NOTE

Phylogenetic relationships of *Anaplasma marginale* and 'Ehrlichia platys' to other *Ehrlichia* species determined by GroEL amino acid sequences

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The heat-shock protein (GroEL) genes of *Anaplasma marginale*, *Ehrlichia muris* and 'Ehrlichia platys' were sequenced and compared with the GroEL of other species of *Ehrlichia*. The GroEL amino acid sequences of *A. marginale* and 'E. platys' were most similar to the GroEL sequence of *Ehrlichia phagocytophila*, with which they formed one group with 6–10% divergence. The *E. muris* GroEL was most closely related to the GroEL of two unclassified strains (HF-565 and Anan), then to *Ehrlichia chaffeensis*, *Ehrlichia canis*, *Ehrlichia ewingii* and *Cowdria ruminantium*, forming a second distinct group (0.3–8.6% divergence). The GroELs of *Ehrlichia risticii* and *Ehrlichia sennetsu* were very similar to one another (only 2% divergence), forming the third group. The first two groups were relatively closely related (17–20% divergence), while the third group was only distantly related to the first two groups (62–73% divergence).

Keywords: *Anaplasma*, *Cowdria*, *Ehrlichia*, *Wolbachia*, heat-shock protein

Ehrlichia are obligately intracellular Gram-negative bacteria that grow within membrane-bound vacuoles. Currently, classification of ehrlichial organisms is based on morphology and host cell tropism rather than genetic phylogeny. *Ehrlichia*, *Cowdria* and *Anaplasma* are classified in different families or tribes (Ristic & Huxsoll, 1984; Ristic & Kreier, 1984). However, molecular analysis of the 16S rRNA gene has shown that they are phylogenetically related (Anderson *et al.*, 1991; Dame *et al.*, 1992; Pretzman *et al.*, 1995; Weisburg *et al.*, 1991; Wen *et al.*, 1995). The availability of the complete database led to previous phylogenetic analysis of *Ehrlichia* based on the 16S rRNA gene. However, phylogeny using a single gene may be misleading due to evolutionary variation of individual genes among related organisms. Comparison of multiple genes increases the accuracy of such phylogenetic relationships. Recent studies (Masui *et al.*, 1997; Sumner *et al.*, 1993, 1997, 2000; van Vliet *et al.*, 1992; Zhang *et al.*, 1997) have shown that the *groEL* gene (the 60 kDa heat-shock protein gene) is an

appropriate target gene for phylogenetic analysis of *Ehrlichia*. However, the GroEL phylogenetic tree of *Ehrlichia* was incomplete because the *groEL* gene in some *Ehrlichia* members had not been sequenced. To construct a complete *Ehrlichia* GroEL phylogenetic tree, we sequenced the *groEL* gene of *Anaplasma marginale*, 'Ehrlichia platys' and *Ehrlichia muris*.

The ehrlichial *groEL* DNA sequences obtained from GenBank and their accession numbers were as follows: *Cowdria ruminantium*, CRU13638; *Ehrlichia canis*, ECU96731; *Ehrlichia chaffeensis*, L10917; *Ehrlichia ewingii*, AF195273; *Ehrlichia phagocytophila*, U96729; *Ehrlichia risticii*, U96732; *Ehrlichia sennetsu*, U88092; *Ehrlichia* sp. strain Anan, AB032711; *Ehrlichia* sp. strain HF-565, AB032711; and *Wolbachia* sp., AB002286. The ehrlichial 16S rRNA gene sequences obtained from GenBank included: *A. marginale*, M60313; *C. ruminantium*, AF069758; *E. canis*, M73221; *E. chaffeensis*, M73226; *E. ewingii*, U96436; strain HF-565, AB024928; strain Anan, AB028319; *E. phagocytophila*, M73224; 'E. platys', M82801; *E. sennetsu*, M73225; *E. risticii*, AF036649; and *Wolbachia pipientis*, AF179630. The sequences of the *Rickettsia prowazekii* GroEL protein (CAB40143) and 16S rRNA gene (M21789) were used to root the trees.

The GenBank accession numbers for *Anaplasma marginale groEL* and 'Ehrlichia platys' *groEL* are AF165812 and AY008300, respectively.

Fig. 1. Phylogenetic relationships of *A. marginale*, *C. ruminantium*, *Ehrlichia* and *Wolbachia* based on the amino acid sequences of GroEL (on the left) and the DNA sequences of the 16S rRNA gene (on the right). The length of each pair of branches represents the distance between sequence pairs. The number on the branch indicates the bootstrap value.

E. muris was provided by Yasuko Rikihisa (Ohio State University, Columbus, OH, USA). *E. muris* DNA from the blood of infected mice was extracted by using an IsoQuick nucleic acid extraction kit (ORCA Research) according to the manufacturer's instructions. *A. marginale* genomic DNA was provided by Anthony F. Barbet (College of Veterinary Medicine, University of Florida, Gainesville, FL, USA). Platelet-rich plasma from a dog infected with '*E. platys*' was provided by Richard E. Corstvet (Louisiana State University, Baton Rouge, LA, USA). The *groEL* consensus sequence was obtained by aligning *E. canis*, *E. chaffeensis*, *E. phagocytophila* and *C. ruminantium* *groEL* sequences using the CLUSTAL W method of the Megalign program (DNASTar). Forward primers GroELf4 (CAT AGT G/TAT GAA A/GGA A/GAG TG) and Pf (GCT ATT AGT AAA CCT TAT GG) and reverse primers Pr (TTA CGT TCTTTA ACC TCA ACT TC) and GroELr3 (GTA/T GCA TGT AAA/T GCA TCT TC) were manually designed from the consensus sequences. PCR amplification was performed for 30 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min.

A 1.4 kb DNA fragment of *A. marginale* and *E. muris* was amplified by using primers GroELf4 and GroELr3 and a 1.1 kb DNA fragment of '*E. platys*' was amplified by using primer pair Pf and Pr. DNA was sequenced with an ABI Prism 377 DNA Sequencer (Perkin-Elmer Applied Biosystems). The GenBank accession numbers for DNA sequences obtained in this study are: *A. marginale*, AF165812; and '*E.*

platys', AY008300. The GroEL sequence of *E. muris* was published while our manuscript was under review (Sumner *et al.*, 2000). The amino acid sequence we obtained was identical to the *E. muris* GroEL sequence (AF210459) in GenBank; therefore, we did not deposit the sequence for *E. muris*.

The maximum-parsimony method of the PAUP 4.0b4a (PPC) software (Swofford, 1998) was used to perform the phylogenetic analysis. Bootstrap values for the consensus tree were based on analysis of 200 replicates. Phylogenetically, *A. marginale* and '*E. platys*' were most closely related to *E. phagocytophila* (9.3 and 6% divergence, respectively) (Fig. 1), *Ehrlichia equi* and the human granulocytotropic ehrlichiosis agent (data not shown). *E. equi* and the human granulocytotropic ehrlichiosis agent were not included in the phylogenetic tree because they were identical to *E. phagocytophila* as described previously (Sumner *et al.*, 1997). *E. muris* was closest to two unclassified ehrlichial isolates (HF-565 and Anan strains) (0.3% divergence) and then to *E. chaffeensis* (0.9% divergence). *Ehrlichia* can be separated into three groups by an arbitrary 10% divergence of GroEL. Members of the *E. chaffeensis* group including strain HF-565, strain Anan, *E. chaffeensis*, *E. canis*, *E. muris*, *E. ewingii* and *C. ruminantium* were 1–5% divergent from one another, and *C. ruminantium* was somewhat more distant from the other species of *Ehrlichia* in the group with 8–9% divergence. Members of the *A. marginale* group including *E. phagocytophila*, '*E. platys*' and *A. marginale* were 6–10% divergent from each other. The organisms

in the *E. risticii* group including *E. risticii* and *E. sennetsu* were 2% divergent from one another. The genetic divergence among the organisms in different groups was 17–73% (17–20% between the *E. chaffeensis* group and the *A. marginale* group; 62–66% between the *E. chaffeensis* group and the *E. risticii* group; 71–73% between the *A. marginale* group and the *E. risticii* group). *Wolbachia* were placed between the *A. marginale* and the *E. risticii* groups, and were 34–40% divergent from the *E. chaffeensis* and *A. marginale* groups and 60% divergent from the *E. risticii* group.

Our results were consistent with previous phylogenetic analysis of *Ehrlichia* by utilizing the 16S rRNA gene sequences (Wen *et al.*, 1995). For a side-by-side comparison with the GroEL phylogenetic tree, we reconstructed the 16S rRNA gene phylogenetic tree of *Ehrlichia* (Fig. 1). The branches of the 16S rRNA gene phylogenetic tree were almost identical to the branches of the GroEL phylogenetic tree (Fig. 1). With a cutoff of 7% divergence of nucleotide sequences, the 16S rRNA gene phylogenetic analysis also separated *Ehrlichia* into three groups corresponding to the groups in the GroEL tree.

Phylogenetic analysis using both the GroEL and 16S rRNA genes indicated that *E. sennetsu* and *E. risticii* are very distantly related to the genus *Ehrlichia*. In contrast, *A. marginale* and *C. ruminantium* are very closely related to the genus *Ehrlichia*. Moreover, *Wolbachia* is more closely related to *Ehrlichia sensu stricto* and *A. marginale* than to *E. sennetsu* and *E. risticii*. Our data and previous data indicated that the genus *Ehrlichia* may require reclassification. The organisms in each phylogenetic group may be classified as a single genus. Therefore, *Ehrlichia* may be classified into three genera: *Anaplasma*, *Ehrlichia* and *Neorickettsia*. Genus *Anaplasma* includes *A. marginale*, '*E. platys*' and *E. phagocytophila*; genus *Ehrlichia* includes *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, *C. ruminantium* and two new strains of *Ehrlichia*, HF-565 and Anan. Strains HF-565 and Anan are not differentiated from *E. muris* by GroEL sequence or 16S rRNA gene sequence (Shibata *et al.*, 2000). Therefore, they may represent a single species. *E. sennetsu* and *E. risticii* should be combined with *Neorickettsia helminthoeca* into a single genus as previously suggested by Pretzman *et al.* (1995).

The similarities and differences among *A. marginale*, *Cowdria* and *Ehrlichia* are also illustrated by their life cycles and antigenic relationships. The organisms in the first two groups are tick-borne intracellular bacteria, and the organisms in the third group are associated with trematodes that parasitize fish and aquatic snails (Reubel *et al.*, 1998). The first two groups of organisms are antigenically related. A 28 kDa outer-membrane protein multigene family has been found in all organisms in the second group. An MSP-2 outer-membrane protein family which shares significant homology with the 28 kDa protein family

was found in *A. marginale* and *E. phagocytophila*. The protein families were not investigated in '*E. platys*', but '*E. platys*' may contain an MSP-2 protein family rather than the 28 kDa protein family based on its phylogenetic relationship with *Anaplasma*. Neither of these outer-membrane protein families was found in *E. sennetsu* and *E. risticii* (Yu *et al.*, 1999). *E. sennetsu* and *E. risticii* are closely related to *Neorickettsia helminthoeca* genetically (Pretzman *et al.*, 1995) and antigenically (Rikihisa, 1991).

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