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# History of the ADP/ATP-Translocase-Encoding Gene, a Parasitism Gene Transferred from a *Chlamydiales* Ancestor to Plants 1 Billion Years Ago

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**Nonmitochondrial ADP/ATP translocase is an energy parasite enzyme. Its encoding gene, *tlc*, is found only in *Rickettsiales*, *Chlamydiales*, and plant and alga plastids. We demonstrate the presence of *tlc* in *Parachlamydia acanthamoebae*. This gene shares more similarity with the *tlc*1 gene of *Chlamydiaceae* and the *tlc* of plant and alga plastids than with the *tlc*2 gene of *Chlamydiaceae*. Phylogenetic analysis, including all other *tlc* homologs found in GenBank, showed that *tlc* was duplicated in a *Chlamydiales* ancestor before the appearance of multicellular eukaryotes. A time scale, calibrated with seven independent time points obtained from fossil estimates and from the 16S rRNA molecular clock, was congruent with the molecular clock provided by *tlc*. Plant and alga plastids acquired *tlc* approximately when *Parachlamydiaceae* and *Chlamydiaceae* diverged, at the eucaryotic radiation time, ca. 1 billion years ago.**

Nonmitochondrial ADP/ATP translocase is a very unique enzyme that exchanges bacterial ADP for ATP from the host cell and allows energy parasitism (17, 29, 30). Of its encoding gene, five and two copies were found in the *Rickettsiales* and *Chlamydiaceae* genomes, respectively, two clades of obligate intracellular bacteria (4, 23, 24, 27). The presence of the *tlc* gene in these distantly related bacterial clades was explained by either horizontal transfer, convergent evolution, or as a result of the common origin of *Chlamydiales* and *Rickettsiales* (7, 8, 21, 27, 30, 31). Only plant plastids are also known to possess this gene and encode for a protein that exchanges plastid ADP for ATP present in the eukaryotic cytoplasm (19). Since several other proteins of *Chlamydiales* were phylogenetically related to plant proteins (27), it has been proposed that the ADP/ATP translocase gene (*tlc*) was acquired from a plant genome by *Chlamydiaceae* and subsequently transferred to *Rickettsiales* (30). However, it makes more sense that a protein allowing energy parasitism originated in a clade of obligate intracellular bacteria parasitizing eucaryotes than in the eucaryotic host itself, since the new protein provides ATP to the intracellular bacteria and represents a selective advantage that allows its encoding gene to be fixed in the genome. Moreover, recent studies suggest that the ancestral *Chlamydiales* may have participated in the ancient chimeric events that led to the formation of the plant lineages and might be related to the cyanobacterium-chloroplast lineage (6, 10).

*Parachlamydia* is a new genus within *Chlamydiales* (2) that presents *Chlamydia*-like developmental stages (15) and shares 80 to 90% similarity of 16S rRNA genes with *Chlamydiaceae* (12, 14). In contrast to the *Chlamydiaceae*, which naturally infect multicellular organisms such as mammals and birds, *Parachlamydia acanthamoebae* naturally infects free-living amoebae and has probably never been a parasite of multicellular organisms. Moreover, since *Parachlamydiaceae-Chlamydiaceae* divergence was contemporary with the eukaryotic radiation about 1 billion years ago (26), the presence of the *tlc* gene within the genome of *P. acanthamoebae* would preclude the hypothesis of a transfer of the *tlc* gene from plants and might, on the contrary, suggest its transfer from *Chlamydiales* to plants. Therefore, in the present study, we investigated whether the *tlc* gene was present within the genome of *P. acanthamoebae* and evaluated the genetic and phylogenetic relationships of the nonmitochondrial ADP/ATP translocase coding sequences from an evolutionary perspective.

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## MATERIALS AND METHODS

***Parachlamydia* culture, purification, and DNA extraction.** *P. acanthamoebae* strain Hall coccus and *Acanthamoeba polyphaga* Linc-AP1 were kindly provided by T. J. Rowbotham (Public Health Laboratory, Leeds, United Kingdom). *Parachlamydia* sp. was grown for 6 days at 32°C within *Acanthamoeba polyphaga* in peptone-yeast extract-glucose broth (15). Harvested bacteria were purified by centrifugation and ultracentrifugation onto a 10% sucrose barrier. To improve purification, the pellet was suspended in phosphate-buffered saline, loaded onto a discontinuous Gastrografine (Schering, Lys-Lez-Lannoy, France) gradient, and ultracentrifuged at 140,000 × *g*. Parachlamydiae, which clustered in a large lower band, were collected, centrifuged at 5,800 × *g*, and resuspended in phosphate-buffered saline twice. DNA was extracted by using the QIAamp DNA Mini-Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions.

**PCR amplification and sequencing of *P. acanthamoebae tlc* gene.** The strategy for determining the sequence of the gene encoding the ADP/ATP translocase (i.e., *tlc*) of *P. acanthamoebae* consisted of (i) amplifying a segment of the targeted gene with degenerate primers and (ii) completing the full-length sequence by the genome-walking approach. adpF1 5′-GAAGC(TA)AAACGTTT(CT)TACGCTCT, adpF11 5′-TGTTCTGGGGGTTAGCCAA, and adpR4 5′-C(AG)TCAATAGC(AG)GCTTTICCTTT-, all designed by alignment of the *tlc* genes of *Chlamydia muridarum*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae* J138, and *Rickettsia conorii*, succeeded in amplifying 910-bp (adpF11-R4) and 980-bp (adpF1-R4) nucleotides. These PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) and sequenced by using the dRhodamine terminator cycle sequencing ready reaction with AmpliTaq DNA (Perkin-Elmer Biosystems, Warrington, United Kingdom). Sequences were determined on 3100 ABI Prism automated sequencer (Applied Biosystems, Courtaboeuf, France). Sequences derived from each primer were aligned, compared, and combined in a single sequence by using Autoassembler

software version 2.1 (Applied Biosystems). The unknown 5′ and 3′ ends of this partial sequence were amplified by using the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, Calif.). The validity of the sequence obtained was assessed by comparison with two additional sequences obtained by PCR amplification with primers designed from the sequence immediately flanking the open reading frame: Adp65F (5′-GATCCACGAAAGCACTCTTATT) Adp62R (5′-GGCAATCTATCACGTAATTGAAAAT).

**Presence of the *tlc* gene in additional clades.** The nucleotide sequence of *Parachlamydia tlc* (GenBank accession number AF490592) and the corresponding amino acid sequence were compared to sequences available in the GenBank database by using the BLASTN and BLASTP 2.2.6 programs available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) (1). An iterated profile search was also performed by using position-specific iterated BLAST (PSI-BLAST) (1).

**Genetic and phylogenetic analysis.** After alignment with CLUSTAL W (28), genetic distances of nucleotides and amino acids sequences were calculated by using MEGA 2.1 software (18). With the same software, we inferred neighbor-joining (p-distance), minimum evolution (p-distance), and parsimony trees (standard parsimony) by using the amino acid and nucleotide sequences.

**Time scale.** The molecular clock of the *tlc* gene was calibrated with seven points obtained from fossil estimates and from the estimated time of divergence of bacterial species on the basis of the 16S rRNA sequence divergence, assuming a rate of evolution of 1 to 2% per 50 million years (20). The 16S rRNA sequences were edited by removal of the longer 5′ and 3′ ends so that their lengths matched that of the shortest sequence. The percentage of 16S rRNA sequence divergence was calculated by using CLUSTAL W program (28), supported by the PBIL website (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl). The time of divergence of green plants and red algae, estimated by Sogin and Silberman (26), the time of divergence of monocotyledons (*Oryza sativa*) and dicotyledons (*Citrus* spp., *Solanum tuberosum*, and *Arabidopsis thaliana*) estimated by Gale et al. (13), and the estimated times of divergence of bacterial species (assuming a rate of evolution of 1.5% of 16S rRNA sequence per 50 million years) were plotted as a function of the proportion (p) of amino acid sites at which the sequence is different from that of the ancestral ADP/ATP translocase sequence, i.e., the p-distance between each node and the node that separated the more divergent sequences. The equation of the regression line, its standard error, and its $r^2$ coefficient were calculated with Stata 7.0 (Stata Corp., College Station, Tex.) and Microsoft Excel 97 (Microsoft Corp., Redmond, Wash.). To estimate the time of divergence of red algae and green plants and of monocotyledons and dicotyledons, regression was performed similarly, using only the time scale inferred from 16S rRNA genes and without using the times estimated by Sogin and Silberman (26) and by Gale et al. (13). Similar analyses were performed by using a minimum evolution tree inferred from amino acid sequences. To further test the reliability of the time scale, we performed an omit test, which assessed how the time estimates were modified by the omission of each of the calibration point.

## RESULTS

**Presence of the *tlc* gene in *P. acanthamoebae*.** We succeeded in amplifying a segment of the *tlc* gene of *P. acanthamoebae* with degenerate primers and completed the full-length sequence by the genome-walking approach (25). The *tlc* gene sequence of *P. acanthamoebae* strain Hall coccus has been deposited in the GenBank database under accession number AF490592.

**Presence of *tlc* gene in *Galdieria sulfuraria* (a red algae), *Citrus* hybrid cultivar (citrus), *Oryza sativa* (rice), *Holospora obtusa* and *Caedibacter caryophilus* (endosymbionts of *Paramecium*), *Encephalitozoon cuniculi* (microsporidia), and *Medicago sativa* (alfalfa).** By using the basic local alignment search tool (BLAST) with the amino acid sequence of the *Parachlamydia* ADP/ATP translocase as input, we found *tlc* gene sequences in additional clades, including *G. sulfuraria* (a red algae; GenBank accession number AJ251356 [236 of 468 identities]), *Citrus* hybrid cultivar (GenBank accession number AY098893 [231 of 480 identities]), *O. sativa* (rice; GenBank accession number AP003234 [236 of 496 identities]), *H. obtusa* (GenBank accession number AY120885 [183 of 465 identities]), *Caedibacter caryophilus* (GenBank accession number AJ441310 [45 of 53 identities]), and *M. sativa* (GenBank accession number AF416339 [52 of 81 identities]). However, when gap and extension penalties of 11 and 1, respectively, were used, BLAST analysis did not detect the *tlc* gene sequences in *Wolbachia* sp. (an endosymbiont of *Drosophila melanogaster*), cyanobacteria, protozoa, or animals. A BLAST search also identified four *E. cuniculi* proteins of unknown functions (GenBank accession numbers NP_586157 to NP_597260 [93 of 443 to 111 of 473 identities]). With the exception of the short *tlc* sequence of *M. sativa* (317 bp), we used all of these *tlc* sequences in the genetic and phylogenetic analyses.

**Proteins of unknown functions related to the ADP/ATP translocase in *Chlamydiales*, cyanobacteria, and plant pathogen (*Xylella fastidiosa*).** By PSI-BLAST, we identified proteins of unknown function with significant alignments within the proteomes of two cyanobacteria (*Nostoc* sp. and *Trichodesmium erythraeum*), within that of *Chlamydiales*, and within different γ-proteobacteria (including *X. fastidiosa*, a plant pathogen). We performed phylogenetic analysis with the amino acid sequences of all of these proteins of unknown function and with the four microsporidium proteins (which exhibit sequence similarity with ADP/ATP translocase but whose functions are also unknown) and with the *Chlamydiales*, *Rickettsiales*, and plant plastid ADP/ATP translocase. The tree topology was similar to that shown in Fig. 1, except that the *E. cuniculi* proteins and the other proteins of unknown function rooted deeply, being phylogenetically far from the ADP/ATP translocase (data not shown).

**Genetic analysis of amino acid and nucleotide sequences.** *P. acanthamoebae tlc* shared greater amino acid sequence similarity with *tlc*1 of *Chlamydiaceae* (62 to 63%) and with *tlc* of plant plastids (53 to 54%) than with *Rickettsiales* (34 to 43%) and *tlc*2 of *Chlamydiaceae* (36 to 38%) (Table 1). Similarly, *P. acanthamoebae tlc* shared greater nucleotide sequence similarity with *tlc*1 of *Chlamydiaceae* and with that of plant plastids than with *Rickettsiales* and *tlc*2 of *Chlamydiaceae* (data not shown). Conversely, plant plastid *tlc* shared greater nucleotide and amino acid sequences similarity with *tlc* of *P. acanthamoebae* and *tlc*1 of *Chlamydiaceae* than with those of *Rickettsiales* and *tlc*2 of *Chlamydiaceae*. Alignment of the amino acid sequences also showed that the ADP/ATP translocase of green plants and red alga plastids present a N-terminal peptide transit of about 90 and 150 amino acids, respectively, whereas that of bacteria do not present this peptide. Such transit peptides are present in most plastids proteins and are necessary to target the protein encoded by nuclear genes to plastids (5).

**Phylogenetic analysis.** Phylogenetic analysis generated robust trees with significant bootstrap values. Figure 1 shows a neighbor-joining tree inferred from amino acid sequences and rooted with the four *E. cuniculi* proteins identified by BLAST. Its topology was similar to that of the neighbor-joining tree inferred from the nucleotides sequences and from both minimum evolution and parsimony trees, with the only exception being that in both parsimony trees the *tlc* of *Holospora* sp. clustered with that of *Rickettsiales.* In neighbor-joining, minimum-evolution, and parsimony trees inferred from amino acid sequences, the node separating the *tlc*1 of *Chlamydiales* and the *tlc* of plant and alga plastids from the *tlc* of the *Rickettsia* spp. was supported by bootstrap values of 95, 92, and 44%,

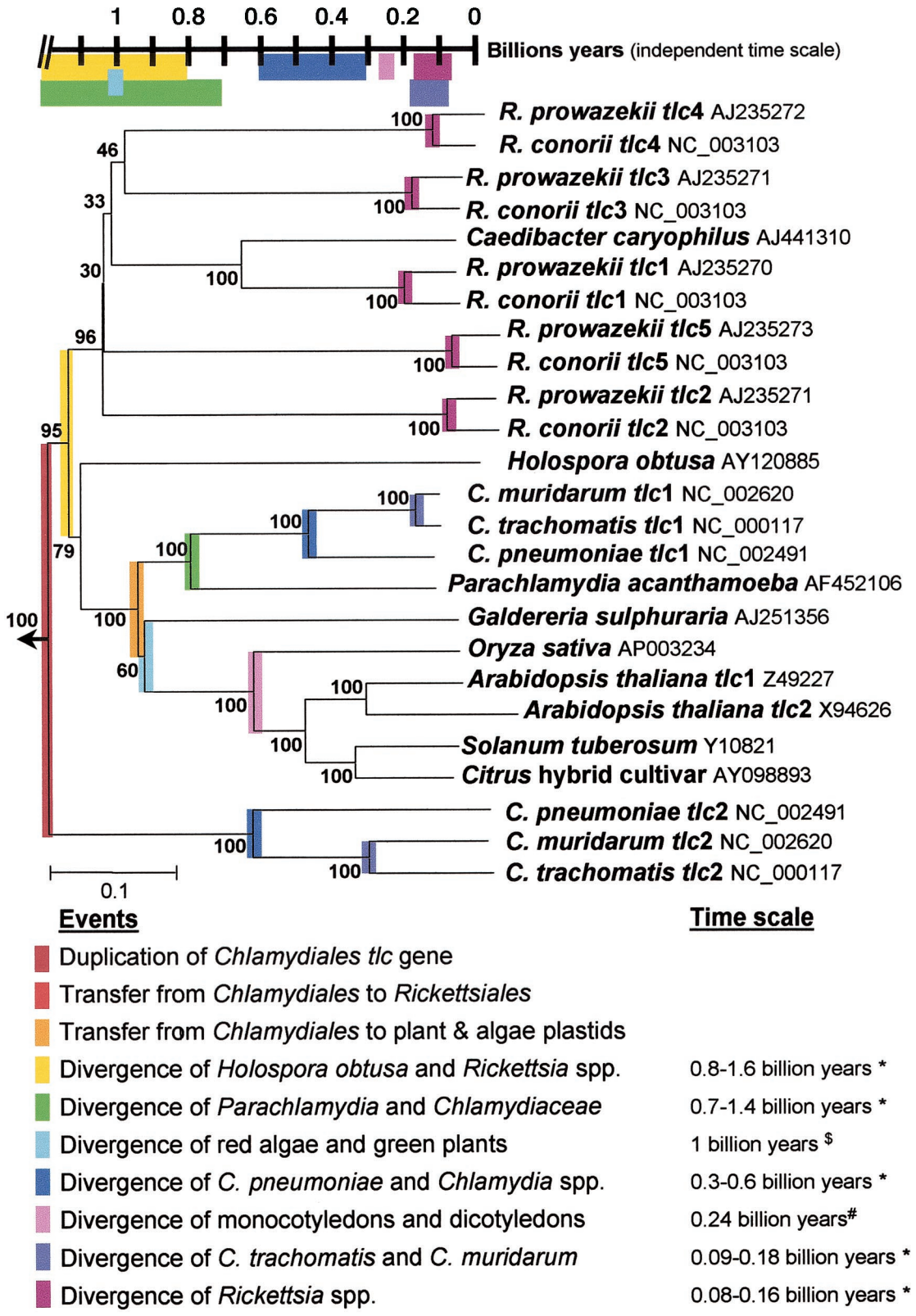


FIG. 1. p-Distance neighbor-joining tree inferred from amino acid sequences of the ADP/ATP translocase of *P. acanthamoebae*, *Chlamydiaceae*, *Rickettsiales*, and plant and alga plastids. Bootstrap values resulting of 100 replications are present at branch points. The tree was rooted with four proteins present in *E. cuniculi*, whose functions are unknown and were identified by BLAST of nonmitochondrial ADP/ATP translocase. The time scale was derived from estimates obtained from the literature (13, 26) and on the basis of 16S rRNA gene sequence divergence (20). Note the congruence between the node of a given divergence (see neighbor-joining tree) and its estimated time (see time scale). ⁕, Estimated from 16S rRNA divergence (20); $, estimated by Sogin et al. (26); #, estimated by Gale et al. (13).

TABLE 1. Comparison of similarities of complete amino acid sequences (excluding alignment gaps) of ADP/ATP-translocase-encoding genes (*tlc*) of *Chlamydiales*, *Rickettsiales*, and plant and alga plastids

| Sequence no. | Species | % Similarity of sequence[a]: | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| 1 | *Parachlamydia* sp. | — | | | | | | | | | | | | | | | | | | |
| 2 | *C. muridarum* 1[b] | 63 | — | | | | | | | | | | | | | | | | | |
| 3 | *Chlamydophila pneumoniae* 1 | 62 | 80 | — | | | | | | | | | | | | | | | | |
| 4 | *C. trachomatis* 1 | 63 | 96 | 80 | — | | | | | | | | | | | | | | | |
| 5 | *C. muridarum* 2 | 36 | 38 | 39 | 37 | — | | | | | | | | | | | | | | |
| 6 | *Chlamydophila pneumoniae* 2 | 38 | 40 | 41 | 40 | 66 | — | | | | | | | | | | | | | |
| 7 | *C. trachomatis* 2 | 37 | 39 | 39 | 38 | 84 | 68 | — | | | | | | | | | | | | |
| 8 | *R. conorii* 1[c] | 36 | 34 | 36 | 34 | 28 | 29 | 27 | — | | | | | | | | | | | |
| 9 | *R. conorii* 2 | 38 | 37 | 38 | 37 | 31 | 31 | 31 | 41 | — | | | | | | | | | | |
| 10 | *R. conorii* 3 | 40 | 40 | 40 | 40 | 31 | 30 | 31 | 39 | 45 | — | | | | | | | | | |
| 11 | *R. conorii* 4 | 34 | 33 | 34 | 33 | 30 | 29 | 29 | 36 | 40 | 40 | — | | | | | | | | |
| 12 | *R. conorii* 5 | 43 | 43 | 43 | 43 | 31 | 31 | 30 | 39 | 42 | 47 | 41 | — | | | | | | | |
| 13 | *H. obtusa* | 40 | 40 | 40 | 40 | 31 | 28 | 30 | 34 | 35 | 36 | 33 | 36 | — | | | | | | |
| 14 | *A. thaliana* 1 | 53 | 52 | 52 | 52 | 34 | 33 | 33 | 33 | 36 | 36 | 32 | 40 | 38 | — | | | | | |
| 15 | *A. thaliana* 2 | 46 | 46 | 45 | 46 | 29 | 30 | 29 | 28 | 30 | 30 | 27 | 34 | 32 | 84 | — | | | | |
| 16 | *S. tuberosum* | 54 | 52 | 53 | 52 | 34 | 33 | 34 | 34 | 37 | 38 | 33 | 39 | 39 | 86 | 76 | — | | | |
| 17 | *Citrus* hybrid cultivar | 53 | 51 | 52 | 51 | 35 | 34 | 34 | 33 | 36 | 37 | 33 | 39 | 38 | 86 | 77 | 91 | — | | |
| 18 | *O. sativa* | 53 | 52 | 53 | 51 | 35 | 34 | 35 | 33 | 37 | 38 | 34 | 39 | 38 | 80 | 72 | 82 | 83 | — | |
| 19 | *G. sulphuraria* | 53 | 52 | 52 | 52 | 33 | 33 | 33 | 36 | 36 | 39 | 33 | 41 | 38 | 59 | 51 | 59 | 59 | 61 | — |

[a] The number at the top of each column corresponds to the sequence as defined in column 1. —, 100% similarity.
[b] Isoenzyme 1.
[c] Similar rates obtained for *R. prowazekii*.

respectively, whereas the node separating the *tlc*1 of *Chlamydiales* from the *tlc* of plant and alga plastids was strongly supported by bootstrap values of 100, 100, and 65%, respectively. In all trees inferred from amino acid sequences, the node separating *tlc*1 of *Chlamydiales* from *tlc*2 of *Chlamydiales* was strongly supported by bootstrap values of 100%.

**Time scale.** The divergences of the 16S rRNA sequence between *Chlamydiales* and *Rickettsiales*, *P. acanthamoebae* and *Chlamydiaceae*, *H. obtusa* and *Rickettsia* spp., *Chlamydophila pneumoniae* and *Chlamydia* spp., and *R. conorii* and *Rickettsia prowazekii* were of 55, 14, 16, 6, and 1.59%, respectively. Thus, if we assume a rate of evolution of 1 to 2% per 50 million years (20), the respective divergences of these organisms may have occurred more than 2.75 billions years ago, 0.7 to 1.4 billion years ago, 0.8 to 1.6 billion years ago, 300 to 600 million years ago, and 80 to 160 million years ago (see Fig. 1).

**Congruence of p-distance and time scale.** The dependence of the p-distance of the neighbor-joining tree inferred from the amino acid sequences of the *tlc* genes on the time estimated for bacterial species on the basis of 16S rRNA sequence divergence (20) and for plastids from the time of divergence of monocotyledons and dicotyledons (13) and of *G. sulfuraria* (a red algae) and *Viridiplantae* (green plants) was nearly linear, as shown by the low extent of deviation from the regression line ($r^2 = 0.84$) (Fig. 2). The equation of the regression line is: $t = -4.11p + 1.28$, where $t$ is the time from now in billion years and "p" is the proportion of amino acid sites at which the sequence is different from that of the ancestral *tlc* sequence at the time of its duplication in *Chlamydiales*, i.e., the p-distance from a given node to the middle of the neighbor-joining tree (see Fig. 1 and 2). If $p = 0$, the estimated time of duplication of the *tlc* gene in *Chlamydiales* will be ca. 1.28 billion years. The reliability of this estimated time was supported by the omit test. By omitting any of each of the calibration point, the time of duplication of the *tlc* gene ranged from 1.20 to 1.33 billion years, with a mean of 1.28 ± 0.04 billion years. The estimated times of transfer of the *tlc* gene from *Chlamydiales* to *Rickettsiales* and to plant and alga plastids would be ca. 1.16 and 0.98 billion years, respectively. Similarly, the estimated times of duplication of the *tlc* gene in *Rickettsia* spp. and in *A. thaliana*

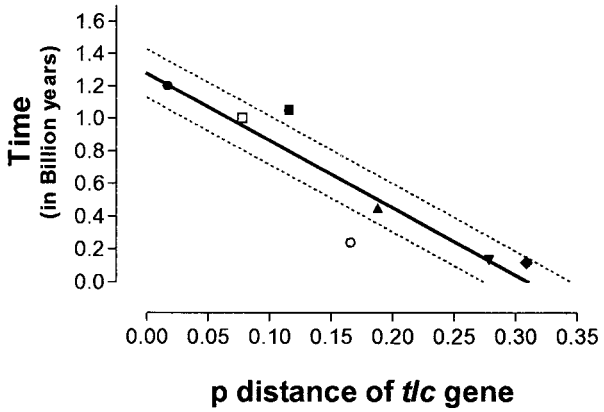


FIG. 2. Congruence ($r^2 = 0.88$) of the time scale and the p-distance of the *tlc* gene. The time scale was derived from independent estimates obtained from the literature (13, 26) and on the basis of 16S rRNA gene sequence divergence (20). The p-distance between each node and the midpoint of the neighbor-joining tree inferred from the amino acid sequences of the ADP/ATP translocase represent the proportion (p) of amino acid sites at which the sequence is different from that of the ancestral sequence, at the time of duplication of the *Chlamydiales* ADP/ATP-translocase-encoding gene (*tlc*). Symbols: ●, divergence of *H. obtusa* and *Rickettsia* spp.; ■, divergence of *Parachlamydia* spp. and *Chlamydiaceae*; □, divergence of red algae and green plants; ▲, divergence of *C. pneumoniae* and *Chlamydia* spp.; ○, divergence of monocotyledons and dicotyledons; ▼, divergence of *C. trachomatis* and *C. muridarum*; ◆, *Rickettsia* spp. Lines: solid line, regression line ($t = -4.11p + 1.28$); dotted lines, confidence intervals ($t = -4.11p + 1.13$ and $t = -4.11p + 1.43$).

TABLE 2. Date of duplication of *Chlamydiales tlc* gene and transfers of the *tlc* gene from *Chlamydiales* to *Rickettsiales* and from *Chlamydiales* to plant and alga plastids[a]

| Event | Date (in billion yr)[b] estimated by: | |
|---|---|---|
| | Neighbor joining | Minimum evolution |
| Duplication of *Chlamydiales tlc* gene | 1.28 | 1.27 |
| Transfer from *Chlamydiales* to *Rickettsiales* | 1.16 | 1.15 |
| Transfer from *Chlamydiales* to plant and alga plastids | 0.98 | 0.97 |
| Divergence of red algae and green plants | 0.97 | 0.95 |
| Divergence of monocotyledons and dicotyledons | 0.51 | 0.50 |

[a] Estimated with ADP-ATP translocase amino acid-sequences by using neighbor-joining and minimum-evolution trees with a time scale derived from the literature (13, 26) and on the basis of 16S rRNA sequence divergence.

[b] The dates of divergence of red algae from green plants and of monocotyledons from dicotyledons were estimated by using a time scale calibrated only on the basis of 16S rRNA sequence divergence.

would be about 1,017 to 1,095 and 223 million years, respectively. The extent of deviation of the regression line was lower ($r^2 = 0.94$) when only the time of divergence of bacterial species estimated on the basis of 16S rRNA sequence divergence was used as a time scale (20). The equation of the regression line was then: $t = -4.13p + 1.34$. The p-distance was 0.089 between the node separating the *G. sulfuraria* from *Viridiplantae* and the midpoint of the neighbor-joining tree (Fig. 1); thus, their time of divergence is about 0.97 billion years. Similarly, the time of divergence for monocotyledons and dicotyledons was estimated to be about 507 million years. Using the minimum evolution tree inferred from the amino acid sequence, the date of duplication of the *tlc* gene, the date of transfer of the gene from *Chlamydiales* to plants, the date of divergence of algae from green plants, and the date of divergence of monocotyledons and dicotyledons are close to those estimated with the neighbor-joining tree (Table 2). By omitting any of the calibration points and using the minimum-evolution tree inferred from the amino acid sequence, the time of duplication of the *tlc* gene ranged from 1.19 to 1.33 billion years, with a mean of 1.27 ± 0.05 billion years, further supporting the reliability of the estimated times.

## DISCUSSION

The sequence of the *tlc* gene in *P. acanthamoeba* was detected in the present study and demonstrated to have been present about 1 billion years ago when *Parachlamydiaceae* and *Chlamydiaceae* are estimated to have diverged. By BLAST analysis, we also detected the sequences of the *tlc* genes in *G. sulfuraria* (red algae), *Citrus* hybrid cultivar (dicotyledons), *O. sativa* (monocotyledons), *H. obtusa* (*Rickettsiales* exclusively growing within the nucleus of some *Paramecium* spp.), and *Caedibacter caryophilus* (another endosymbiont of *Paramecium* spp. belonging to *Rickettsiales*). We also detected four proteins of unknown function in *E. cuniculi* (microsporidia), which shared some degree of similarity with the *tlc* gene. With these sequences, those of the five *tlc* paralogs of *R. conorii* (23) and those used by Wolf et al. (30), we performed genetic analysis and generated robust trees with significant bootstrap values. The topology of these trees is similar to that recently published by Amiri et al. (3). The deep branching of the four *E. cuniculi* proteins suggests that the ADP/ATP translocase of *Chlamydiales*, *Rickettsiales*, and plant plastids share a common ancestor with these proteins. The presence of additional proteins of unknown function in the genome of *Chlamydiales*, cyanobacteria, and plant pathogens (such as *X. fastidiosa*), which exhibited similar sequences profiles with ADP/ATP translocase and, like *E. cuniculi* proteins, branched deep in the phylogenetic tree, suggests that the ADP/ATP translocase shares a common ancestor with another transmembrane transport protein that is present in both cyanobacteria and *Chlamydiales*. The function of these proteins, like the one found by BLAST in *E. cuniculi* genome, remains to be determined. We focused here on analysis of the evolutionary relationship of the ADP/ATP translocase, whose function has been confirmed only for *Rickettsiales*, *Chlamydiales*, and plant plastids. The closer genetic similarity of the *tlc* of red algae and plant plastids with that of *Chlamydiales* compared to those of *Rickettsiales* suggested that the *tlc* gene has been exchanged between eucarya and the ancestral *Chlamydiales*. The tree shown in Fig. 1 supports our hypothesis that *tlc* originated in the *Chlamydiales* ancestor and not in plants. Indeed, if the two *tlc* genes found in *Chlamydiaceae* resulted from gene duplication, this event predates the divergence of the *Parachlamydiaceae* and *Chlamydiaceae* and the transfer of *tlc*1 to eucaryotes (Fig. 1). This tree inferred from amino acid sequences was representative of the topology obtained with parsimony and with other datasets.

Our study provides a scale of time derived from the divergence of 16S rRNA gene sequences (20), with or without additional time estimates obtained from the literature (13, 26). The congruence of the p-distance derived from amino acid sequences of the ADP/ATP translocase with these time scales ($r^2$ coefficients of 0.84 and 0.94, respectively) confirms the value of the tree presented in Fig. 1. This congruence confirms the value of the calibration of the molecular clock, performed based on both a fossil estimate and another molecular clock. The congruence of the p-distance derived only from *tlc* nucleotide sequences with the scale of time derived from the divergence of 16S rRNA sequences is further confirmed by the estimated time of divergence of red algae from green plants of 0.97 billion years that is similar to the 1 billion years estimate of Sogin and Silberman (26). The reliability of these results is further confirmed by the fact that dates estimated by using the neighbor-joining tree inferred from the ADP/ATP translocase amino acid sequences were really close to those estimated by using the minimum-evolution tree (Table 2) and by the fact that omission of any of the calibration point of the time scale only slightly modified the estimated time of duplication of the *tlc* genes in the *Chlamydiales* (standard deviations of 0.04 and 0.05 billions years, respectively).

The time scale analysis suggests that the ancestral sequence duplicated in *Chlamydiales* 1.27 to 1.28 billion years ago (see Table 2). Since the estimated time of transfer of the *tlc* gene to *Rickettsiales* was about 1.15 to 1.16 billion years, the presence of *tlc* genes is probably due to horizontal transfer and not to the speciation of *Chlamydiales* from *Rickettsiales*, which is estimated to have occurred more than 2.75 billion years ago. Although horizontal transfer of genes between *Rickettsiales* and *Chlamydiaceae* is unlikely to occur today, since these clades do not share common host cells, such a transfer may

have occurred more than one billion years ago, at a time when these bacteria may have been facultatively intracellular and may have shared a common ancestral cell host, such as a free-living amoeba. The latter hypothesis is supported by the fact that a branch of evolution of both clades still parasitizes *Acanthamoeba* spp. (2, 11). The absence of the *tlc* gene in *Wolbachia* sp. (a clade that diverged from *Rickettsia* spp. 100 to 200 million years ago) is probably due to subsequent gene loss (22). The *tlc*1 gene was apparently transferred to eucarya 0.97 to 0.98 billion years ago, i.e., around the time when *Chlamydiaceae* and *Parachlamydiaceae* diverged (0.7 to 1.4 billion years ago). The presence of *tlc*1 in red algae and in higher plant plastids and its absence in the sequenced genomes of protozoan and animals show that transfer occurred after the divergence of red algae and plants from other eucaryotes but before that of *Rhodophyta* (red algae) from *Viridiplantae* (green plants). The latter divergence is contemporary with the eukaryotic radiation, which has also been estimated to have occurred about 1 billion years ago (9, 26). Since these time evaluations are congruent with our tree representations, we believe that they reflect the true time of transfer of *tlc*1 to eukaryotes.

Everett et al. (10) suggested that the common *Chlamydiales* ancestor might be related to the cyanobacterium-chloroplast lineage; the absence of the *tlc* gene in the genome of *Synechocystis* sp., *Nostoc* sp., and *T. erythraeum* (three cyanobacteria) (16), being then explained by a subsequent gene loss (22) or by the appearance of that gene in the *Chlamydiales* genome after their speciation from cyanobacteria. Brinkman et al. (6) showed that the vast majority of plant-like genes in *Chlamydiales* correspond to plant genes that are derived from and function in the chloroplast, suggesting that the ancestral *Chlamydiales* might have been involved in the endosymbiotic origin of chloroplasts or have at least played a role in the chimeric events that led to the formation of plant lineages.

The existence of the *tlc* gene and its duplication, long before the radiation of eukaryotes, including plants and animals, demonstrates the long history of parasitism of *Chlamydiales* with unicellular eukaryotes and is the oldest evidence of bacterial parasitism. One branch of *Chlamydiales* still parasitizes protozoa, whereas the other is associated with multicellular animals.

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