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A Characteristic Difference among GroEL Homologs from Intracellular Symbionts of Closely-Interrelated Species of Aphid

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ABSTRACT—Nucleotide sequences encoding GroEL homologs of intracellular symbionts in three closely interrelated aphids were compared with one another and that for GroEL. It was suggested that in these proteins a particular position is highly susceptible to amino acid substitution, through which the GroEL homologs of symbionts seemed to have acquired a unique function on top of the activity as molecular chaperone. This may represent a rare example of non-neutral evolution of molecule under the positive selection pressure.

INTRODUCTION

A considerable number of insect species harbor intracellular symbionts that are vertically transmitted through host generations (Buchner, 1965). One type of them is the so-called mycetocyte symbionts, which are harbored by the host's mycetocytes, or bacteriocytes, huge cells differentiated for this purpose. In general, host and its mycetocyte symbionts are closely mutualistic and indispensable to each other for their growth and reproduction (Ishikawa *et al.*, 1992). The mycetocyte symbiosis is typically observed with homopterous insects, and among others, those of aphid species are the best studied in terms of both the nature of the interaction between host and symbiont (Ishikawa, 1984; Baumann *et al.*, 1995) and their evolutionary histories, as revealed by molecular phylogenetics (Moran *et al.*, 1993).

Phylogenetical studies on the 16S rDNA sequences indicated that all the prokaryotic symbionts from different aphid species examined belong to a single, well-supported clade within the γ-3 subgroup of Proteobacteria and have *Escherichia coli* and related bacteria as their closest relatives (Moran *et al.*, 1993). The formal designation, *Buchnera aphidicola,* applies to this symbiont clade (Munson *et al.*, 1991). In addition, it has been shown that the sequence-based phylogeny of *Buchnera* is completely concordant with the morphology-based phylogeny of the corresponding aphid species. This suggests that a single original infection in a common ancestor of the aphid has been followed by cospeciation of aphids and *Buchnera* to give rise to extant symbionts. By comparative sequence analyses of *Buchnera* 16S rDNAs, with the aphid fossil record in mind, the age of the common ancestor has been estimated at about 180-250 million years (Moran and Baumann, 1994).

One significant finding by us with *Buchnera* from the pea aphid, *Acyrthosiphon pisum* was that, when housed in the host cell, it selectively synthesizes symbionin (Hara *et al.*, 1990), a stress protein homologous to *E. coli* GroEL (Hendrix, 1979). Bacterial and organellar proteins homologous to GroEL function as molecular chaperone (Ellis and van der Vies, 1991), and are collectively called chaperonin (Hemmingsen *et al.*, 1988). It has been demonstrated that symbionin functions not only as molecular chaperone *in vitro* (Kakeda and Ishikawa, 1991) and *in vivo* (Ohtaka *et al.*, 1992), but also as an enzyme that transfers the phosphate group from ATP to substrates through its autophosphorylation in an energy coupling manner (Morioka *et al.*, 1993). This unique property of symbionin is reminiscent of histidine protein kinase serving as a sensor molecule in the two-component pathway of signal transduction (Gross *et al.*, 1989; Morioka *et al.*, 1994).

Although GroEL is more than 85% identical with symbionin at the amino acid sequence level (Ohtaka *et al.*, 1992), it apparently lacks the phosphotransferase activity observed with symbionin. It is conceivable that the GroEL homolog of *Buchnera* has aquired this unique activity through its adaptation to the intratacellular environment in the course of evolution. In an effort to gain an insight into the origin of the phosphotransferase activity of symbionin, in this study we determined the nucleotide sequences of symbionin genes of two other *Buchnera* species from the aphids closely related to *A. pisum.*

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MATERIALS AND METHODS

Insect materials

Both long-established parthenogenetic clones of pea aphids, *Acyrthosiphon pisum*, and potato aphids, *Acyrthosiphon solani*, were raised and maintained on young broad bean plants at 15°C under a long day regime of 16 hr light and 8 hr dark (Ishikawa, 1982). Kondo aphids, *Acyrthosiphon kondoi,* were raised and maintained on clover leaves under the same conditions as above.

Aphids were removed from plants and their mycetocytes were immediately dissected manually (Sasaki and Ishikawa, 1995). The isolated mycetocytes were crushed by passing through a capillary pipette, and the released symbionts, after washing and centrifugation, were heated in PBS at 95°C for 90 sec, and used as template DNA for the polymerase chain reaction (PCR).

PCR

The symbionin gene (*symL*) is led by the gene encoding SymS (*symS*) , a GroES homolog, with a short intergenic sequence between them, the two constituting the so-called *sym* operon, an *E. coli groE* homologous operon (Ohtaka *et al.*, 1992). In the present study, we amplified the nearly entire lengths of *symL* and the intergenic sequence, and part of *symS* by PCR. Primers used for this purpose were A: 5'-AAATTCGTCCATTGCATGATCG-3' (nt 6-27) and B: 5'-TACATCATTCCACCCATGCCACCC-3' (nt 1646-1623), which were constructed according to the sequences known for *symS* and *symL* from *A. pisum* (Ohtaka *et al.*, 1992). Numerals following the oligonucleotide sequences designate *symS* and *symL* nucleotide (nt) numbers, respectively. The reaction mixture (10 μl) consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.05 % (v/v) W-1 (BRL), 0.1 mM each of dATP, dGTP, dTTP, and dCTP, and 0.25 μM each of the two primers. A trace amount of template DNA was added into the reaction mixture, followed by incubation at 94°C for 5 min. To start PCR, 0.25 U of Ampli Taq DNA polymerase (Perkin Elmer) was added, and the mixture was overlaid with mineral oil. For amplification of the DNA segment of the *sym* operon, thermal cycles for PCR consisted of 45 sec at 94°C, 60 sec at 55°C, and 90 sec at 73°C, and total of 30 cycles were performed, followed by a 5-min extension cycle at 73°C.

Cloning and sequencing

The PCR-amplified DNA segments, which were approximately 2 kbp in length, were purified on 0.8% agarose gel electrophoreses using the Geneclean II kit (Bio101). The purified DNA segments were subjected to blunting and 5'-phosphorylation with T4 DNA polymerase (Takara) and polynucleotide kinase (Epicentre), and ligated to *Eco*RV-digested pBluescriptSK$^-$ using the Takara ligation kit, version 2.0 (Sambrook *et al.*, 1989). In order to clone the 2 kbp insert, the recombinant plasmids were transformed into *E. coli* DH5*α* cells. Nucleotide sequences of the insert were determined on both strands using a Hitachi SQ-5500 DNA sequencer with the ΔTaq fluorescent dye-primer cycle sequencing kit (Amersham) (Sanger *et al.*, 1977). The Internet provided-program CLUSTAL V (Higgins *et al.*, 1992) was used to align nucleotide sequences.

RESULTS AND DISCUSSION

In the present study, we determined the nucleotide sequences of the greater part of the *sym* operons of intracellular symbionts (*Buchnera* sp.) from two aphid species, *A. kondoi* and *A. solani,* and compared their *symL* sequences with that of *Buchnera* from *A. pisum*, and the *groEL* sequence of *E. coli.* It is evident from their morphology that the three *Acyrthosiphon* species are closely interrelated (Moritsu, 1983). In particular, some strains of *A. kondoi* are scarcely distinguishable morphologically from *A. pisum*.

In Fig. 1, amino acid sequences of the three symbionins, together with that of *E. coli* GroEL, which were predicted from the nucleotide sequences determined, were aligned. These sequences represent 540 out of the total 548 amino acid residues of the four proteins, which only lack the 8 C-terminal residues. Just as expected from the close relationship among the host insects, the three symbionin sequences were more than 99% identical with one another, while GroEL was about 86% identical with any one of the symbionins. It has been known that the 8 C-terminal amino acids, which were not determined here, are completely conserved between GroEL and the symbionin of *A. pisum* (Ohtaka *et al.*, 1992). The three symbionin genes were 92-94% identical with one another at the nucleotide sequence level, while *groEL* was 73-75% identical with those genes.

It was previously shown that His-133 of symbionin from *A. pisum* had been substituted for Ala at the same position of GroEL (Ohtaka *et al.*, 1992). It has been also demonstrated that the His-133 is the phosphorylation site of the symbionin, suggesting that the amino acid substitution at this position endowed the protein with the phosphotransferase activity (Morioka *et al.*, 1994). In addition, the chaperoning activity of the symbionin was dramatically augmented in response to its phosphorylation at the His-133 (Morioka *et al.*, 1993). One significant finding in this study was that the His-133 had not been conserved in other symbionins from the aphid species that are closely related to *A. pisum*. As noted in Fig. 1, any one of the three symbionins was different from the other two at only 5 amino acid positions. One of them was the position 133 where, in *A. kondoi* and *A. solani*, Asn had been substituted for His in *A. pisum*.

Figure 2 represents nucleotide sequences coding for the region around the position 133 of GroEL and symbionins. As demonstrated previously, the histidine codon at the position 133 of the *A. pisum* symbionin has been created as a result of three consecutive base substitutions between the symbionin gene and *groEL*. In considering that nearly all the other base substitutions between the two genes are synonimous or analogous, such non-neutral, consecutive substitutions are a very rare event (Ohtaka and Ishikawa, 1993). These base substitutions resulted in a creation of the phosphorylation site in the *A. pisum* symbionin, which not only improved the protein as the molecular chaperone (Morioka *et al.*, 1993), but also endowed it with the phosphotransferase activity (Morioka *et al.*, 1994). Therefore, it is well grounded to assume that mutations at these positions have been positively selected in the course of evolution of *Buchnera*. In this context, it was rather unexpected that in the two other symbionin genes from *A. kondoi* and *A. solani* the first base of the codon 133 had been further replaced by A to code for a still different amino acid, asparagine.

Interestingly enough, however, asparagine is also subject to phosphorylation to give rise to unstable phosphoasparagine. In this regard, it should be emphasized

```
                           20                  40
GroEL    MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTIT
A.pisum  MAAKDVKFGNEARIKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPSIT
A.kondoi MAAKDVKFGNEARIKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPSIT
A.solani MAAKDVKFGNEARIKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPSIT
         **********.**.*********************************.**
                 60                  80                 100
GroEL    KDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLAQAII
A.pisum  KDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATLLAQSIV
A.kondoi KDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATLLAQSIV
A.solani KDGVSVAREIELEDKFENMGAQMVKEVASKANDAAGDGTTTATLLAQSIV
         *******************************************.***.*.
                          120                 140
GroEL    TEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTI
A.pisum  NEGLKAVAAGMNPMDLKRGIDKAVISAVEELKHLSVPCSDSKAITQVGTI
A.kondoi NEGLKAVAAGMNPMDLKRGIDKAVISAVEELKNLSVPCSDSKAITQVGTI
A.solani NEGLKAVAAGMNPMDLKRGIDKAVISAVEELKNLSVPCSDSKAITQVGTI
          *********************** .****** ***********.*****
                160                 180                 200
GroEL    SANSDETVGKLIAEAMDKVGKEGVITVEDGTGLQDELDVVEGMQFDRGYL
A.pisum  SANADEKVGSLIAEAMEKVGNDGVITVEEGTGLQDELEVVKGMQFDRGYL
A.kondoi SANADEKVGSLIAEAMEKVGNDGVITVEEGTGLQDELEVVKGMQFDRGYL
A.solani SANADEKVGSLIAEAMEKVGNDGVITVEEGTGLQDELEVVKGMQFDRGYL
         ***.** ** ******.***..******.********.** *********
                          220                 240
GroEL    SPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAKAGKPLLII
A.pisum  SPYFINKPETGIVELENPYILMADKKISNVREMLPILESVAKSGKPLLII
A.kondoi SPYFINKPETGIVELENPYILMADKKISNVREMLPILESVAKSGKPLLII
A.solani SPYFINKPETGIVELENPYILMADKKISNVREMLPILESVAKSGKPLLII
         *********** ****.*.**.*******.*****.**.***.*******
                260                 280                 300
GroEL    AEDVEGEALATLVVNTMRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGTV
A.pisum  SEDLEGEALATLVVNSMRGIVKVAAVKAPGFGDRRKAMLQDISILTGGSV
A.kondoi SEDLEGEALATLVVNSMRGIVKVAAVKAPGFGDRRKAMLQDISILTGGSV
A.solani SEDLEGEALATLVVNSMRGIVKVAAVKAPGFGDRRKAMLQDISILTGGSV
         .**.***********.**************************. ****.*
                          320                 340
GroEL    ISEEIGMELEKATLEDLGQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIR
A.pisum  ISEELAMELEKSTLEDLGQAKRVVISKDTTTIIGGVGEKHSIQSRISQIR
A.kondoi ISEELAMELEKSTLEDLGQAKRVVISKDTTTIIGGVGEKHSIQSRISQIR
A.solani VSEELAMELEKSTLEDLGQAKRVVISKDTTTIIGGVGEKHSIQSRISQIR
         .***..*****.*************.*******.****  .**.*..***
                360                 380                 400
GroEL    QQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKARVEDAL
A.pisum  QEIQEATSDYDKEKLNERLAKLSGGVAVLKVGAATEVEMKEKKARVEDAL
A.kondoi QEIQEATSDYDKEKLNERLAKLSGGVAVLKVGAATEVEMKEKKARVEDAL
A.solani QEIQEATSDYDKEKLNERLAKLSGGVAVLKVGAATEVEMKEKKARVEDAL
         *.*.*******.***.**.***.*****.*********************
                          420                 440
GroEL    HATRAAVEEGVVAGGGVALIRVASKLADLRGQNEDQNVGIKVALRAMEAP
A.pisum  HATRAAVEEGVVAGGGVALVRVAGKIADLRGQNEDQNVGIRVALRAMEAP
A.kondoi HATRAAVEEGVVAGGGVALVRVAGKISNLRGQNEDQNVGIRVALRAMEAP
A.solani HATRAAVEEGVVAGGGVALVRVAGKISNLRGQNEDQNVGIRVALRAMEAP
         *******************.***.*...************.*********
                460                 480                 500
GroEL    LRQIVLNCGEEPSVVANTVKGGDGNYGYNAATEEYGNMIDMGILDPTKVT
A.pisum  LRQIVSNSGEEPSVVTNNVKDGKGNYGYNAATDEYGDMIDFGILDPTKVT
A.kondoi LRQIVSNSGEEPSVVTNNVKDGKGNYGYNAATDEYGDMIDFGILDPTKVT
A.solani LRQIVSNSGEEPSVVTNNVKDGKGNYGYNAATDEYGDMIDFGILDPTKVT
         ***** * *******.* **.* *********.***.*** *********
                          520                 540
GroEL    RSALQYAASVAGLMITTECMVTDLPKNDAADLGAAGGMGG
A.pisum  RSALQYAASVAGLMITTECMVTDLPKEDKSSDSSSSPAGG
A.kondoi RSALQYAASVAGLMITTECMVTDLPKEDKSSDSNSSPAGG
A.solani RSALQYAASVAGLMITTECMVTDLPKEDKSSDSSSSPAGG
         **************************.* .  . ..  **
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Fig. 1. Comparison of the deduced amino acid sequences of GroEL and *Buchnera* symbionins from three aphid species. The greater parts of the *sym* operons of intracellular symbionts from *A. kondoi* and *A. solani* were amplified by PCR, and their nucleotide sequences were determined. Amino acid sequences deduced for the symbionins were aligned with those of GroEL and the symbionin from *A. pisum* (Ohtaka *et al.*, 1992). Asterisks mark positions of amino acid residues conserved in the four chaperonins. Dots indicate positions where analogous amino acid substitutions between GroEL and symbionins were observed. In the boxed positions, amino acid residues were varied among symbionins. Sequences do not contain the 8 C-terminal residues.

Fig. 2. Comparison of the nucleotide sequences encoding the region around the position 133 of the four chaperonins. Sequences were divided into codons by dotted lines. Asterisks indicate positions of nucleotides conserved in the four chaperonin genes. Bold asterisk marks the position 133 where codon is highly susceptible to variation.

that many chaperonins, both bacterial and organellar, so far examined contain lysine at the corresponding site (Gupta, 1990). The amino acid is also phosphorylated to form the phosphoramidate bond. Since both phosphoasparagine and phospholysine, just like phosphohistidine, tend to have a high standard free energy of hydrolysis, it is likely that these chaperonins also have the energy-coupling phosphotransferase activity, though not evidenced yet. All taken together, it is conceivable that the codon 133 of chaperonin genes is a "hot spot" of molecular evolution, and that through frequent substitutions of bases in this codon chaperonin is in the process of evolutionary change from a mere chaperone to a multifunctional protein. Probably, this represents a rare example of non-neutral evolution of molecule, in which a protein has acquired a novel function under the positive selection pressure.

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