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Gene Organization and Transcriptional Analysis of the *Spiroplasma citri rps*B/*tsf*/*x* Operon

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Abstract. The nucleotide sequence of a 6863-bp *Spiroplasma citri* DNA fragment comprising the spiralin gene was determined. Sequence analysis revealed eight putative ORFs that encode ribosomal protein S2, elongation factor Ts, spiralin, 6-phosphofructokinase, pyruvate kinase, and three unidentified proteins (A, B, and X). The gene organization reported here is different from that previously published. Northern blot analysis of *rps*B, *tsf*, and *x* transcripts indicates that these genes are organized into a single transcriptional unit (operon). However, the detection of an additional transcript corresponding to the *rps*B gene alone suggests that a transcriptional mechanism should occur in the 3′ region of the *rps*B gene, allowing a conditional transcription termination.

Ribosomal protein S2 and the translational elongation factor Ts (Ef-Ts), respectively encoded by *rps*B and *tsf* genes, are both components of the translational apparatus in prokaryotes. These genes are adjacent and co-transcribed in *Escherichia coli* [2, 3] and *Bacillus subtilis* [9], whereas in the genome of the two mollicutes *Mycoplasma genitalium* [7] and *Mycoplasma pneumoniae* [8] they reside at different locations, and thus each of them may constitute monocistronic transcriptional units or be part of two different polycistronic operons.

Sequence analysis of a 6.8-kbp DNA fragment of the phytopathogenic mollicute *S. citri* [11] 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). In *S. citri rps*B and *tsf* are adjacent, and the lack of rho independent transcriptional termination signal between *rps*B and *tsf*, and between *tsf* and *x*, suggests that *rps*B, *tsf*, and *x* represent a single transcriptional unit. We report here the transcriptional analysis of the *rps*B, *tsf*, and *x* genes, which confirmed the above statement and revealed a particular feature concerning the *rps*B transcription.

It must be noted that the gene organization deduced from sequence analysis of the *S. citri* 6.8-kbp DNA fragment, which was originally selected [10] for its ability to express in *E. coli* the major *S. citri* membrane protein [17], i.e., the spiralin, does not agree with that previously deduced from the analysis of a subclone [5]. We report the corrected gene organization, along with a Southern verification on the *S. citri* chromosome.

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Materials and Methods

DNA manipulation and sequencing. Plasmid pES1 was digested by *Eco*RI, and the restricted DNA fragments were ligated into pBS$^+$ plasmid (Stratagene Cloning Systems, La Jolla, CA). Ligation products were used to transform *E. coli* XL1 Blue strain (Stratagene) by electroporation. Two recombinant plasmids, named pBES10 and pBES11, contained, as expected (see pES1 Fig. 1 and Fig. 2B), DNA fragments of 1.7 kbp and 1.55 kbp, respectively. The sequences of these two inserts were determined with the T7 sequencing kit (Pharmacia Biotechnology Inc.) as well as the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Corp.). Sequence of the *rps*B gene was obtained by sequencing parts of the pES1 insert from relevant primers. Restricted DNA was analyzed by Southern analysis as previously described [13].

Sequence analysis. Sequences were analyzed with the Wisconsin Package (version 9.0) of software programs from Genetics Computer Group (GCG, Madison, WI) [6]. Potential ORFs were examined by codon bias analysis [14] from Sequaid II software with the codon frequency table of *S. citri* based on previously sequenced genes. The proteins deduced from the ORFs were submitted for BLASTP [1]

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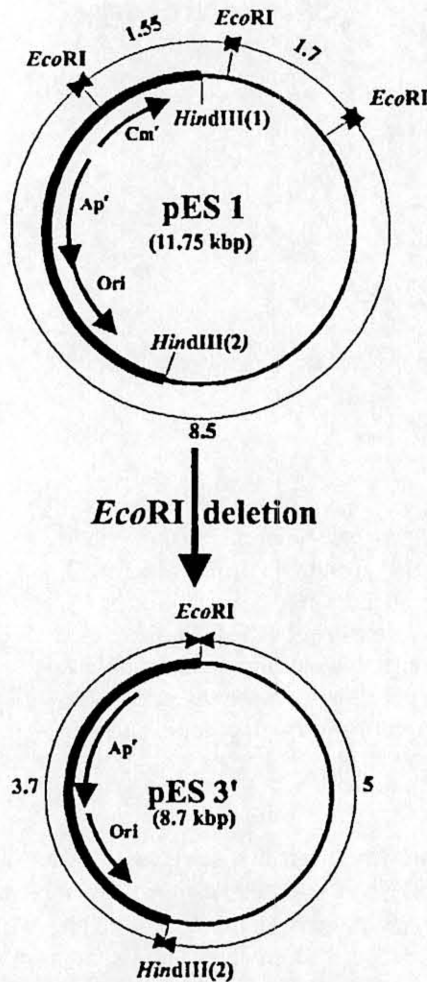

Fig. 1. Restriction map of recombinant plasmid pES1 and pES3'. The heavy line represents the pBR328 vector. The digestion fragments are designated by their size in kilobase pairs. *Hin*dIII(1) and *Hin*dIII(2): *Hin*dIII borders of pBR328 in pES1 and pES3'.

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Northern blot analysis. Total *S. citri* RNA was extracted from *S. citri* cells in accordance with the supplier's specifications (Rneasy Kit, Quiagen), separated through a 1.2% (wt/vol) denaturing agarose gel, and transferred to nitrocellulose C-extra membranes (Amersham). The protocols of Sambrook et al. [12] were followed for the hybridization analysis of *S. citri* RNA. Radiolabeled probes were hybridized with the membranes for 16 h at 37°C with 100 μg/ml of denatured salmon sperm DNA. The membranes were washed four times for 15 min at 37°C with 2 × SSC containing 0.5% (wt/vol) SDS, and two times for 30 min at 60°C with 0.1 × SSC containing 0.1% (wt/vol) SDS.

Nucleotide sequence accession number. The DNA sequence data reported here have been submitted to the GenBank database and have been assigned the accession number AF012877. The deduced amino acid sequences of the ORFs described herein are also available under this accession number.

Results and Discussion

DNA sequence analysis. The complete nucleotide sequence of a 6.8-kbp *S. citri* DNA segment was determined by sequencing the insert of the recombinant plasmid pES1. This plasmid, selected from a library of cloned genomic DNA sequences from *S. citri* strain R8A2 constructed in *E. coli* [10], was subcloned into plasmid pES3' by *Eco*RI digestion (Fig. 1) [5].

Results of the pES1 sequence analysis are shown in Table 1 and Fig 2B. The first potential ORF (ORF a) is truncated at its 5' end and codes for a polypeptide without significant similarity with any proteins in the databases. The second potential ORF (ORF b) encodes a putative protein of 258 residues with no significant similarities with proteins in the databases. The third ORF, ORF I (*rps*B), encodes a protein of 359 amino acids, which is highly similar to bacterial ribosomal protein S2. This mycoplasmal protein with 359 amino acids is much larger than its eubacterial counterparts (about 250 amino acids), and could have extraribosomal functions [4, 15, 16]. Analysis of ORF II (*tsf*), III (*x*), IV (*spiralin*), V (*pfk*), and VI (*pyk*) has been described previously [5]. The sequence analysis of the 6.8-kbp insert of pES1 (Fig. 2B) shows that the gene organization of pES1 does not agree with that previously described for the pES3'subclone (Fig. 2A):

(i) the last 333 nucleotides of the presumed end of the *pyk* gene on the pES3' insert (Fig. 2A: thick black line) are located on the opposite end on the pES1 insert (Fig. 2B: thick black line). Deduced amino acid sequences of *pyk* genes of pES1 and pES3' were submitted for BLASTP analysis. The most similar protein is the pyruvate kinase of *Bacillus psychrophilus* (acc. number p51182). Alignments showed a greater similarity between pyruvate kinases from pES1 and *B. psychrophilus* (48.25% identity) than between those from pES3' and *B. psychrophilus* (29.28% identity). This strongly suggests that the real sequence of the *S. citri pyk* gene is that found on pES1 insert rather than that found on pES3' insert.

(ii) the *rps*B and *tsf* genes are transcribed in the same direction on the pES1' insert while they were divergently transcribed on the pES3' insert. The organization of *rps*B, *tsf*, and *x* in pES1 and in *S. citri* genomic DNA was found to be identical, as shown by Southern blot analysis (Fig. 3). Positions of probes A, B, and C from the pES3' insert are shown in Fig. 2A. Only hybridization with probe A revealed a 1.7-kbp *Eco*RI fragment with both pES1 (Fig. 3A, lane 1) and genomic DNA (Fig. 3A, lane 2), as expected if organization of *rps*B, *tsf*, and *x* in pES1 and in *S. citri* genomic DNA is identical (Fig. 2B and 2C). Also, as expected, hybridization with probes B and C (Fig. 3, B and C) revealed no differences between pES1 and genomic DNA.

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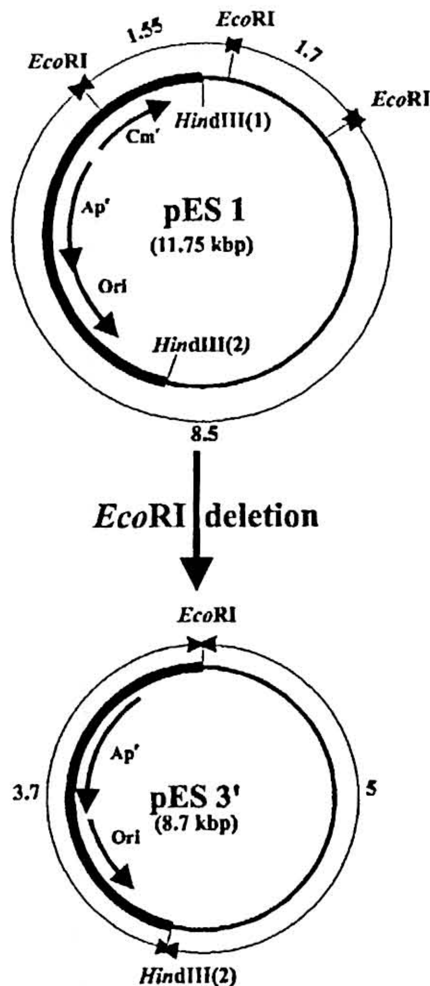

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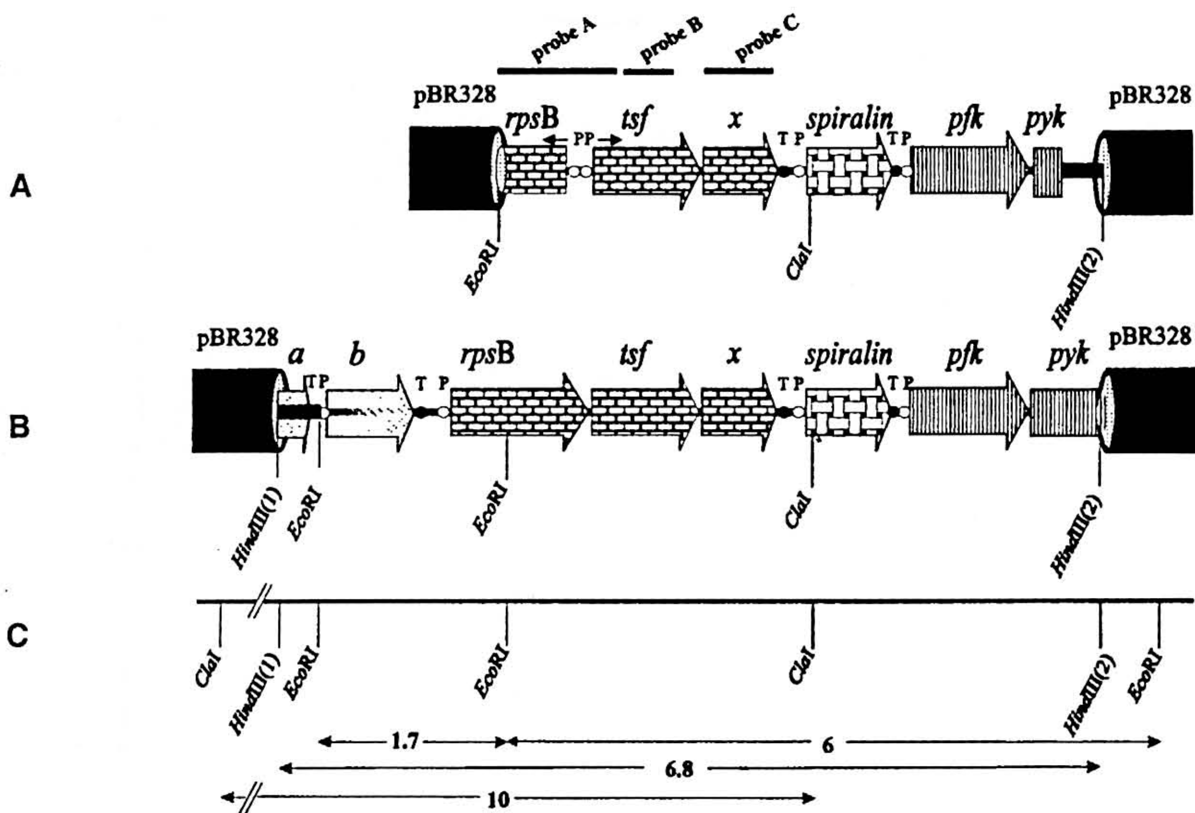

Fig. 2. (A) Organization of *S. citri* genes on the DNA insert of plasmid pES3′ and positions of the probes A, B, and C used in Southern hybridization. (B) Organization of *S. citri* genes on the DNA insert of plasmid pES1. (C) Restriction map of the chromosomal DNA of *S. citri* in the spiralin gene region. Possible promoters (P) and terminators (T) are represented by open and solid circles respectively. Sizes are indicated in kilobase pairs.

Table 1. ORFs and putative proteins of *Spiroplasma citri* DNA insert of plasmid pES1 and homologous genes and proteins from eubacteria

ORF	Location on spiroplasmal DNA: First nucleotide	Location on spiroplasmal DNA: Last nucleotide	Size of putative spiroplasmal protein (no. of amino acids)	Homologous protein (origin)	Size (no. of amino acids)	% Identity	Corresponding gene	Accession number
a	1[a]	177	>58					
b	270	1044	258					
I	1530	2607	359	Ribosomal protein S2 (*Pediococcus acidilactici*)	261	47.1	*rps*B	p49668
II	2679	3567	296	Elongation factor Ts (*Mycoplasma genitalium*)	298	40.0	*tsf*	p47246
III	3601	4234	211					
IV	4479	5202	241					
V	5355	6336	327	PFK (*Haemophilus influenzae*)	321	47.8	*pfk*	p43863
VI	6392	6863[b]	>157	PYK (*Bacillus psychrophilus*)	586	48.3	*pyk*	p51182

[a] 5′ end of ORF a is truncated.
[b] 3′ end of ORF VI is truncated.

Northern blot analysis. The organization and relative orientation of *rps*B and *tsf* on pES1 is analogous to that reported for *E. coli* [2, 3] and *B. subtilis* [9]. In *E. coli*, *rps*B and *tsf* genes form a single transcriptional unit, and an attenuation mechanism is suspected to explain the Ef-Ts/ribosomal protein S2 ratio of 1/3 [2]. In *B. subtilis* a potential terminator is found between *rps*B and *tsf*.

Northern blot analysis of *S. citri* RNA with probes

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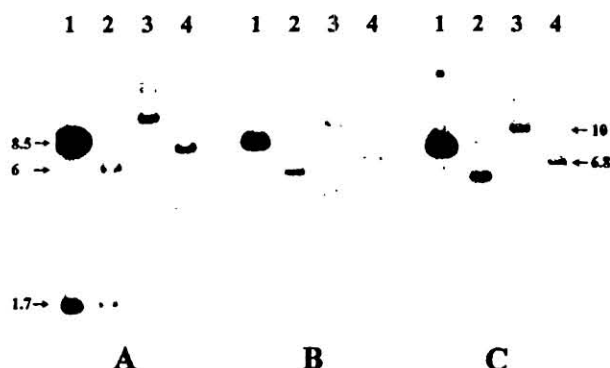

Fig. 3. Southern hybridization analysis of pES1 (lane 1) and *S. citri* genomic DNA (lanes 2, 3, 4) with probes A (panel A), B (panel B), and C (panel C) from pES3′ insert (Fig. 2A). Lanes 1 and 2, DNA restricted with *Eco*RI; lane 3, DNA restricted with *Cla*I; lane 4, DNA restricted with *Hin*dIII. Sizes are indicated in kilobase pairs.

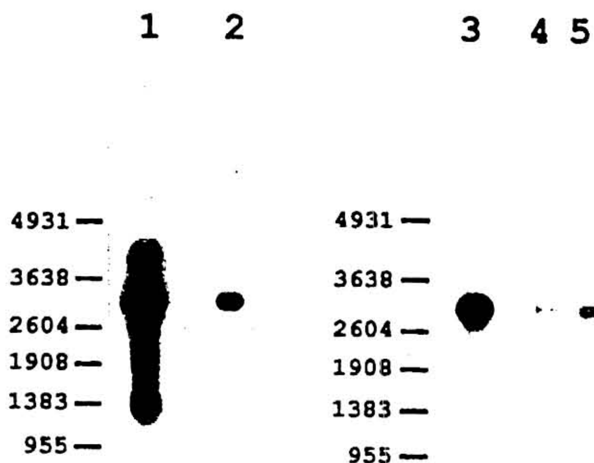

Fig. 4. Northern hybridization of *rps*B, *tsf*, and *x* transcripts. Hybridization was performed with probes specific for *rps*B (lane 1), *tsf* (lanes 2 and 3), and *x* (lanes 4 and 5). Equal quantities (3 μg) of *S. citri* RNA were analyzed by electrophoresis on two denaturing gels (lanes 1 and 2 on one gel; lanes 3, 4, and 5 on a second gel). RNA markers (Promega) were used as molecular mass markers. Sizes are indicated in nucleotides.

specific for *rps*B, *tsf*, and *x* genes gave a signal whose size, about 2.9 kb (Fig. 4), is consistent with the predicted length of the transcript comprising the three genes. The presence of a promoter-like sequence upstream of *rps*B gene and the presence of a terminator-like sequence at the end of the *x* gene, along with the absence of a rho-independent termination signal in the spacer region between *rps*B and *tsf*, and *tsf* and *x*, agree with this result. This indicates that *rps*B, *tsf* and *x* constitute a single transcriptional unit in *S. citri*, whereas in *M. genitalium* [7] and *M. pneumoniae* [8], *rps*B and *tsf* genes reside at different locations of the genome. However, the hybridization with the *rps*B probe alone (Fig. 4, lane 1) revealed an additional transcript of about 1.3 kb. This size is consistent with the predicted length of *rps*B transcript alone.

The significance of the weaker signal (about 4 kb) detected in Northern blot with the three probes is actually unclear.

These results indicate that a regulatory mechanism may act at the transcriptional level at the spacer region between *rps*B and *tsf* in *S. citri*. The only "regulatory" sequence found in this region was a 20-bp inverted repeat sequence at the 3′ end of the *rps*B gene. This sequence could be a binding site for a regulatory DNA-binding protein. Results of experiments confirming this hypothesis will form the basis of a subsequent report. However, the mechanism by which this sequence could influence the premature termination of transcription at the end of *rps*B is still unknown.

ACKNOWLEDGMENTS

Support for L. Le Dantec was provided by the Ministère de l'Education Nationale, de L'Enseignement Supérieur et de la Recherche. We thank S. Jarach for technical assistance in the Northern blot hybridization experiments.

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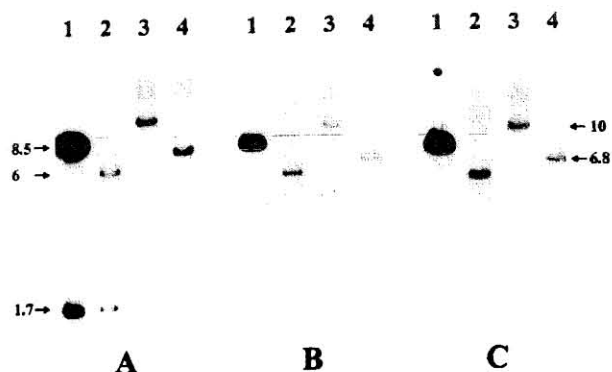

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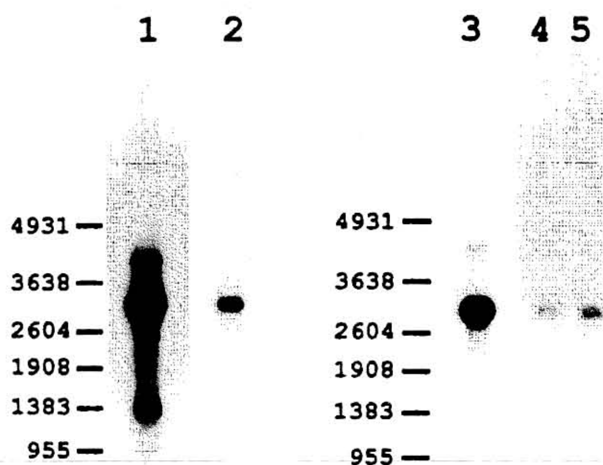

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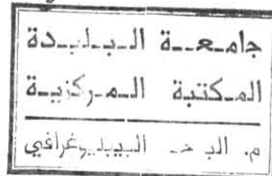

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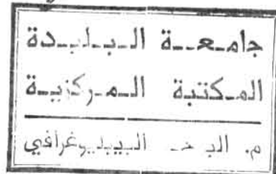

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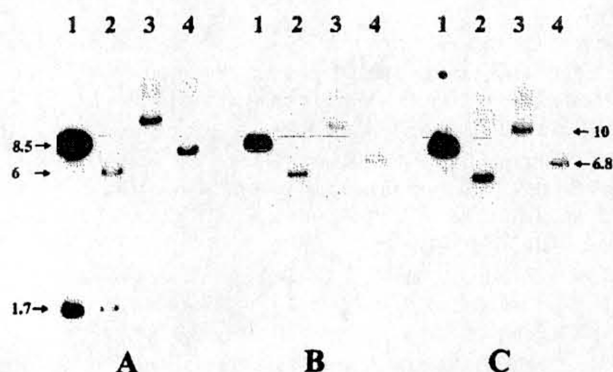

Fig. 3. Southern hybridization analysis of pES1 (lane 1) and *S. citri* genomic DNA (lanes 2, 3, 4) with probes A (panel A), B (panel B), and C (panel C) from pES3′ insert (Fig. 2A). Lanes 1 and 2, DNA restricted with *Eco*RI; lane 3, DNA restricted with *Cla*I; lane 4, DNA restricted with *Hind*III. Sizes are indicated in kilobase pairs.

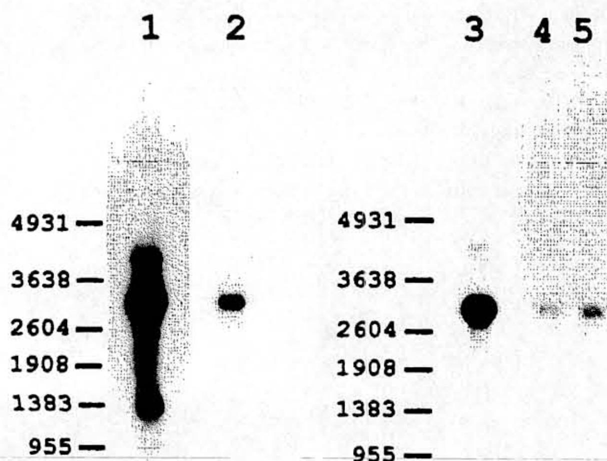

Fig. 4. Northern hybridization of *rps*B, *tsf*, and *x* transcripts. Hybridization was performed with probes specific for *rps*B (lane 1), *tsf* (lanes 2 and 3), and *x* (lanes 4 and 5). Equal quantities (3 μg) of *S. citri* RNA were analyzed by electrophoresis on two denaturing gels (lanes 1 and 2 on one gel; lanes 3, 4, and 5 on a second gel). RNA markers (Promega) were used as molecular mass markers. Sizes are indicated in nucleotides.

specific for *rps*B, *tsf*, and *x* genes gave a signal whose size, about 2.9 kb (Fig. 4), is consistent with the predicted length of the transcript comprising the three genes. The presence of a promoter-like sequence upstream of *rps*B gene and the presence of a terminator-like sequence at the end of the *x* gene, along with the absence of a rho-independent termination signal in the spacer region between *rps*B and *tsf*, and *tsf* and *x*, agree with this result. This indicates that *rps*B, *tsf* and *x* constitute a single transcriptional unit in *S. citri*, whereas in *M. genitalium* [7] and *M. pneumoniae* [8], *rps*B and *tsf* genes reside at different locations of the genome. However, the hybridization with the *rps*B probe alone (Fig. 4, lane 1) revealed an additional transcript of about 1.3 kb. This size is consistent with the predicted length of *rps*B transcript alone.

The significance of the weaker signal (about 4 kb) detected in Northern blot with the three probes is actually unclear.

These results indicate that a regulatory mechanism may act at the transcriptional level at the spacer region between *rps*B and *tsf* in *S. citri*. The only "regulatory" sequence found in this region was a 20-bp inverted repeat sequence at the 3′ end of the *rps*B gene. This sequence could be a binding site for a regulatory DNA-binding protein. Results of experiments confirming this hypothesis will form the basis of a subsequent report. However, the mechanism by which this sequence could influence the premature termination of transcription at the end of *rps*B is still unknown.

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