Evolutionary Relationships among Eubacterial Groups as Inferred from GroEL (Chaperonin) Sequence Comparisons

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The essential GroEL proteins represent a subset of molecular chaperones ubiquitously distributed among species of the eubacterial lineage, as well as in eukaryote organelles. We employed these highly conserved proteins to infer eubacterial phylogenies. GroEL from the species analyzed clustered in distinct groups in evolutionary trees drawn by either the distance or the parsimony method, which were in general agreement with those found by 16S rRNA comparisons (i.e., proteobacteria, chlamydiae, bacteroids, spirochetes, firmicutes [gram-positive bacteria], and cyanobacteria-chloroplasts). Moreover, the analysis indicated specific relationships between some of the aforementioned groups which appeared not to be clearly defined or controversial in rRNA-based phylogenetic studies. For instance, a monophyletic origin for the low-G+C and high-G+C subgroups among the firmicutes, as well as their specific relationship to the cyanobacteria-chloroplasts, was inferred. The general observations suggest that GroEL proteins provide valuable evolutionary tools for defining evolutionary relationships among the eubacterial lineage of life.

The study of macromolecules emphasizing the historical information contained in their sequences has resulted in profound changes in our conception of the evolution of life on our planet, with its attendant consequences for the classification of living organisms (27, 30, 43, 53). In particular, comparisons of the 16S rRNA sequences from a large number of species have been pivotal in providing evidence of three primary lines of descent, two of them leading to the prokaryotic lineages (*Eu*) *Bacteria* and *Archaea* (27, 28, 30, 53). These studies also indicate that the eubacterial lineage has evolved into (at least) 10 distinct divisions, although the specific relationships among (and sometimes within) them have yet to be convincingly determined (28, 30, 31, 43, 53, 56).

Given the limitations inherent in the assumptions on which current phylogenetic methods are based, phylogenies based on a single macromolecule may not necessarily reflect the true phylogeny of the lineages in which it occurs (5, 9, 15, 30, 43, 44, 56). Therefore, it is becoming increasingly evident that resolution of the evolutionary relationships between organisms (especially prokaryotes) undoubtedly requires comparative analysis of data from different macromolecules showing useful features as molecular chronometers (5, 30, 43).

The GroEL, or Hsp60 (the common major antigen in numerous eubacterial genera [7]), chaperonins constitute a family of highly conserved housekeeping proteins. These proteins are ubiquitously distributed among eubacteria and eukaryotic organelles and possess functions essential for the survival of cells in physiological, as well as stressful, situations (7, 10, 13). The similarities between evolutionary trees drawn from a limited set of these molecules and those of 16S rRNA have been noted previously (7, 10). We extended this phylogenetic analysis by using an expanded GroEL data base and found that these proteins represent valuable molecular chronometers.

MATERIALS AND METHODS

GroEL as an evolutionary chronometer. Widely different base compositions in the different lineages under study have been reported to constitute potential sources of inconsistencies when nucleotide sequences (including those of rRNA) are compared for phylogenetic studies (15, 30, 44). Since this substitutional bias is minimized in highly conserved proteins (15, 20), inferences based on comparisons of their amino acid sequences have been proposed to be more reliable than those based on the corresponding nucleotide sequences (15).

Analysis of the GroEL proteins of the organisms listed in Table 1 indicates that the tendencies seen in nonconserved proteins (i.e., correlation of low G+C base content with increases in Ile, Lys, Phe, and Tyr on the one hand and high G+C content with Ala, Arg, and Gly on the other [20]) are minimized in these highly conserved proteins (49) and that most of the amino acid changes result in conservative substitutions (10, 49). Moreover, the size (ca. 550 amino acid residues), as well as the highly conserved function, of these proteins (10, 13) appears to include most of the desirable features of a molecular chronometer (30, 43, 53). Therefore, we used comparisons of GroEL amino acid sequences rather than the corresponding nucleotide sequences for inferences of eubacterial phylogenies.

Data sources and data base searches. The organisms from which *groEL* genes have been characterized, their affiliations according to 16S rRNA analysis, and the sources of information are provided in Table 1. DNA and protein data base searches were performed at the National Center for Biotechnology Information by using the BLAST network service (2).

Data analysis. Alignments of the 58 GroEL protein sequences indicated in Table 1 were done as described previously (10), and final adjustments were decided after visual inspection. To calculate evolutionary distances, 525 aligned positions were employed after removal of ambiguous alignments that include in all sequences a stretch of nine amino acids equivalent to *Escherichia coli* GroEL positions 427 to 435), the C-terminal portion (starting at the position equivalent to *E. coli* GroEL position 531), and transit peptides from eukaryotic sequences. Evolutionary distances were computed by using the amino acid conversion table (PAM 001) compiled by Dayhoff

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TABLE 1. Organisms, corresponding affiliations, and sources of Hsp60 sequences used in this work

Organism and affiliation ^a	GenBank accession no.	Reference
α-Proteobacteria		
<i>Bartonella bacilliformis</i>	M98257	UD ^b
<i>Brucella abortus</i>	L09273	14
<i>Agrobacterium tumefaciens</i>	X68263	39
<i>Rhizobium leguminosarum</i>	L20775	UD
<i>Rhizobium meliloti</i> A ^c	M94192	35
<i>Rhizobium meliloti</i> C	M94190	35
<i>Rhizobium meliloti</i> C ^c	M94191	35
<i>Bradyrhizobium japonicum</i> 2 ^c	Z22604	11
<i>Bradyrhizobium japonicum</i> 3 ^c	Z22603	11
<i>Zymomonas mobilis</i>	L11654	UD
<i>Ehrlichia chaffeensis</i>	L10917	46
<i>Rickettsia tsutsugamushi</i>	M31887	45
β-Proteobacteria		
<i>Neisseria gonorrhoeae</i>	Z23008	UD
<i>Neisseria flavescens</i>	Z22955	UD
<i>Neisseria meningitidis</i>	Z22956	UD
γ-Proteobacteria		
<i>Coxiella burnetii</i>	M20482	10
<i>Pseudomonas aeruginosa</i>	M63957	42
<i>Haemophilus ducreyi</i>	M91030	32
<i>Salmonella typhi</i>	U01039	UD
<i>Escherichia coli</i>	X07850	16
Symbiont P of <i>Acyrtosiphon pisum</i>	X61150	10
<i>Yersinia enterocolitica</i>	X68526	UD
<i>Chromatium vinosum</i>	M99443	10
<i>Legionella pneumophila</i>	M31918	18
<i>Legionella micdadei</i>	X57520	10
Symbiont of <i>Amoeba proteus</i>	M86549	1
δ/ε-Proteobacteria		
<i>Helicobacter pylori</i>	X73840	23
Bacteroids		
<i>Porphyromonas gingivalis</i>	D17398	UD
Chlamydiae		
<i>Chlamydia trachomatis</i>	M31739	10
<i>Chlamydia pneumoniae</i>	M69217	21
<i>Chlamydia psittaci</i>	X51404	10
Spirochetes		
<i>Leptospira interrogans</i>	L14682	3
<i>Borrelia burgdorferi</i>	X54059	40
<i>Treponema pallidum</i>	X54111	17
Firmicutes (gram-positive bacteria), low G+C content		
<i>Clostridium perfringens</i>	X62914	10
<i>Clostridium acetobutylicum</i>	M74572	10
Thermophilic bacterium PS-3	P26209	10
<i>Bacillus stearothermophilus</i>	L10132	38
<i>Bacillus subtilis</i>	M81132	10
<i>Lactococcus lactis</i>	X71132	UD
<i>Staphylococcus aureus</i>	S62126	29
Firmicutes (gram-positive bacteria), high G+C content		
<i>Streptomyces albus</i>	M76658	25
<i>Mycobacterium leprae</i>	M14341	26
<i>Mycobacterium tuberculosis</i>	M17705	41
<i>Mycobacterium paratuberculosis</i>	X74518	UD
<i>Streptomyces albus</i> ^c	M76657	25
<i>Streptomyces coelicolor</i> ^c	X75206	UD
<i>Mycobacterium leprae</i> ^c	S25181	34
<i>Mycobacterium tuberculosis</i> ^c	X60350	UD

Continued

TABLE 1. Continued

Organism and affiliation ^a	GenBank accession no.	Reference
Cyanobacteria		
<i>Synechococcus</i> sp. strain PCC 7942	M58751	10
<i>Synechocystis</i> sp. strain PCC 6803 ^c	D12677	10
<i>Synechocystis</i> sp. strain PCC 6803	M57517	22
Chloroplasts		
<i>Cyanidium caldarium</i>	X62578	24
<i>Triticum aestivum</i> α ^d	X07851	16
<i>Ricinus communis</i> α	X07852	16
<i>Brassica napus</i> α	M35599	10
<i>Brassica napus</i> β ^d	M35600	10
<i>Arabidopsis thaliana</i> β	JT0901 ^e	55

^a As indicated by 16S rRNA analysis (28, 31).^b UD, unpublished data.^c *groEL* is present in a *groESL* operon (this applies only in cases in which more than one *groEL* gene has been reported in a particular species).^d α/β indicates the type of polypeptide that composes the plant chloroplast GroEL chaperonin (10).^e PIR accession number.

et al. (8). For construction of phylogenetic trees, we employed the neighbor-joining distance method (36), which has been shown in model studies to be relatively consistent compared to other methods, even in the presence of unequal rates of evolution (36). For comparisons, phylogenetic trees were also inferred by using the PROTPARS maximum-parsimony method (9). Confidence limits for the inferences obtained were placed by using the "bootstrap" procedure (9).

The programs PROTDIST, NEIGHBOR, PROTPARS, SEQBOOT, and CONSENSE, present in the PHYLIP package (9), version 3.5 (kindly provided by J. Felsenstein, University of Washington, Seattle), were employed in this work. GroEL protein alignments and evolutionary distances were provided for the reviewing process and are available from us on request.

RESULTS AND DISCUSSION

The evolutionary relationships between the eubacterial species listed in Table 1, as inferred from their GroEL proteins, are shown in Fig. 1. Our analyses by the distance (Fig. 1) and parsimony (data not shown) methods indicate the presence of two defined clusters, one of which includes proteobacteria, chlamydiae, spirochetes, and the eubacterial species *Porphyromonas gingivalis* and *Helicobacter pylori* and appears to be clearly separated from another cluster formed by cyanobacteria-chloroplasts and firmicutes. Detailed comparison of the amino acid sequences indicated that the homologs from the former cluster (i.e., proteobacteria, chlamydiae, spirochetes, *P. gingivalis*, and *H. pylori*) contain a Lys residue at the position equivalent to *E. coli* GroEL position 51, whereas an Asn residue is present at the equivalent position in those from the cyanobacteria-chloroplasts-firmicutes cluster (49).

We have analyzed the results shown in this work at two levels, both within and between each of the distinct groups depicted in Fig. 1, contrasting our observations with the evolutionary relationships based on 16S rRNA analysis (4, 6, 28, 30, 31, 33, 37, 43, 48, 51–54, 56) as follows.

Proteobacteria. This eubacterial division contains a large number of species whose outstanding attribute is their diversity of phenotypes (56). The proteobacteria (as a whole) could not be defined by a simple signature in 16S rRNA comparisons;

only phylogenetic trees based on this molecule define this group (43, 53, 56). Since some degree of uncertainty has been found in these inferences, their corroboration by the use of other molecules is important from an evolutionary perspective (43). Our analysis based on GroEL sequence comparisons tends to support rRNA-based inferences, including the separation of proteobacterial species into distinct subdivisions depicted in rRNA trees, as well as the internal relationships found in these analyses within them (4, 6, 28, 30, 31, 33, 37, 43, 48, 51–54, 56), as discussed below.

α -Proteobacteria. We observed that the species assigned to the α -proteobacterial subdivision by rRNA analysis (4, 6, 28, 31, 37, 43, 51–54, 56) also clustered in GroEL-derived trees drawn by either the distance (Fig. 1) or the parsimony (data not shown) method. Our analysis revealed a clearly defined subgroup within the α -proteobacteria which includes two species of rhizobia, as well as *Agrobacterium tumefaciens*, *Brucella abortus*, *Bartonella bacilliformis*, *Bradyrhizobium japonicum*, and *Zymomonas mobilis* (Fig. 1). This group has also been observed in rRNA trees and designated the *Agrobacterium-Rhizobium* cluster (51). Interestingly, we observed within this cluster a closer relationship between the GroEL proteins of *B. bacilliformis*, *B. abortus*, *Rhizobium leguminosarum*, and *A. tumefaciens* and the protein encoded by *groESL* operon A of *R. meliloti* (35) (Fig. 1). This particular affiliation was also supported by a peculiarity in the protein sequences which occurs only among the latter five species at the position equivalent to *E. coli* GroEL position 427 (a Thr [or Ser] residue followed immediately by an amino acid deletion [49]). It is worth mentioning that similar affiliations (i.e., a closer relationship between *Agrobacterium*, *Rhizobium*, *Brucella*, and *Bartonella* species and the presence of *Bradyrhizobium* species as loosely related to the bacteria mentioned among the α -proteobacteria) have been determined by 16S rRNA analysis (4, 37, 51, 52, 54).

Phylogenetic trees derived by both distance (Fig. 1) and parsimony (data not shown) analyses of GroEL suggest that the aforementioned *Agrobacterium-Rhizobium* cluster is distant from but specifically related to a fast-evolving subgroup that includes *Ehrlichia chaffeensis* and *Rickettsia tsutsugamushi* (family *Rickettsiaceae* [51]). Although the unique phenotypic traits of *R. tsutsugamushi* have made its classification uncertain (7, 45), recent 16S rRNA analysis has indicated that this organism is closely affiliated with species of the genus *Rickettsia* (6), which in turn form a cluster with those of the genus *Ehrlichia* (4, 6, 31), in agreement with our observations (Fig. 1). The latter results are particularly relevant to the observation that mitochondrial GroEL homologs are closely affiliated with those of the aforementioned *Rickettsia-Ehrlichia* cluster (50), which not only supports the proposed α -proteobacterial ancestry for the mitochondrion (5) but also focuses on the nature of its putative closest ancestors, as discussed elsewhere (50).

The situation of *R. meliloti* and *B. japonicum* merits comment, given that several *groEL* genes have been identified in these bacteria (11, 35). Our results (Fig. 1) suggest that the *B. japonicum groEL* genes likely represent products of a duplication event. On the other hand, two of the *R. meliloti* GroEL proteins (those encoded by gene C and *groESL* operon C [35]) were found in our analysis not immediately affiliated to their homolog present in operon A but related to those of *B. japonicum* (although bootstrap analysis did not provide high confidence for this particular affiliation; Fig. 1). Given that only the product of *groESL* operon A shows the sequence idiosyncrasy mentioned above, as well as the close similarity between these particular affiliations and those obtained for the

same species by 16S rRNA analysis (4, 37, 52, 54), it seems reasonable to propose that the aboriginal *R. meliloti groESL* genes are represented by operon A. The two extra *groE* genes (gene C and that of operon C [35]) may have been acquired by lateral gene transfer, a situation not totally unexpected among soil bacteria. Nevertheless, alternative explanations for these results would be based on assuming either the loss of some duplicated genes in all of the proteobacterial species examined (with the exception of *B. japonicum* and *R. meliloti*) or differential evolution of *R. meliloti groEL* genes after duplication.

β - and γ -Proteobacteria. GroEL from species of the β - and γ -proteobacterial subgroups (as defined by 16S rRNA analysis) clearly clustered in our analysis (Fig. 1), and the phylogenetic tree obtained closely resembles those obtained by 16S rRNA analyses (28, 31, 43, 48, 53, 56). For instance, a clearly defined cluster formed by *Haemophilus ducreyi*, *Yersinia enterocolitica*, *E. coli*, *Salmonella typhi*, and symbiont P of *Acyrtosiphon pisum* was observed (Fig. 1), therefore reinforcing the specific relationships between these bacterial species found in 16S rRNA trees (28, 31), as well as the affiliation of the latter symbiont with the *Y. enterocolitica*-enteric bacteria cluster among the γ -proteobacteria (48). On the other hand, the endosymbiont of *Amoeba proteus* (X-bacterium [1]) was found in close affiliation with a distinct cluster within the γ -proteobacteria formed by *Legionella* species (Fig. 1). This represents the first classification of the latter bacterium based on a comparison of molecular sequences.

The specific affiliations of *Chromatium vinosum*, *Coxiella burnetii*, and *Pseudomonas aeruginosa* within the γ -proteobacteria could not be defined precisely in our study, as judged by the bootstrap values obtained in these cases (Fig. 1). In any case, the presence of the phototroph *C. vinosum* in this proteobacterial subdivision reinforces the idea that all of these species evolved from free-living, phototrophic ancestors (10, 53). In turn, the presence of *C. burnetii* within the γ -proteobacteria, in addition to the aforementioned affiliation of *B. bacilliformis* with α -proteobacterial species (Fig. 1), supports the proposed polyphyletic nature of the order *Rickettsiales*, as well as the family *Rickettsiaceae* (4, 51).

Interestingly, although GroEL sequences from only a single β -proteobacterial genus (i.e., *Neisseria*) were available for analysis, our results (Fig. 1) are in line with the presence in rRNA trees of the β -proteobacteria as a subgroup (albeit deeply branching [43, 53]) of the γ -proteobacteria.

Chlamydiae. The chlamydiae constitute a group of obligate intracellular organisms that are differentiated from other bacteria by their unique developmental cycles (53). In GroEL-based phylogenetic trees drawn by either the distance (Fig. 1) or the parsimony (data not shown) method, *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* formed a clearly defined cluster, an observation that sustains results based on rRNA analyses (12, 28, 30, 31, 43, 53, 56). Moreover, our results also reinforce the closer affiliation of *C. pneumoniae* with *C. psittaci* (12), despite the low degree of homology between these organisms on the DNA level.

Spirochetes. GroEL chaperonins from the spirochetal species analyzed (*Leptospira interrogans*, *Treponema pallidum*, and *Borrelia burgdorferi*) formed a cluster separate from those of the other eubacterial groups by either the distance (Fig. 1) or the parsimony (data not shown) method, a result that reinforces the conclusions drawn from comparisons of their phenotypes, as well as rRNA analysis (28, 30, 31, 43, 53, 56), including the early branching of the leptospires in these trees (31).

H. pylori and P. gingivalis. *H. pylori* and *P. gingivalis* have been assigned by rRNA analysis to the δ/ϵ -proteobacteria (*H.*

FIG. 1. Evolutionary relationships between eubacterial species and groups based in GroEL sequence comparisons. An unrooted phylogenetic tree was constructed by the neighbor-joining distance method (36) as described in Materials and Methods. The number at end branch indicates the number of times that the adjacent two groups it defines occurred, as obtained by the bootstrap procedure from 100 replicated trees (9). The designations [operon] and [gene] indicate the presence of more than one *groEL* gene in a particular species, either linked to a *groES* gene in a *groESL* operon ([operon]) or in a form ([gene]) which is not immediately linked to *groES*. The length of each branch is proportional to the calculated evolutionary distance, and the scale is indicated at the bottom. The denomination of the major eubacterial groups is that of Table 1.

pylori [28, 31]) and to the bacteroids among the *Cytophaga-Flavobacter-Bacteroides* group (*P. gingivalis* [33]). Although in these cases the number of GroEL sequences is quite restricted and bootstrap analysis did not provide enough confidence for these particular affiliations to allow definite conclusions, it is worth mentioning that the branching orders in Fig. 1 corresponding to proteobacteria, chlamydiae, spirochetes, and *P. gingivalis* were reproducibly formed and resemble those observed for the corresponding groups in recently published 16S rRNA trees (28, 31).

In any case, a larger *groEL* data base, especially from δ/ϵ -proteobacterial species, as well as from the bacteroids and related groups (28, 31), may be necessary to clarify these particular affiliations.

Cyanobacteria-chloroplasts. GroEL from *Synechococcus* and *Synechocystis* species, as well as those from several plastids, formed a clearly defined cluster in either distance or parsimony analysis (Fig. 1 and data not shown). This result agrees with inferences based on rRNA comparisons concerning the putative affiliation of the symbiont that generated the chloroplasts among all of the photosynthetic eukaryotes (including red algae) in the cyanobacterial lineage (5, 50, 53). A detailed discussion of these results has been published elsewhere (50).

Firmicutes. The species of the firmicutes group have been referred to as gram-positive bacteria (28, 30, 43, 53, 56) or posibacteria (5). In turn, in the 1984 edition of *Bergey's Manual of Systematic Bacteriology*, these organisms are classified as firmicutes, a term that is indicative of the rigid, dense cell walls of most of the species that form this eubacterial division (27) and which we have used in this work.

Either distance (Fig. 1) or parsimony (data not shown) analysis clearly separated the species of firmicutes with high G+C content from those with low G+C content. The bacterial relationships within the low-G+C content subgroup closely resembled those of rRNA trees (28, 31, 43), e.g., the clustering of clostridial species and their separation from the group formed by *Lactococcus lactis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *B. stearothermophilus* (Fig. 1). It is also worth noting the specific affiliation of thermophilic bacterium PS-3 with the *Bacillus* group (specifically, with *B. stearothermophilus*), which represents the first classification of this bacterium based on molecular terms.

Concerning the high-G+C content subgroup of firmicutes, a duplication that specifically involves the *groEL* gene seems to have occurred in the last ancestor of actinomycetes and mycobacteria (19). Interestingly enough, a clear separation between the high-G+C content species of firmicutes in which *groEL* genes are linked to *groES* in an operon (labeled [operon] in Fig. 1) and those in which the genes are separated from *groES* (labeled [gene]) was observed. Moreover, the former genes encode GroEL proteins with unusually high His residue contents at their C termini (25, 34, 49), a deviation from the classical Gly/Met-rich region found in these proteins (including those encoded by *groEL* [gene] in these bacteria [10]). The significance of this particular idiosyncrasy (25, 34), as well as the physiological role of the extra *groEL* genes (which have evolved differentially [19]), remains obscure.

There exist some controversies concerning the specific relationship between and within the gram-positive bacteria in recent 16S rRNA-based trees drawn by different methods (28, 31). Our results (Fig. 1) suggest that the high-G+C content and low-G+C content subgroups of firmicutes shared a last common ancestor. Although bootstrap analysis does not produce much confidence for this particular result, comparisons of polypeptide sequences indicate the existence of a particular deletion (equivalent to *E. coli* GroEL position 154) which is shared only by the homologous proteins present in firmicutes (49). Therefore, the overall observations support the notion of last common ancestry for the high-G+C content and low-G+C content subgroups of firmicutes, in agreement with maximum-likelihood-derived 16S rRNA trees (31).

Concerning the affiliation of firmicutes with the other eubacterial groups analyzed in this work, our analysis by either the distance (Fig. 1) or the parsimony (data not shown) method indicated that cyanobacteria-chloroplasts and firmicutes are specifically affiliated, a result that was supported by the bootstrap test. Although this particular relationship was also suggested previously by rRNA sequence signatures (53), it is not evident in rRNA-based phylogenetic trees (28, 31, 43, 53).

The overall observations of this work are summarized in Fig. 2. Our results indicate a clear separation between the branches that lead to cyanobacteria-firmicutes on the one hand and spirochetes-chlamydiae-bacteroids-proteobacteria on the other. The uncertainty in defining specific affiliations (branching orders) between certain eubacterial groups is also indicated in Fig. 2 by the shaded circle.

For a number of reasons, protein sequence comparisons

FIG. 2. Evolutionary relationships between eubacterial groups as inferred from GroEL chaperonins. The distinct eubacterial clusters are depicted as triangles in which the base is proportional to the number of GroEL protein sequences and the height represents the average distance (calculated from the data of Fig. 1) separating the terminal nodes from the deepest branching point within the cluster. Branches in which bootstrap support was lower than 50 of 100 replicated trees have been collapsed, and this uncertainty in the branching order is represented by the shaded circle. The evolutionary distance scale is shown at the bottom.

have not made a large contribution to the field of bacterial systematics (43). In this sense, it appears advantageous to use the identification of GroEL proteins as powerful tools, complementary to rRNA (and other molecules), to define evolutionary relationships among eubacterial species and groups. This emerges as particularly relevant in cases in which affiliations between some eubacterial groups appear controversial, as seems to be the case for 16S rRNA trees inferred by different methods (28, 31). Since *groEL* genes have been identified in species of seven of the major groups that compose the eubacterial lineage (i.e., cyanobacteria, firmicutes, spirochetes, chlamydiae, proteobacteria, bacteroids, and *Thermus thermophilus* [47]) and no sequence-related GroEL proteins have been found in species of *Archaea* (albeit functional homologs exist [10]), further characterization of *groEL* genes (especially in the deepest eubacterial branchings [28, 30, 31]) may help to elucidate the evolutionary path of this major lineage of life.

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