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# Cloning and Characterization of the Ribosomal Protein Genes in the *spc* Operon of a Prokaryotic Endosymbiont of the Pea Aphid, *Acyrthosiphon kondoi*

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**Abstract**

To correlate a prokaryotic endosymbiont in the pea aphid, *Acyrthosiphon kondoi*, with the endosymbionts in related aphid species as well as with free-living bacteria and subcellular organelles, and to study the mode of its gene expression within aphid cells, we have cloned and characterized the genes encoding ribosomal proteins S3, L16, L29, S17, L14, L24, L5, S14, S8, L6, L18, S5, L30, L15 and secretion protein Y (Sec Y) from the S10 and *spc* ribosomal protein gene operons of this endosymbiont. The organization of these genes is identical to that in *Escherichia coli*, and their nucleotide sequences are highly similar (87% identity) to the corresponding *E. coli* genes. They are much less similar to the corresponding chloroplast and mitochondrial genes. The guanine plus cytosine G+C content of the genes of the *A. kondoi* endosymbiont is much higher than those of the endosymbionts in related aphid species reported so far. It appears either that the *A. kondoi* endosymbiont is derived from an ancestral bacterium different from those in other aphids or that its G+C content increased in a relatively short time after the evolutionary divergence of its host.

**Key words:** pea aphid; prokaryotic endosymbiont; nucleotide sequence; S10 and spc ribosomal protein operons; gene organization

## 1. Introduction

Almost all aphids are known to harbor one or two species of prokaryotic endosymbionts which are classified as primary and secondary endosymbionts. The former is thought to be present in almost all species of aphids,[1] while the latter has been found in only some aphid species such as the pea aphid, *Acyrthosiphon pisum*.[2,3] The primary symbiont is gram-negative and has a spherical to slightly oval shape. It resides in specialized cells, termed mycetocytes, within the body cavity of the aphid that are aggregated into an organ-like structure called mycetome.[3] The rod-shaped secondary symbiont in *A. pisum* has been reported to be associated with the sheaf cells surrounding the mycetocytes.

From the nucleotide sequence analysis of the 16S ribosomal RNA (rRNA) of the primary symbionts in eleven species of aphids including *A. pisum* and *Schizaphis graminum*, it has been suggested that the primary symbiont is a member of the gamma-3 subdivision of *Proteobacteria*, which includes such well-studied species as *Escherichia coli and Pseudomonas putida*. It was also indicated that the symbiont became associated with a common ancestor for the present four aphid families.[4–6] Thus, the primary symbionts in these aphid families have been considered to be of common origin, and hence were named *Buchnera aphidicola*.[1] The rod-shaped secondary symbiont in *A. pisum* has been reported to constitute a lineage different from that of the primary symbiont within the gamma-3 subdivision of the class of *Enterobacteriaceae*.[5,6]

The primary symbiont is transferred into a next generation of aphid at an early stage of embryogenesis without passing a free living stage outside the insect body. In fact, the primary symbiont can not be successfully cultivated outside the host. Conversely, an aposymbiotic aphid that has lost its endosymbiont as a result of treatment, for example, with prokaryote-specific antibiotics or heat is remarkably undersized and sterile.[7] It has been suggested that the role of the primary symbiont is to supply essential amino acids to the host insect to compensate for the imbalance of amino acid composition in phloem sap which the aphid feeds on.[8] The aphid and the primary symbiont are thus dependent on each other. For these reasons, the primary symbiont has been said to

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be at a stage of evolution from a free-living bacterium to a subcellular organelle.

Analysis of the genomes of chloroplasts and mitochondria has indicated that transfer of genes into the nucleus occurred during the evolutionary process from the ancestral symbiotic bacteria into the present organelles. For example, the analysis of the ribosomal protein gene clusters in chloroplasts [9–11] and plant mitochondria,[12] corresponding to the S10, *spc* and $\alpha$ operons of *E. coli*, indicated that their organization is highly conserved, but some genes have been deleted from the organellar genome and can be found in the nucleus. A most extreme case is the human mitochondrial genome that has no ribosomal protein gene at all.[13] The mitochondrial genome of the yeast, *Saccharomyces cerevisiae*, on the other hand, contains one ribosomal protein gene, which has a rather unusual amino acid sequence and is not similar to any known bacterial ribosomal proteins.[14] All other ribosomal proteins are encoded in the nuclear genome.[15]

For these reasons, we were interested to analyze the structure and function of the ribosomal protein genes of the primary endosymbionts in pea aphids through molecular characterization and comparison with the corresponding genes in free-living bacteria and subcellular organelles. We wanted to ask as to whether or not the primary endosymbionts in pea aphids can be regarded as being at an evolutionary stage from free-living bacteria to subcellular organelles. As an initial step of such studies, we chose *Acyrthosiphon kondoi* because of its relative easiness in cultivation, and characterized the *spc* operon of its primary endosymbiont as described below.

## 2. Materials and Methods

### *2.1. Insect materials*

A well-established parthenogenetic clone of the pea aphid, *A. kondoi* (strain Kurashiki), was obtained from Dr. Kawata and maintained on young broad bean, *Vicia faba* L., at 15°C in the daily cycle of 16 hr light and 8 hr darkness.

### *2.2. Isolation of endosymbionts*

Primary symbionts were isolated essentially as described by Harrison et al.[16] Pea aphids were first sterilized with 70% ethanol, washed with sterile water and homogenized in 6 volumes of ice-cold buffer A (35 mM Tris-HCl, pH 7.6, containing 0.25 M sucrose, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 5 mM phenylthiourea). The homogenate was passed through two layers of cheese cloth and centrifuged at 8,500 rpm for 20 min at 4°C in a Beckman JA-17 rotor. The pellet was re-suspended in the same buffer, layered over 2.45 M sucrose cushion in buffer A and centrifuged at 12,000 rpm for 30 sec at 4°C in a Tomy MRX-150 micro centrifuge. The supernatant was collected and centrifuged at 15,000 rpm for 2 min at 4°C. The pellet was re-suspended in buffer A and gently layered over a discontinuous Percoll density gradient, containing 9, 18, 27, and 45% Percoll. Percoll solutions were prepared by diluting Percoll with buffer A containing 1% bovine serum albumin, 5% polyethylene glycol 6000, 1% Ficoll, and 8.6% sucrose. The gradient was centrifuged at 2,500 rpm for 20 min at 4°C in a Sakuma R90-23 centrifuge. Endosymbionts concentrated at the boundary between 27% and 45% were recovered, suspended in 3 volumes of buffer A and centrifuged at 8,500 rpm for 5 min at 4°C in a Beckman JA-17 rotor. The resultant pellet was re-suspended in TE (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) and used as an endosymbiont preparation.

### *2.3. Preparation of the genomic DNA from aphid endosymbiont and* E. coli

Isolation of the genomic DNA from the primary endosymbiont preparation as well as from *E. coli* cells was performed essentially according to Saito and Miura.[17]

### *2.4. Preparation of the genomic DNA from whole insect*

Pea aphid was homogenized, filtrated and centrifuged as described above. The pellet was re-suspended in TES (10 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and 1 mM EDTA). 0.3 mg/ml of lysozyme was then added and the resultant solution was incubated at 37°C for 60 min. After the addition of 0.2 volume of 10% sodium dodecyl sulfate (SDS), the mixture was incubated at 37°C for a further 2 hr. Proteinase A was then added to a final concentration of 250 $\mu$g/ml. After an overnight incubation at 37°C, DNA was extracted as described above.

### *2.5. Southern hybridization analyses*

Genomic DNA was digested with one or two restriction enzymes and subjected to electrophoresis in a 0.8% agarose gel. The resultant fragments were transferred to a nylon membrane (Biodyne Transfer Membrane, Pall) and hybridized with a 1.75 kb-long *Eco*RI fragment derived from pSI17[18] that corresponded to a terminal region of the *E. coli spc* operon encoding three ribosomal proteins, S5 (partial), L30 and L15, and part of Sec Y protein. Hybridization was performed overnight at 42°C in 20 mM sodium phosphate buffer, pH 6.5, containing 50% formamide, 5 X SSC, 1 X Denhardt's solution, 100 $\mu$g/ml salmon sperm DNA, 0.2% SDS, and 10% sodium dextran sulfate. The membrane was washed three times with 2 X SSC containing 0.1% SDS for 5 min at room temperature and then once with 0.2 X SSC containing 0.1% SDS for 30 min at 42°C.

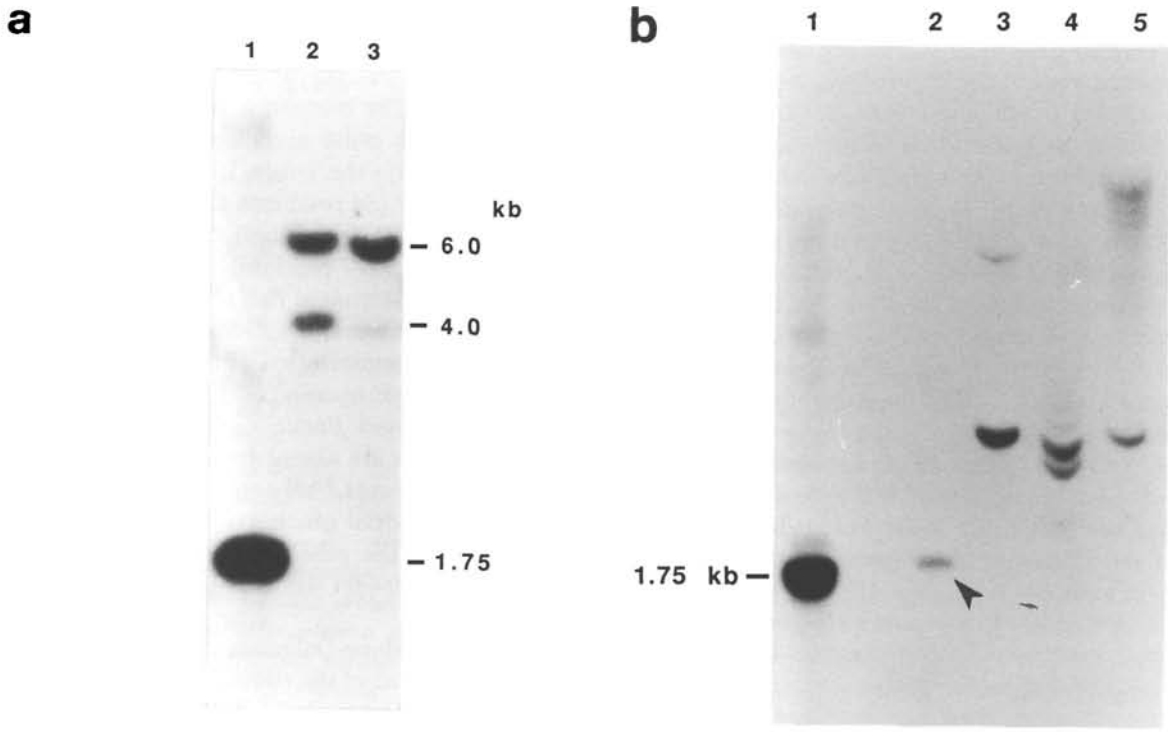



**Figure 1.** Genomic Southern hybridization analysis of the ribosomal protein genes of the *spc* operon. (a) 10 $\mu$g DNA from *E. coli* (lane 1), the whole insect (lane 2), and the primary symbiont of *A. kondoi* (lane 3) were digested with *Eco*RI and hybridized with an ($\alpha$-$^{32}$P)dATP-labelled *E. coli* DNA fragment corresponding to the 3′-terminal region of the *spc* operon. The positions of molecular size markers are indicated at right. (b) 10 $\mu$g DNA from *E. coli* (lane 1) and the primary symbiont (lanes 2 through 5) were digested with *Hin*dIII (lane 3), *Pst*I (lane 5), *Eco*RI and *Hin*dIII (lanes 1 and 2), or *Hin*dIII and *Pst*I (lane 4), and hybridized with the same probe used in (a). Arrowhead in (b) points to the 1.8 kb-long fragment that was cloned and analyzed in this work.

### *2.6. Cloning and sequencing of part of the* spc *operon*

A 1.8 kb-long *Eco*RI-*Hin*dIII fragment was first isolated from the genomic DNA of the primary endosymbiont of *A. kondoi*, by using a 1.75 kb-long DNA fragment of *E. coli* containing part of the *spc* operon genes as a probe in screening by Southern hybridization. A 6.7 kb-long *Eco*RI fragment containing the above-mentioned 1.8 kb *Eco*RI-*Hin*dIII fragment was subsequently cloned into λgt10 and various sub-fragments derived from it as well as from the 1.8 kb *Eco*RI-*Hin*dIII fragment were cloned into M13 phage vectors for nucleotide sequence determination. To introduce deletions for sequencing, a deletion kit from Takara Shuzo (Kyoto) was used. The nucleotide sequence was determined by the dideoxy method,[19] either manually or by using a Hitachi SQ3000 DNA sequencer. The sequence data obtained were stored and analyzed by using the computer programs DATBAS and NUCDAT.[20] The nucleotide sequence data obtained have been deposited to DDBJ under the accession number of D31786.

## 3. Results

### *3.1. Cloning of the spc operon*

To isolate the ribosomal protein genes of the primary symbiont in *A. kondoi*, we first performed genomic Southern hybridization analysis using various *E. coli* DNA fragments containing ribosomal protein genes as probes. When a fragment corresponding to the terminal region of the *spc* operon was used as a probe, a single band of the symbiont DNA digested with *Eco*RI was found to give a rather strong hybridization signal (Fig. 1a). The size of the fragment was approximately 6.5 kb in length. Upon double digestion of the symbiont DNA with *Eco*RI and *Hin*dIII, a fragment of 1.8 kb in length was found to hybridize with the same probe (Fig. 1b). This fragment was accordingly cloned into pUC119, named pEHS5109 and used in subsequent experiments.

To confirm that the DNA fragment in pEHS5109 was indeed derived from the primary symbiont in *A. kondoi*, we performed an additional genomic Southern hybridization analysis using the 1.8 kb-long DNA fragment cloned in pEHS5109 as a probe. An intense band of the same

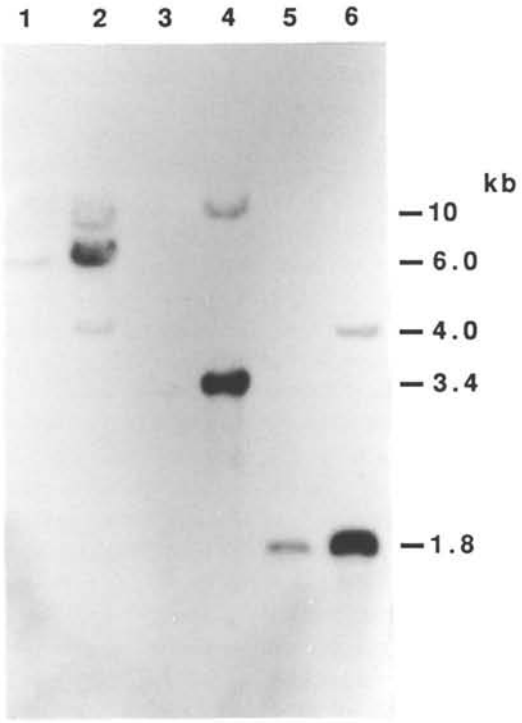



**Figure 2**. Genomic Southern hybridization analysis with the 1.8 kb-long fragment as a probe. 10 $\mu$g DNA from the whole insect (lanes 1, 3 and 5) and from the primary symbiont in *A. kondoi* (lanes 2, 4 and 6) were digested with *Eco*RI (lanes 1 and 2), *Hin*dIII (lanes 3 and 4) or *Eco*RI and *Hin*dIII (lanes 5 and 6), and hybridized with the [$\alpha$-$^{32}$P]dATP-labelled 1.8 kb-long fragment cloned into pEHS5109. The positions of molecular size markers are indicated at right.

size shown in Fig. 1 was detected (Fig. 2). Therefore, we concluded that the cloned DNA fragment was derived from the primary symbiont in *A. kondoi* and analyzed it further. The 6.5 kb-long *Eco*RI fragment was similarly cloned into $\lambda$gt10 by screening with the 1.8 kb-long *Eco*RI-*Hin*dIII fragment as a hybridization probe. The clone was named pES71. Various sub-fragments derived from the two clones, pEHS5109 and pES71, were used for nucleotide sequence determination experiments described below.

### 3.2. *Nucleotide sequence data and gene organization*

The aphid symbiont DNA fragments in clones pEHS5109 and pES71 were analyzed by various restriction enzymes (data not shown). The nucleotide sequence of their sub-fragments was accordingly determined as presented in Fig. 3. Search for sequence similarity was performed against the GenBank database (Release 81.0) which indicated that the sequence was 87% identical to the part of the *E. coli spc* operon as expected. We identified three ribosomal protein genes encoding a C-terminal half of S5 (corresponding to the C-terminal 40 amino acid residues), entire L30 (59 amino acid residues) and L15 (144 residues), and a large portion of Sec Y (356 residues) in the nucleotide sequence data of clone pEHS5109. The nucleotide sequence of additional ribosomal protein genes encoding the C-terminal half of S3 (205 residues), the entire L16 (136 residues), L29 (63 residues), S17 (84 residues), L14 (123 residues), L24 (104 residues), L5 (179 residues), S14 (101 residues), S8 (130 residues), L6 (177 residues), and L18 (117 residues), as well as the N-terminal half of S5 (126 residues) were determined with various sub-fragments derived from clone pES71. The organization of these genes was identical to that of the *spc* operons of *E. coli*[21] (Fig. 4), *Micrococcus luteus*[22] and *Bacillus subtilis*.[23] Interestingly, some of these genes are absent from the genome of the chloroplasts of liverwort,[9] tobacco[10] and rice[11] as well as from the mitochondrial genome of liverwort[12] as indicated. In this respect, the gene organization of the primary symbiont in *A. kondoi* seems is highly similar to free-living eubacteria.

Potential Shine-Dalgarno (SD) sequences were found in front of most of the ribosomal protein genes. The only exception is the gene for L30: there is a nucleotide sequence difference between the symbiont and *E. coli* genes at the end of the previous gene, i.e. the gene encoding S5, as shown in Fig. 3. Consequently, the *E. coli* gene is one amino acid residue longer than the corresponding symbiont gene and the SD sequence for the symbiont gene is likely to be UAA, the stop codon for the S5 gene. UAA is complementary to the 3′ end of the 16S rRNA in both *E. coli* and the primary symbiont in *A. pisum*[5,6] and considered to function as an SD sequence at least in *E. coli*. On the other hand, the SD sequence for the corresponding *E. coli* gene is GGA.

We then analyzed similarity between the deduced amino acid sequences for 14 ribosomal proteins as well as for SecY protein of the symbiont and those of *E. coli* and other bacteria using the PIR database. As shown in Table 1, the identity in the amino acid sequences compared was in the range of 80.1–97.5% between the *A. kondoi* symbiont and *E. coli*, and 33.7–69.1% between the symbiont and gram-positive bacteria. The result thus indicated that the primary symbiont in *A. kondoi* is closely related to *E. coli* as in the case of the endosymbionts in related aphids.

### 3.3. *G+C content and codon usage of the symbiont genes*

From the nucleotide sequence data, the G+C content of the genes in *A. kondoi* endosymbiont was found to be 50.4%. This is in a sharp contrast to those of the genes in *A. pisum* and *S. graminum* endosymbionts: in both

**Table 1.** Similarity of the amino acid sequences for ribosomal proteins and SecY between the primary symbiont of *A. kondoi* and other eubacteria[a]

| Protein | *E. coli* | *B. subtilis* | *B. stearo-thermophilus* | *M. capricolum* |
|---|---|---|---|---|
| **S3** | 98.1 | —[b] | — | 44.6 |
| **L16** | 80.1 | — | — | 48.5 |
| **L29** | 90.3 | 41.7 | 45.0 | 35.6 |
| **S17** | 94.0 | 51.9 | 53.3 | 54.4 |
| **L14** | 96.7 | 65.0 | 69.1 | 52.0 |
| **L24** | 93.2 | 45.0 | 45.0 | 35.6 |
| **L5** | 94.4 | 58.1 | 59.8 | 53.4 |
| **S14** | 91.4 | 55.3 | — | 39.3 |
| **S8** | 93.1 | — | — | 49.6 |
| **L6** | 83.0 | — | 50.0 | 42.8 |
| **L18** | 91.5 | — | 49.2 | 40.5 |
| **S5** | 94.5 | 51.2 | 52.4 | 43.1 |
| **L30** | 88.1 | 46.7 | 47.5 | — |
| **L15** | 87.5 | 45.5 | 47.2 | 45.8 |
| **secY** | 97.5 | 41.5 | — | 33.7 |

[a] The predicted amino sequence data presented in Fig. 3 was compared with that of *E. coli*,[21] *Bacillus subtilis*,[23,28] *Bacillus stearothermophilus*[29–33] and *Mycoplasma capricolum*.[34] Similarity is expressed in percent. [b] Not available.

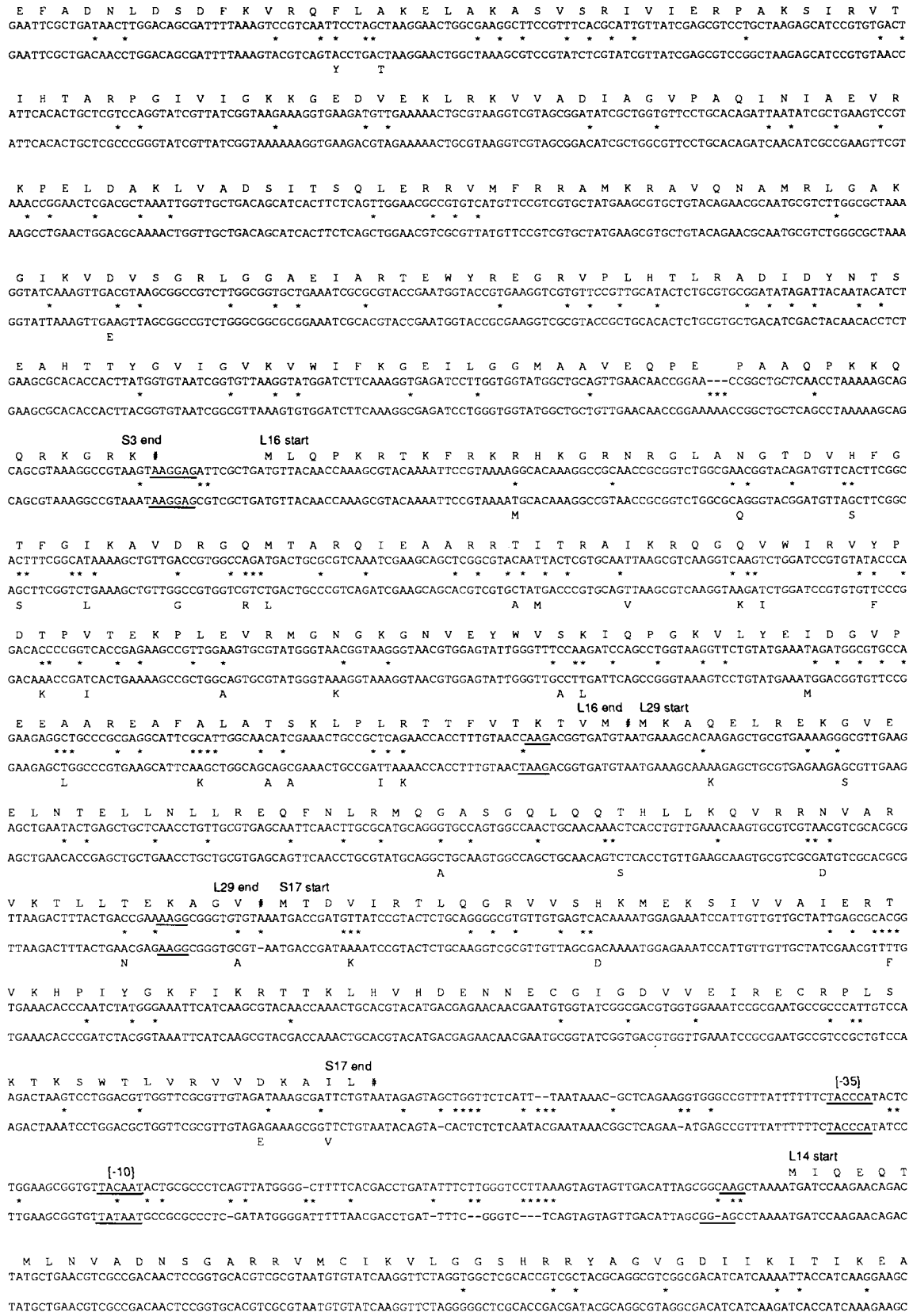
cases the G+C content was reported to be about 30%. Accordingly, we compared the codon usage pattern in those three endosymbionts, because lower G+C content was expected to be reflected by a preference for A or T at the third position, and to a lesser extent at the first position of each codon. The choice of synonymous codons in *A. pisum* and *S. graminum* endosymbionts is clearly different from that in *A. kondoi* endosymbiont: for example, the codon, UUC, for phenylalanine is preferred in the *A. kondoi* endosymbiont, whereas the other codon, UUU for the same amino acid is preferred in *A. pisum* and *S. graminum* endosymbionts. In many cases, the codon usage in the former two symbiont species is distinctly biased for A and T at the third position of each codon. The nucleotide sequence data presented here thus suggest that the primary endosymbiont in *A. kondoi* is different from those in two related aphid species mentioned above.

## 4. Discussion

In this work we have cloned and analyzed the nucleotide sequence of 14 ribosomal protein genes as well as the gene encoding *sec*Y from an endosymbiont of the aphid, *A. kondoi*. Earlier, Isikawa and his colleagues have reported that the endosymbiont of a related aphid, *A. pisum*, produced a single species of protein termed symbionin at the detectable level.[7] Subsequent analysis of this protein showed that it is a homolog of GroEL of *E. coli*.[25] The expression of this gene, therefore, requires the transcriptional and translational apparatus of the symbiont. To analyze the mechanism facilitating the predominant expression of the symbionin gene, we undertook the analysis of the translational apparatus of the endosymbiont of the aphid, *A. kondoi*. As a first step of such an analysis, we have cloned and characterized ribosomal protein genes as described above.

Comparison of the nucleotide sequence data for ribosomal protein genes between the aphid symbiont and *E. coli* revealed that they are highly similar to each other as shown in Fig. 3. Most of the differences between the two organisms occurred at the codons' third positions and are neutral. However, there are cases in which differences are observed at the codons' first positions, resulting in alterations in the amino acid sequences of the corresponding gene products. In addition, a few cases have been observed in which the two sequences are different in their length in such a way that the deduced amino acid sequence of the gene products are longer or shorter by one amino acid residue. For example, the S3 gene of the endosymbiont is shorter than the *E. coli* counterpart by one amino acid residue, because the former lacks one codon near its 3′-terminus (Fig. 3). In this connection, we think it quite likely that the published *E. coli* sequence data[21] contains an error at the 3'-end of the gene encoding S14. There is, among others, one nucleotide extra in the *E. coli* data in comparison with the symbiont data, resulting in the translational termination of the *E. coli* gene with a UAG (TAG) codon as shown in Fig. 3. This results in a frameshift which causes a different C-terminus of S14 proteins: while the *E. coli* protein ends with EIPGLKKG, the symbiont protein is two amino acid residues longer and ends with EVPGLKKASW. Moreover, the translational terminator of the symbiont gene is UAA (TAA) as in the case of all other ribosomal protein genes in this region. While we were performing comparative analysis of the ribosomal protein genes in this work, we noticed the presence of as many as twelve cases of discrepancy between the data in the GenBank nucleotide sequence database (and the corresponding amino acid sequences

**Figure 3.** Nucleotide sequence of the 6.7 kb-long fragment (6,400 bp are shown) containing part of the S10 and *spc* ribosomal protein gene operons. The upper nucleotide sequence indicates that of the *A. kondoi* symbiont determined in this work and the lower sequence represents the corresponding *E. coli* sequence taken from the GenBank nucleotide sequence database (release 80.0). The predicted amino acid sequence for the symbiont data is shown above the upper nucleotide sequence and the amino acid residues of the corresponding *E. coli* proteins that are different are indicated below the *E. coli* sequence.* indicates differences between the two organisms, and # translational terminators. Gaps are placed by inserting hyphens to obtain maximal matches between the two sequences. A putative promoter (denoted as [−35] and [−10]) for the symbiont *spc* operon and likely Shine-Dalgarno sequences are underlined. The sequence data have been deposited to DDBJ (accession number D31786).



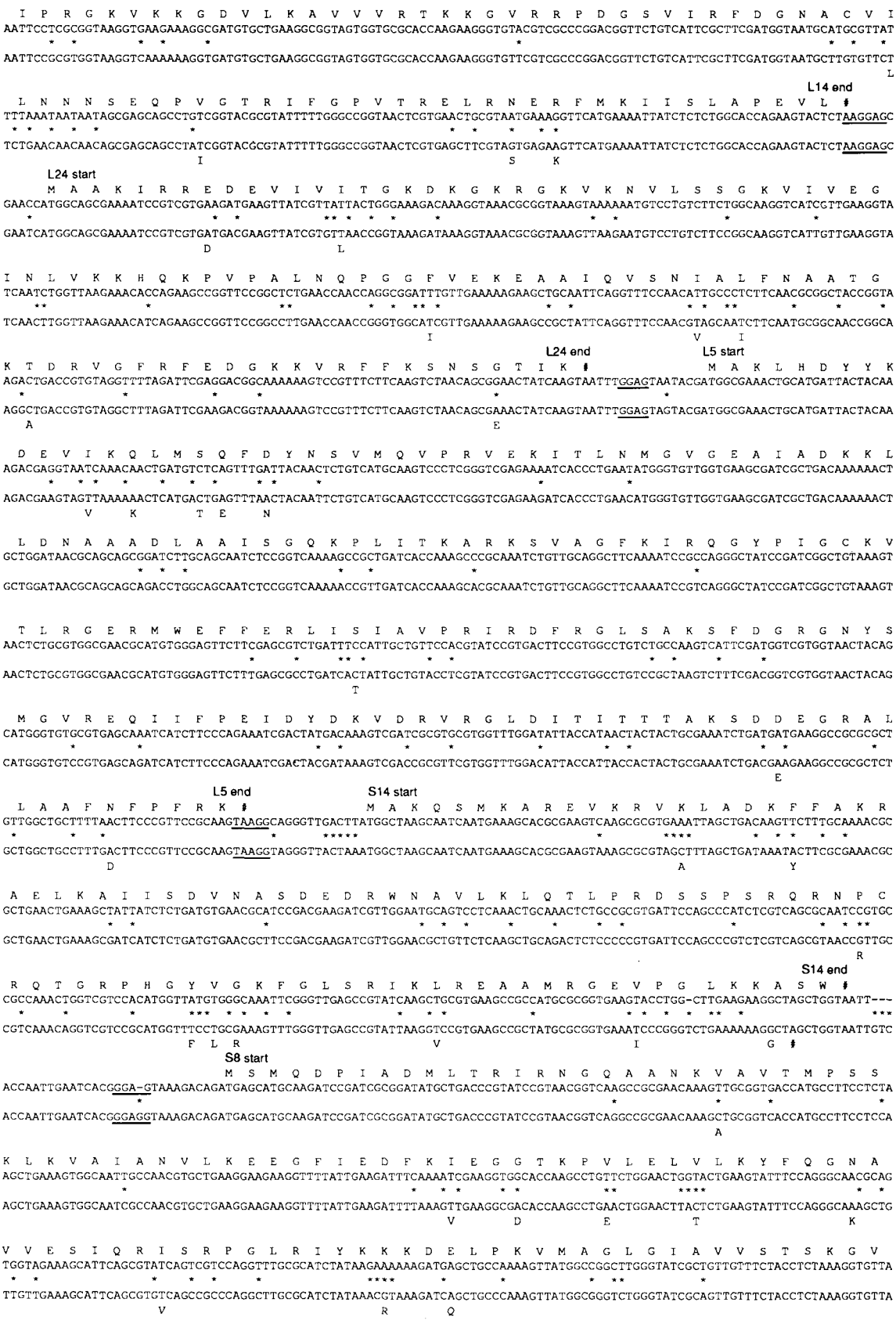
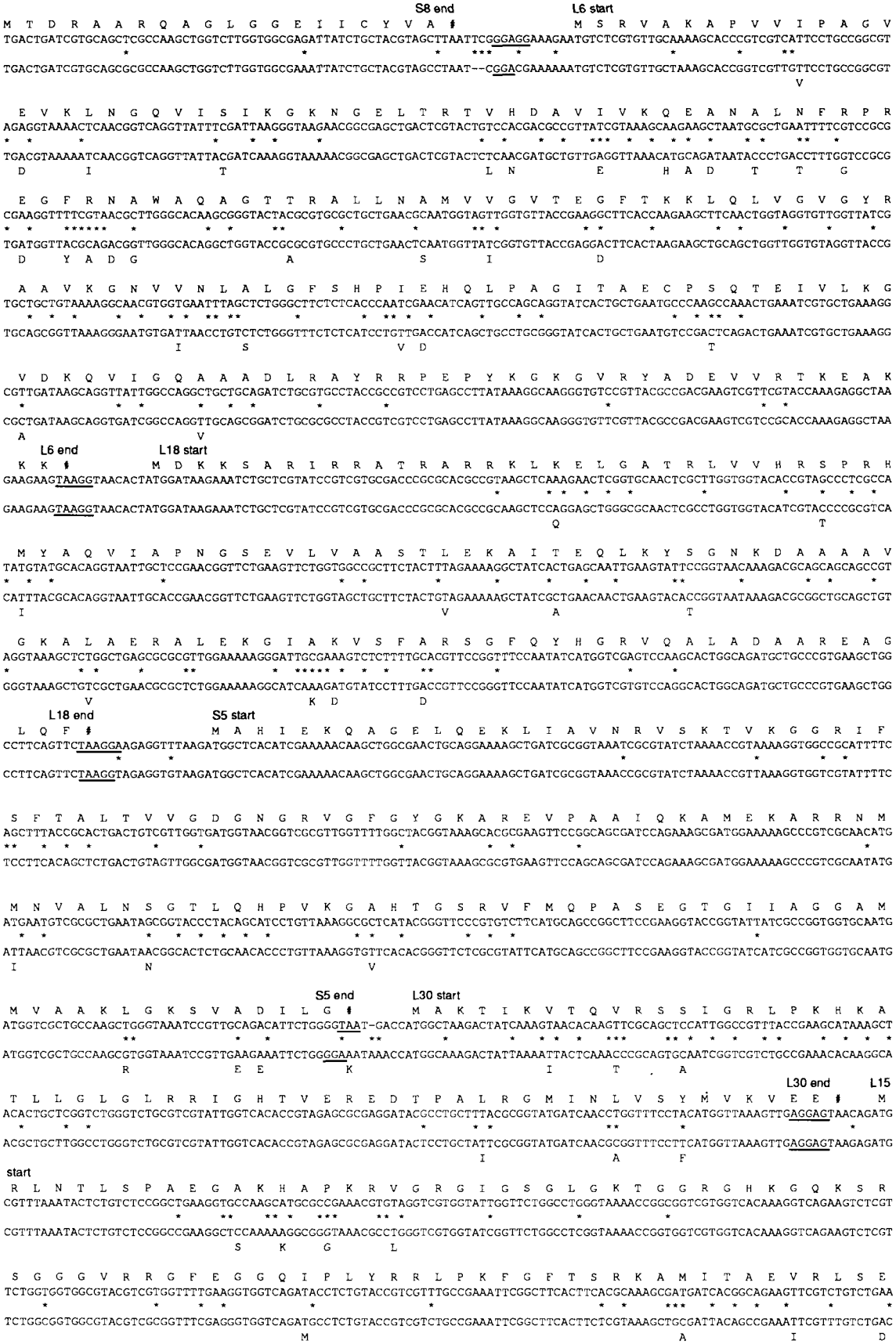



**Figure 3**. Continued

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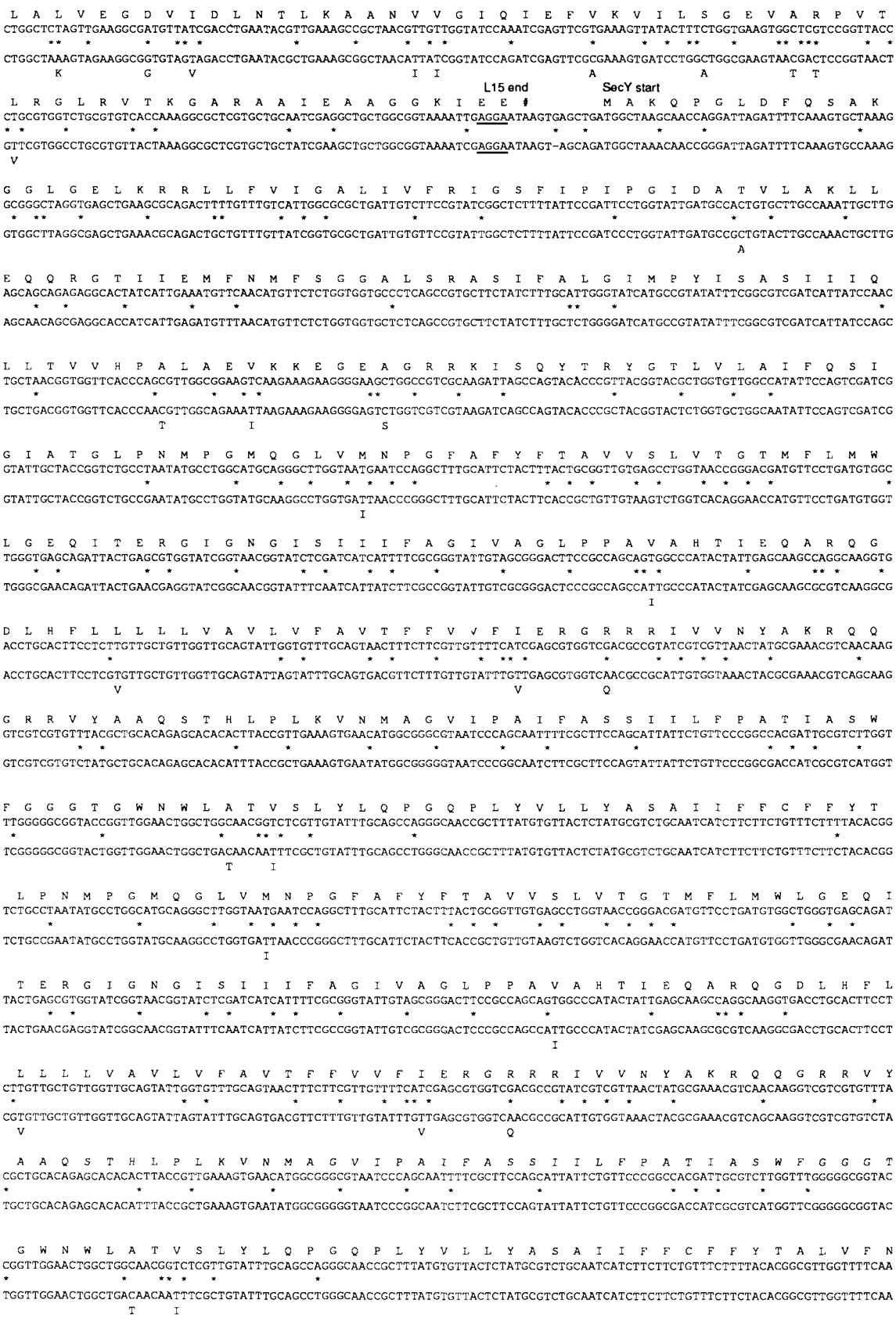


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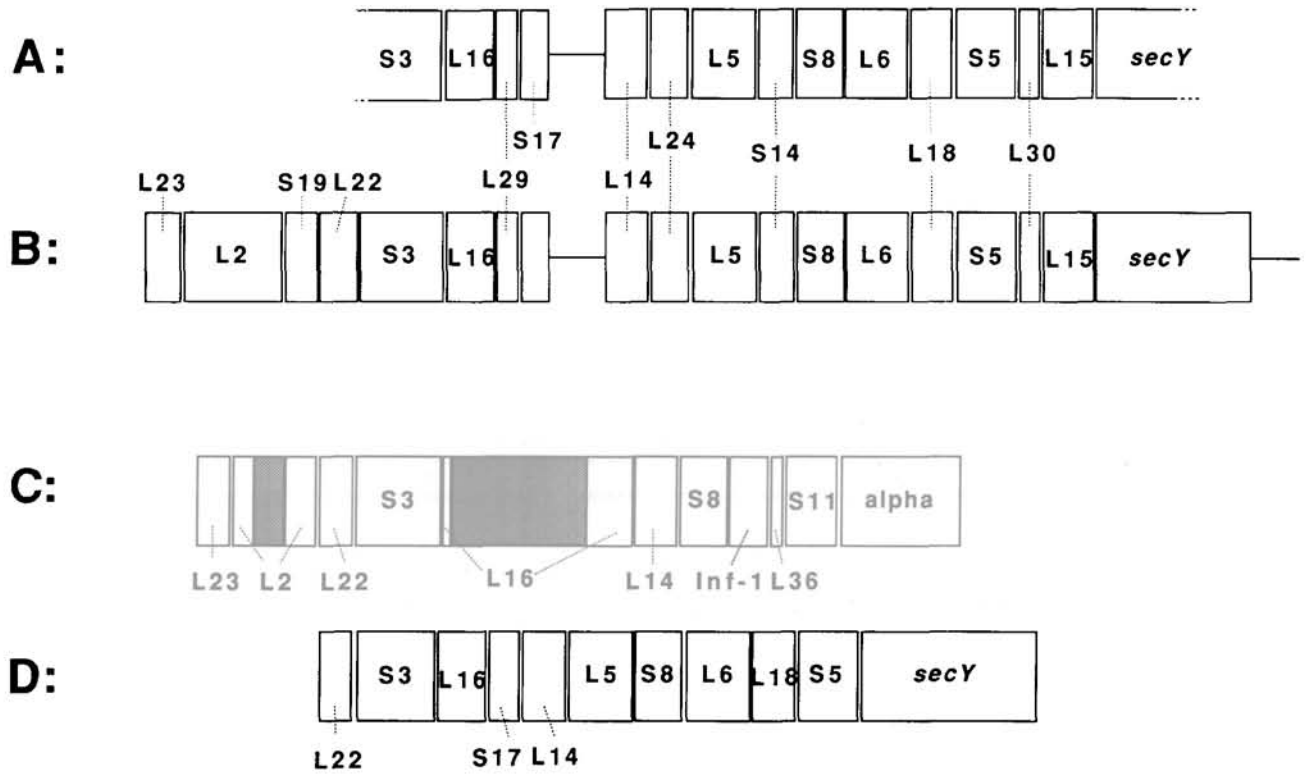


**Figure 4.** Schematic illustration showing the structure of the S10 and *spc* ribosomal protein gene operons from the endosymbiont (**A**), *E. coli* (**B**),[29] liverwort and tobacco chloroplasts (**C**),[9,10] and *Cyanelle paradoxa* (**D**).[35] Stippled regions in the chloroplast genes indicate introns.

deduced from them) and those in the PIR amino acid sequence database. In all cases the amino acid sequence data seem to be mistaken, because our nucleotide sequence data are in complete agreement with the *E. coli* nucleotide sequence data as far as the amino acid residues preticted at these positions are concerned, despite that the nucleotide sequences of the two organisms are different from each other at many positions as shown in Fig. 3.

Despite the nucleotide sequence differences between the *A. kondoi* endosymbiont and *E. coli*, the organization of the genes in the S10 and spc ribosomal protein gene operons is identical in these organisms as shown in Fig. 4. In contrast, the gene organization in another ribosomal protein operon containing the genes for L21 and L27 is quite different between the aphid symbiont and *E. coli* (Yamashita and Isono, manuscript in preparation), although the nucleotide sequence data are as similar as that presented here. It remains to be investigated further to what extent the ribosomal protein genes are conserved in the aphid symbiont.

The difference in the genomic G+C content and in the codon usage between the *A. kondoi* endosymbiont and those in two related aphids raises a question: is it reasonable to assume that the three aphid endosymbionts are the same species and can commonly be named, *Buchnera aphidicola*? Previous electron-microscopic observations suggest that *A. kondoi* harbors an endosymbiont that is morphologically indistinguishable from those in other related aphids.[24] In addition, our own optical and electron-microscopic observation (data not shown) indicated that the primary symbiont isolated from *A. kondoi* had the size and shape indistinguishable from the *A. pisum* symbiont, and was found to be Gram-negative. Contamination of a secondary symbiont or an intestinal bacterial species appeared to be not likely, because (1) the symbionts we isolated from *A. kondoi* insects and used as the primary symbionts of this aphid were the most abundant bacterial symbionts in this aphid, and (2) the genomic Southern hybridization data shown in Fig. 2 indicate that the 1.8 kb-long DNA fragment that was cloned into pEHS5109 showed much stronger hybridization with the symbiont DNA preparation (lanes 2, 4 and 6) than with the whole insect DNA (lanes 1, 3 and 5). Furthermore, no distinct bands were detected in the whole insect DNA other than those detected with the symbiont DNA. The primary symbiont in *A. kondoi* appears, therefore, morphologically the same as *B. aphidicola*, but its G+C

content and codon usage is notably different.

Three alternative ways to explain this phenomenon seem possible: (1) the genomic G+C content of the primary symbiont in *A. kondoi* was originally as low as 30% and increased to 50% through an unknown mechanism;[26] (2) the genomic G+C content of the common ancestor for the endosymbionts present in *A. kondoi* and related aphids was, for example, some 40% and the value steadily became higher in *A. kondoi* symbiont, while it became lower in other symbionts; or, (3) in *A. kondoi*, the original symbiont was *B. aphidicola*, but it was replaced by a morphologically similar bacterial endosymbiont that was more closely related to *E. coli*. If the first explanation is correct, then such a big change in the genomic G+C content as presented here is the first case ever reported. Therefore, it seems more reasonable to postulate that replacement of endosymbiont has occurred in *A. kondoi* during the course of evolution. In this connection it is interesting to note that artificial replacement of an endosymbiont in *Drosophila simulans* with that from a mosquito species resulted in sexual incompatibility of the treated strain with other *D. simulans* strains.[27] Therefore, it would be interesting to analyze whether *A. kondoi* can mate with *A. pisum* to form a hybrid or not.

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