNSGENTAINVK) were used to design two pairs of degenerated primers in order to amplify by polymerase chain reaction (PCR) part of the gene of the P46 protein. An amplified DNA fragment of 700 bp was generated using the P462/P461c primer pair (see “Experimental Procedures”), cloned into pTAG vector, and sequenced. Sequence analysis of the cloned DNA fragment revealed that the amino acid sequence predicted from the amplified product contained the N-terminal sequence of the P1 peptide and the entire sequence of the P3 peptide (KIDLELK3), confirming that this amplified product was generated from the P46 gene.

Cloning of the full-length P46 gene was undertaken to gain further sequence information. A 3.5-kbp EcoRI S. citri DNA fragment was detected by Southern blot analysis of restricted genomic DNA using the 700-bp DNA fragment as a probe. Screening of an EcoRI S. citri genomic library with this probe yielded a clone containing a 3.5-kbp insert which was sequenced. Sequence analysis (details are described under “Experimental Procedures”) of the 3.5-kbp insert gave the following results.

Authenticity of the insert was confirmed by perfect matches with the sequence of the three peptides (P1, P2, and P3) derived from purified P46 (Fig. 5).

The molecular mass deduced from the ORF of the cloned P46 gene was 37 kDa, whereas its apparent molecular mass in SDS-PAGE was 46 kDa.

Sequence similarities between deduced amino acid sequences of the insert and protein data bases (see Table I) showed that the gene coding for the P46 protein was located

Fig. 4. Purification of P46 by gel filtration chromatography. A, elution profile of proteins from the gel filtration chromatography of P46 containing fraction F29. 200 µl of fraction F29 eluted from the heparin-agarose column (Fig. 2) were loaded onto the pre-equilibrated Superose 12 column (see “Experimental Procedures”). The proteins were eluted from the column at 0.2 ml/min, and eluted proteins were monitored by absorbance at 280 nm (A280). The hatched area represents the P46 containing fractions. Ca, A, B, O, Ch, and R indicate the positions of the molecular mass markers catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa), respectively. B, SDS-PAGE analysis of fractions eluted from the Superose 12 column. 100-µl aliquots of eluted fractions from the Superose 12 column were resolved by 10% SDS-PAGE, and proteins were detected by Coomassie Blue staining. C, Southwestern analysis of the fractions eluted from the Superose 12 column. 100-µl aliquots of eluted fractions from the Superose 12 column were assayed for IRS binding activity using Southwestern analysis.
between the rplP and rpsQ genes that code, respectively, for ribosomal proteins L16 and S17. As shown in Fig. 6, comparison with similar operons in *E. coli*, *B. subtilis*, and several mollicutes revealed that P46 gene is at the position of ribosomal protein L29. Only the N-terminal sequence of P46 shares similarities with the sequences of L29 ribosomal proteins in protein data bases. Domain analysis of P46 using the Prodom protein domain families data base (22, 23) revealed a unique feature of this protein among other known L29 proteins. P46, which is much larger than other L29 proteins, could be arranged into three domains (Fig. 7A). The first domain (domain I, from position 1–57 of the P46 sequence) matches the Prodom domain 1463 (Prodom 34.1) which is a common domain of the prokaryotic L29 family (Fig. 7B). The second domain (domain II, from position 58–137 of P46) matches the Prodom domain 24734 (Prodom 34.1) which is the C-terminal sequence of the *M. capricolum* L29 ribosomal protein (Fig. 7C). The third domain, ranging from position 138–339 on the P46 sequence shares no significant similarities with any Prodom domains. However, BlastP analysis of this domain revealed significant similarities with DNA-binding histone H1-like proteins. It exhibits 28.6% amino acid identity to *Pseudomonas aeruginosa* transcriptional regulatory protein AlgP (38) also termed algR3 (39), 31.3% amino acid identity to *Bordetella pertussis* histone H1 homolog BpH1 (40), and 26.3% amino acid identity to

![Fig. 5. Nucleotide and predicted amino acid sequence of P46. Nucleotide residues are numbered from 5' to 3' starting with the first residue of the ATG codon encoding the putative initiation methionine. The deduced amino acid sequence is displayed below the nucleotide sequence in one-letter code starting from the methionine. The three peptide sequences obtained from the purified P46 are highlighted by solid black boxes in the order P2, P3, and P1, respectively. The potential ribosome-binding site is underlined, and the stop codon is indicated by ***.

| Table I
| ORFs and putative proteins of the 3.5-kbp *S. citri* DNA insert, containing the P46 gene (ORF V) and homologous genes and proteins from eubacteria |

| ORF | Location on spiroplasmal DNA | Size of putative spiroplasmal protein (no. amino acids) | Homologous protein (origin) | Size (no. amino acids) | Identity | Corresponding gene | Accession number |
|-----|-----------------------------|------------------------------------------------------|-----------------------------|------------------------|---------|-------------------|-----------------|
| I   | 1<sup>a</sup> 58            | >19                                                  | Ribosomal protein S19 (*M. capricolum*) 88 89.5  | rpsS                   | p10132  |
| II  | 90 426                     | 112                                                 | Ribosomal protein L22 (*M. capricolum*) 111 62.2  | rplIV                  | p10139  |
| III | 448 1204                   | 252                                                 | Ribosomal protein L29 (*M. capricolum*) 138 40.6  | rpsC                   | p02353  |
| IV  | 1206 1617                  | 137                                                 | Ribosomal protein L16 (*M. capricolum*) 137 78.1  | rplP                   | p02415  |
| V   | 1823 2640                  | 339                                                 | Ribosomal protein S17 (*M. capricolum*) 85 75.3  | rpsQ                   | p10142  |
| VI  | 2642 2897                  | 85                                                  | Ribosomal protein L14 (*M. capricolum*) 102 72.4  | rplN                   | p10137  |
| VII | 2921 3287                  | 122                                                 | Ribosomal protein L24 (*M. capricolum*) 108 51.7  | rplX                   | p10141  |
| VIII| 3301 3475<sup>a</sup>      | >58                                                 | Ribosomal protein L24 (*M. capricolum*) 108 51.7  | rplX                   | p10141  |

<sup>a</sup> The genes are truncated.
Chlamydia trachomatis histone H1 homolog Hc2 (41). Histone H1-like proteins exhibit sequence similarities to eucaryotic histones H1 and are involved in bacterial nucleoid organization and/or transcriptional regulation through their interactions with bacterial DNA. These observations suggested that the C-terminal domain (domain III, Fig. 7A) of P46 is implicated in DNA-protein interaction.

Localization of P46—The above results suggested that P46 is the L29 ribosomal protein of S. citri. To confirm this finding, we have analyzed S. citri ribosomal proteins. S. citri ribosomes were partially purified by differential ultracentrifugation (25, 26). Proteins from the supernatant (S-105) and the washed ribosomes were resolved on SDS-PAGE and examined by Southwestern analysis (Fig. 8). A 46-kDa IRS-binding protein was detected in both the S-105 supernatant (Fig. 8, lane 1) and in the washed ribosome fraction but in much larger quantities in the latter (Fig. 8, lane 2). These results indicated that P46 is present in the ribosomal fraction. Three other IRS-binding protein species of approximately 25, 26, and 38 kDa were detected in the ribosome fraction (Fig. 8, lane 2). These proteins are probably the DNA-binding proteins detected in crude extracts of spiroplasmal cells and in the eluted fractions of the heparin-agarose affinity column (Fig. 2C). The binding affinity of these proteins has not been investigated.

DISCUSSION

Despite the large amount of work on the regulation of ribosome synthesis (see Ref. 42 for a review), molecular mechanisms of regulation of some ribosomal protein operons have not been elucidated completely. This is the case of the E. coli rpsB-tsf operon for which an attenuation mechanism was proposed but has not been demonstrated (3). In S. citri, rpsB, tsf, and x represent the three genes of a single transcriptional unit, and a potential regulatory sequence (a 20-bp inverted repeat sequence) was found at the 3’ end of the rpsB gene (70). In the study reported in this paper we have detected, by Southwestern blot analysis, a protein (P46) that binds with preferential affinity to a 30-bp oligonucleotide (IRS) containing the 20-bp inverted repeat sequence. We have partially purified the P46 protein, cloned, and sequenced its gene.

The deduced amino acid sequence from the cloned gene indicated that the N-terminal end of P46 shared significant similarity with the L29 ribosomal protein family. In addition, sequence analysis of the whole cloned DNA insert carrying the P46 gene indicated that the P46 gene is located within the S10-spc operon, and more precisely between the rpsL and rpsQ genes coding, respectively, for ribosomal proteins L16 and S17 (Fig. 6). Thus, the P46 gene is located at the position of the L29 gene in the previously described S10-spc operon and represents a ribosomal protein gene. Indeed, Southwestern analysis of S. citri ribosomal proteins revealed the presence of the 46-kDa IRS-binding protein (Fig. 8). These findings suggested that P46 is the L29 ribosomal protein of S. citri.

The native form of P46 has an apparent molecular mass of approximately 180 kDa, as estimated by gel filtration chromatography. This represent about four times the value of 46 kDa determined by SDS-PAGE and five to six times the theoretical molecular weight of P46 (36,559). Hence, the native form of P46 should be homomultimeric. The multimeric form of P46 is seen as that existing out of the ribosome (extraribosomal form) but not within the ribosome. Indeed, the only multi-copy ribosomal protein found in eubacterial ribosomes is the L7/L12 ribosomal protein with four copies per ribosome (43, 44). The extraribosomal form of L7/L12 is a stable dimer (45–47). We have no experimental proof that P46 binds the IRS as a multimer; as in our Southwestern analyses, samples containing P46 were submitted to an SDS denaturing step before electroblotting. However, it is known that renaturation to the native conformation during electrotransfer of the protein from the gel to the cellulose membrane does occur (27).

The apparent molecular mass of P46 observed in SDS-PAGE (46 kDa) is larger than that calculated from the amino acid sequence (36,559 Da). Abnormal electrophoretic migration in SDS-PAGE has been described for other ribosomal proteins for which the apparent molecular mass in SDS gels is up to 30% higher than that calculated from the primary sequence (48). The molecular mass of P46 (apparent or calculated) is larger than that of all other eubacterial L29 proteins. P46 could be divided into three domains. The N-terminal domain of P46 (domain I) showed significant similarity with the L29 ribosomal protein family (60% identity with the N-terminal domain of M. capricolum L29 ribosomal protein) (Fig. 7, A and B). The internal domain of P46 (domain II) matched the C-terminal domain of M. capricolum L29 ribosomal protein (Fig. 7, A and C). The C-terminal domain of P46 (domain III) (Fig. 7A) shared significant similarities with the histone H1-like proteins found in some bacterial species such as Hc2 from C. trachomatis (41) and AlgP (algR3) (38, 39) from P. aeruginosa. The C. trachomatis Hc2 histone H1-like protein, initially identified by Southwestern blotting of chlamydial lysates (49), has been implicated in DNA binding, nucleoid compaction, and in vitro transcription/translation repression (41, 50, 51). The AlgP (algR3) histone H1-like protein is a DNA-binding protein involved in the transcriptional activation of algD, a necessary step for the establishment of mucoidy in P. aeruginosa (38, 39, 52). Repeated tetrapeptides like KPAA and variants are found in eukaryotic H1 histones and AlgP. Such repeated sequences appear to be crucial for DNA binding by AlgP (38). Repeats of such KPAA motifs and variants are also found, to a lower extent, within the C-terminal domain of P46. With these observations, it is tempting to associate the third domain of P46 with the IRS-binding property of the protein. We have made some preliminary experiments in order to confirm this hypothesis. The presence of a unique asparaginyl-glycyl peptide bond between position 138 and 139 of P46, i.e. between domain II and III (Fig. 7A), should allow cleavage of P46 at this position with hydroxylamine (53–57). This cleavage should generate an 138-amino acid peptide (domains I and II) and a 201-amino acid peptide (domain III). Keeping in mind the altered electrophoretic mobility of the P46, the 201-amino acid peptide should behave on SDS-PAGE as a polypeptide of apparent molecular mass of 28 kDa. Southwestern analysis of peptides generated by the action of hydroxylamine on P46 has indeed revealed only one IRS-binding peptide of approximately 29 kDa.3 Altogether,

3 L. Le Dantec, C. Saillard, and J. M. Bouvé, unpublished results.
FIG. 7. Comparison of P46 with several eubacterial ribosomal proteins L29. A, a schematic representation of primary structure and potential domain organization of P46 in comparison with those of several eubacterial ribosomal protein L29s. Numbers in parentheses refer to the length, in amino acid residues, of the protein. Scale at the bottom is in amino acids. I, II, and III represent the potential N-terminal, internal, and C-terminal domains of P46, respectively. Boxes with similar internal motifs represent the same domain in the Protod protein domain families data base. B, alignment of amino acid sequence of domain I of P46 with that of five L29 ribosomal proteins from several eubacterial species. Numbers in parentheses refer to the number of residues preceding the first residue shown in the alignment. Conserved residues that are present in all or most of these proteins are indicated by the black areas. Hyphens represent gaps introduced for maximal alignment. C, alignment of amino acid sequence of domain II of P46 with that of M. capricolum ribosomal protein L29.

these data support the role of the C-terminal domain of P46 in DNA-protein interaction. Therefore P46 could be a bifunctional protein. The N-terminal domain has a ribosomal function as it has high homology with the L29 ribosomal protein family. The C-terminal domain is seen as the one involved in IRS binding and might have regulatory function at the IRS of the genomic DNA. The bifunctional nature and the presence of DNA binding motifs in some ribosomal proteins has been reported and has led to speculations on the origin of the ribosomal proteins (58–64). It is interesting to note that in B. subtilis the HPB12-L24 protein has been described as a bifunctional ribosomal protein (L24) with histone-like properties and DNA-binding activity (63, 65, 66).

Mollicutes are the smallest and simplest self-replicating organisms, and the currently dominating hypothesis is that they have evolved by degenerative (regressive) evolution from Gram-positive bacteria with low guanine + cytosine genomes (1, 67, 68). In term of evolution strategy, mollicutes may have concentrated two different functions in a single gene during genome size reduction. Evidence for a single gene affording dual enzymatic function (malate/lactate dehydrogenase) has been described in M. genitalium by Cordwell and co-workers (69). Similarly, analysis of the complete sequence of the M. genitalium genome led Fraser and co-workers (6) to state that some M. genitalium proteins may have become adapted to perform more than one function. P46 has most likely a ribosomal protein function by its N-terminal L29 domain and a putative regulatory function on the IRS of the rpsB/tsf/tsx operon by its C-terminal domain. S2 and EF-Ts are components of the translational machinery, and P46 may play an interconnecting role in the coordinated regulation of the components of the translational apparatus. However, the mechanism by which the binding of P46 on the IRS could influence the transcription of the rpsB/tsf/tsx operon is still unknown. In vitro studies of the transcriptional regulation of the rpsB/tsf/tsx operon with and without the presence of the P46 protein may help understand the extraribosomal function of P46.

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