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Revisiting bacterial phylogeny

Natural and experimental evidence for horizontal gene transfer of 16S rRNA

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Current methods used for phylogenetic classification of prokaryotes largely rely on the sequences of 16S rRNA genes that are ubiquitously present in the cell. Theoretical basis of this methodology is based on the assumption that 16S rRNA genes are only vertically inherited and are thus indigenous to each species. However, microbial genomic analysis has revealed the existence of prokaryotic species containing two types of rRNA (*rrn*) operons of seemingly different origins. It has also been reported that some bacteria contain 16S rRNA that are mosaics of sequences from multiple species. This suggests that horizontal gene transfer (HGT) occurred for 16S rRNA genes. In addition, a recent HGT experiment mimicking the natural HGT process has shown that a wide range of foreign 16S rRNA genes can be transferred into *Escherichia coli*, including those from different phylogenetic classes (with a minimum sequence identity of 80.9% to the *Escherichia coli* 16S rRNA gene). Thus, in contrast to the complexity hypothesis that states informational genes are rarely horizontally transferred between species, 16S rRNA is occasionally amenable to HGT. Results of the current method for rapid identification and classification of prokaryotes based on the 16S rRNA gene should thus be carefully analyzed and interpreted.

Introduction

Conventional genetics assumes that each gene is "vertically" inherited from an ancestor to its descendants. However, recent development of genomics and bioinformatics has shown that "horizontal" gene transfer (HGT) frequently occurs, particularly among prokaryotes. These studies have revealed that prokaryotic genomes are mosaics of DNA fragments from different origins.[1-4] HGT involves the addition or exchange of genomic fragments from one species to another[1-5] through four known mechanisms: (1) transfer of DNA mediated by bacteriophage infection (transduction), (2) transfer of DNA by mating of different species (conjugation), (3) uptake of extracellular DNA fragments due to the natural competency of the cell (transformation) and (4) transfer of a DNA fragment mediated by a virus-like element (gene transfer agents). Once incorporated into the genome, the transferred genomic fragments can be vertically inherited by descendants, generating species diversity. HGT often promotes accelerated evolution or drastic phenotypic changes. For example, 20% of the genomic DNA of the enterohemorrhagic *Escherichia coli* strain O157:H7 is transferred from non-*E. coli* species primarily by transduction to obtain pathogenic characteristics.[6]

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How are Prokaryotic Species Defined?

In organisms that reproduce sexually, a species is often defined as a group of organisms that are capable of interbreeding and producing fertile offspring. This raises the questions regarding prokaryotic species, which reproduce asexually but often exchange genes between organisms through HGT. Historically, prokaryotic species have been defined for convenience according to their phenotypes such as morphology, metabolism and staining properties; these criteria do not fit well with the definition of a species as in sexual organisms. Prokaryotic species are currently defined relatively arbitrarily according to criteria. For example, a species of prokaryotes is defined as a group of microorganisms that share at least 70% homology according to DNA-DNA hybridization studies.[7]

Use of 16S rRNA Sequence for Classifying Prokaryotes

In 1970s, researchers began to challenge the taxonomic classification of prokaryotes based on the nucleotide sequence of 5S rRNA or amino acid sequences of proteins such as cytochrome *c* and ferredoxin. In 1977, Carl R. Woese classified prokaryotes based on the oligonucleotide catalog of 16S rRNA that make up the 30S (small) ribosomal subunit, revealing that prokaryotes are composed of 2 different domains, bacteria and archaea.[8,9] Development of recombinant DNA technology and sequencing

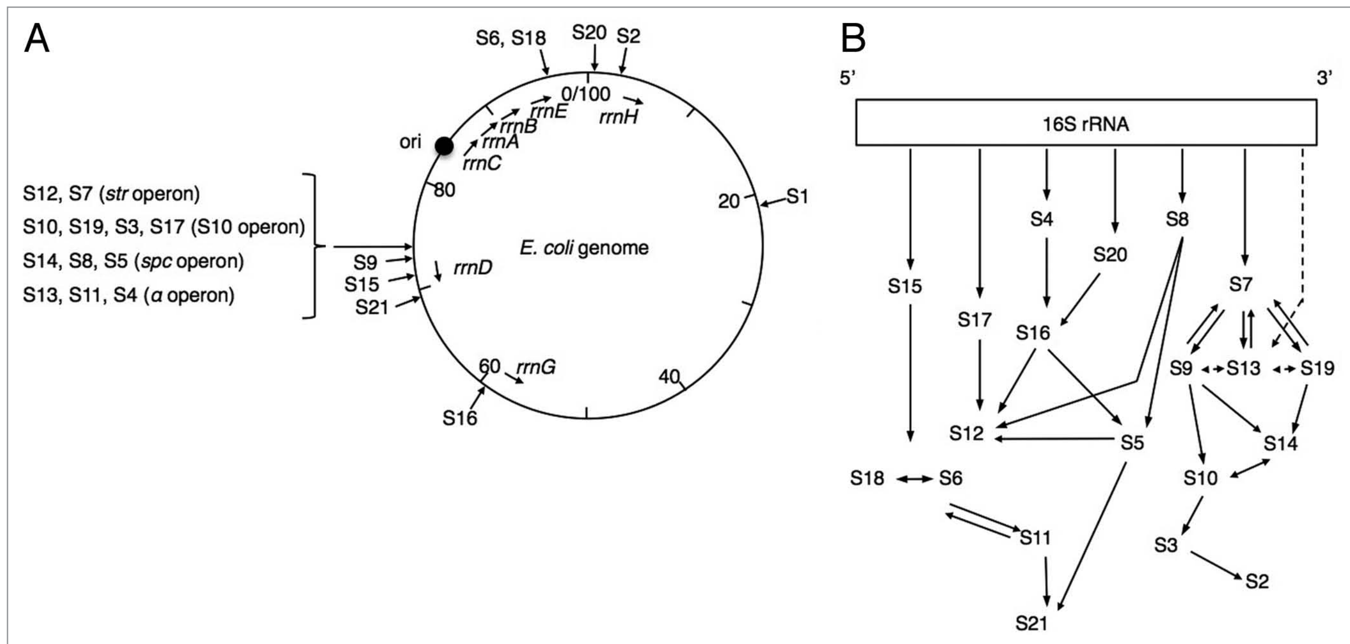

Figure 1. (**A**) Genome-wide distribution of *rrn* operon and ribosomal protein-encoding genes. Location of ribosomal protein genes involved in 30S subunit (S1–S21) and 7 *rrn* operons on the genomic map of *E. coli* (0–100 min). (**B**) Assembly map of the 30S subunit. Assembly order of ribosomal protein to 16S rRNA is shown by arrows. Figure was constructed as descried by Culver et al.[17]

methods enabled rapid comparison and classification of 16S rRNA genes between species.[8,9] Empirically, two organisms with 70% homology according to DNA-DNA hybridization experiments show approximately 97% homology for the 16S rRNA sequence.[10] Recently, metagenomic approaches have enabled the prediction of taxonomical positions of uncultured, non-isolated organisms.[11,12] There are six reasons why the 16S rRNA-based approach has become the gold standard for phylogenetic analysis:

(1) 16S rRNA genes are least likely to have undergone HGT between species.

(2) 16S rRNA genes are ubiquitous in prokaryotes.

(3) 16S rRNA contains approximately 1500 bases, which is adequate (or sufficient) for analysis.

(4) Sequences of 16S rRNA genes are highly conserved and thus comparable between distantly related species.

(5) The 16S rRNA gene also contains variable regions, enabling comparison between closely related species.

(6) Some regions of the 16S rRNA gene are completely conserved, enabling the use of “universal primers” for PCR detection or sequencing.

Among these reasons, (1) should especially be stressed because, for this reason, 16S rRNA genes are thought to represent the authentic microorganism phylogeny.[8,9,13] The theoretical basis to assume that 16S rRNA genes are not amenable to HGT is due to the “complexity hypothesis”.[14]

Complexity Hypothesis: Theoretical Background for Defining HGT-Resistant Genes

Products of “informational genes” that are involved in DNA replication, transcription and translation (central dogma) often function as essential complexes with multiple components (e.g., the ribosome). The complexity hypothesis[14] assumes that such informational genes generally coevolve with their binding partners in the same complex. For example, let us consider a complex consisting of protein A and A’; if component A is mutated, component A’ may mutate in a compensatory manner so as not to lose complete functionality of the complex. This process makes the relationship between A and A’ more specific. It should be noted that genes encoding elements in a complex (i.e., A and A’) are often dispersed throughout the genome (**Fig. 1A**), and successful HGT of A requires simultaneous HGT of A’ from a different locus. As the number of components in a complex increases, the occurrence of HGT becomes less likely. Informational genes (as compared with single functional genes) in a species are thus thought to represent the species’ genomic background. This makes informational genes more suitable for constructing the tree of life.

Complexity of 16S rRNA in the Ribosome Structure

The ribosome is a universal translational apparatus and its 30S subunit, in which the 16S rRNA is located, plays a role in monitoring recognition of codons on mRNA through the anti-codons of tRNAs.[15] 16S rRNA interacts with as many as 21 different ribosomal proteins (**Fig. 1A**) to construct the 30S subunit. In vitro reconstitution experiments have shown that ribosomal proteins hierarchically interact with 16S rRNA and are incorporated into the intermediate particle of the subunit as shown in the assembly map (**Fig. 1B**),[16] although this hierarchy, known as the “assembly gradient” is not essential in vivo.[17] The crystal structure model of the 30S subunit clearly shows that 16S rRNA

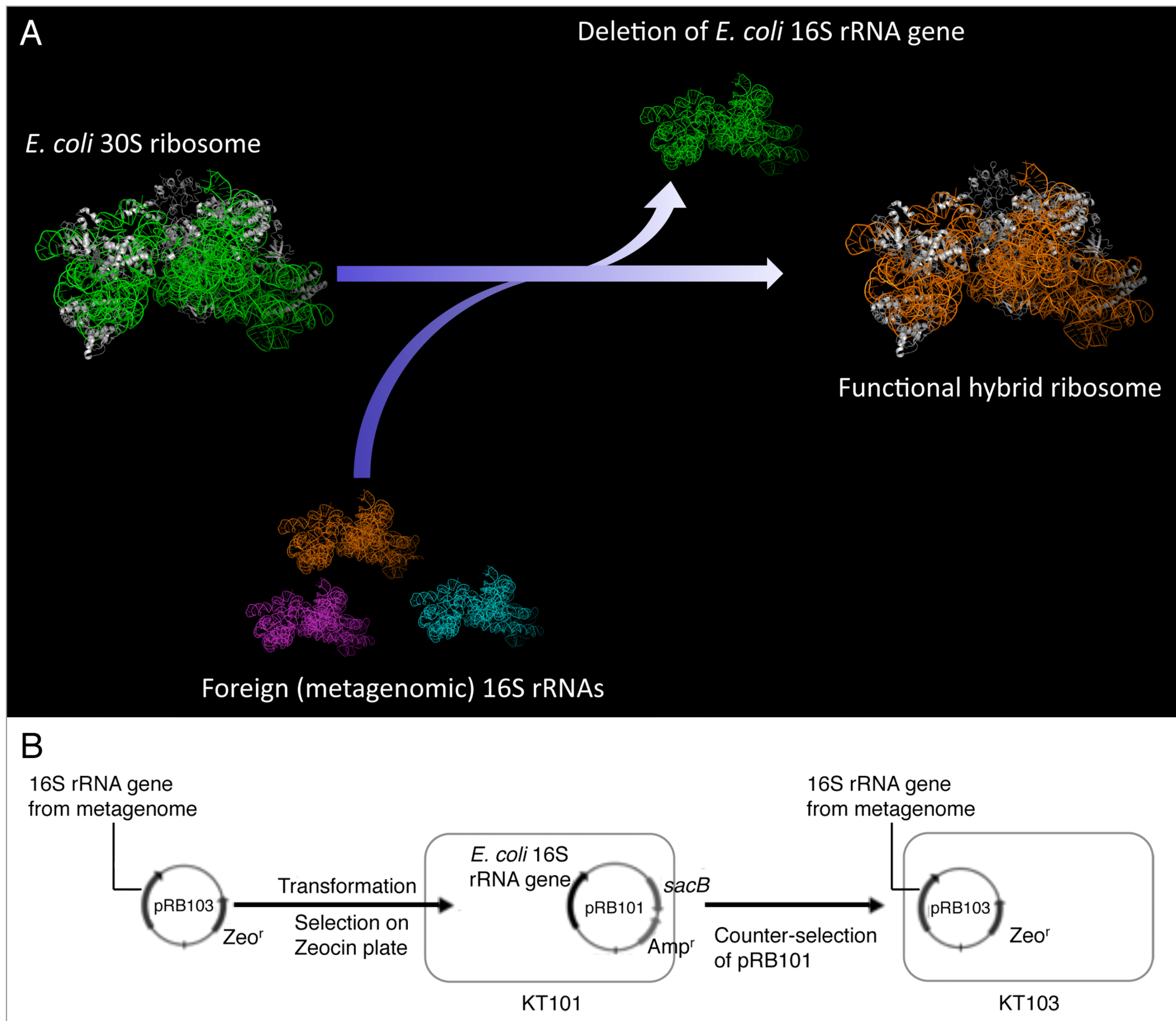

Figure 2. (**A**) Schematic illustration of laboratory HGT experiment of 16S rRNA. *Escherichia coli* ribosomal 30S subunits (green, 16S rRNA; white, ribosomal proteins) were genetically modified to replace 16S rRNA with foreign 16S rRNA (orange, blue and cyan). Through functional screening, 16S rRNA genes that are active in *E. coli* can be selected (orange). (**B**) *Escherichia coli* genetic system for laboratory HGT experiment of 16S rRNA. *Escherichia coli* Δ7 strain KT101 was used to screen for foreign (metagenomically retrieved) 16S rRNA genes compatible with an *E. coli* genetic background. The rescue plasmid contained the *rrnB* operon of *E. coli* and the counterselectable marker *sacB*. Transformants containing pRB103 were referred to as KT103.

forms the subunit structural core.[18,19] The conserved region of 16S rRNA is sensitive to mutation; even subtle mutations (i.e., single point mutation) often render the ribosome completely non-functional.[20] It is thus reasonable for evolutionary biologists to hypothesize that HGT of 16S rRNA does not occur because it requires hundreds of concurrent nucleotide mutations.[8,9,13] In contrast, genetic studies have shown that 16S or 23S rRNA is somewhat flexible when mutations occur within variable regions (regions not conserved between species), including binding sites for some ribosomal proteins.[21,22]

Evidence of HGT of 16S rRNA in Nature: Counterexamples to the Complexity Hypothesis

rRNAs are transcribed as an operon (*rrn* operon), with the transcript processed by various kind of endo- or exoribonucleases to generate 16S rRNA, 23S rRNA, 5S rRNA and some tRNAs, although some exceptions exist.[23] Diversity exists in the copy numbers of *rrn* operons in prokaryotes; e.g., *Mycoplasma pneumonia* has only a single copy, whereas *E. coli* has 7 and *Clostridium paradoxum* has as many as 15 copies.[13] In *E. coli*, differences in

16S rRNA sequences among seven *rrn* operons (*rrnA–H*) are less than 1%. Interestingly, several isolated species of prokaryotes contains *rrn* operons that show significant diversity. For example, the thermophilic bacterium *Thermoanaerobacter tengcongensis* contains four *rrn* operons, and the 16S rRNA sequence of *rrnC* shows only 88.4% homology with other *rrn* operons (*rrnA*, *rrnB* and *rrnD*).[13] The 16S rRNA gene of *rrnC* is not a pseudogene because it correctly forms the conserved secondary structure of 16S rRNA. The sequence is more closely related (95%) to that of other *Thermoanaerobacter* species (*T. subterraneus* SL9 and *T. keratinophilus* 2KXI), suggesting that this gene was transferred into the ancestor of *T. tengcongensis* and became fixed as a functional gene in this organism over evolution.[13] Similar interoperonic diversity is observed in *Desulfotomaculum kuznestovii* (91.7% homology), *Thermobispora bispora* R51 (93.6%), *Thermonospora chromogena* (94%) and *Halobacterium marismortui* (95%).[13] These examples can be regarded as snapshots of ongoing HGT of 16S rRNA in nature and are thought to be clear counterexamples of the complexity hypothesis. Chimeric 16S rRNA genes have also been reported between two distantly related species[24] or closely related lineages,[25-27] suggesting that partial fragments of the 16S rRNA genes are also amenable to HGT.

It remains unclear how HGT of 16S rRNA is driven in the environment. 16S rRNA genes may be propagated by transduction because, according to a previous report, this gene can be incorporated into a broad-host-range, generalized transducing phage (SN-T) at a frequency of 1×10^9 transductants/PFU.[28] The existence of several universally conserved sequences in the *rrn* operon[8,9,11,29] may allow and facilitate recombination of a foreign *rrn* operon with that of the host after incorporation into the genome.[5] An experimental study showed that 16S rRNA can also undergo HGT via transformation by showing that *Helicobacter pylori* acquires a tetracycline-resistant phenotype when the 16S rRNA gene containing multiple point mutations, which render this gene resistant to tetracycline, is added to the medium.[30]

HGT Experiment Revealed Unexpected Robustness of the 16S rRNA Gene

The evidence described above that HGT of 16S rRNA has occurred in nature raises doubt on the basic assumption that 16S rRNA is unlikely to undergo HGT between species. To address this issue, we performed systematic HGT experiments for 16S rRNA using *E. coli* by introducing homologs of this gene that had been amplified from metagenomes extracted from various environments (i.e., soils, fermented foods, sea water and hot springs) (**Fig. 2**). Metagenomes are mixtures of uncultured microbial genomes (and plasmids) in environmental samples rich in microbial diversity.[31] Because both ends (5' and 3') of 16S rRNA genes show high homology among prokaryotes, we amplified 16S rRNA genes from various (phylogenetically diverse) species by PCR using a universal primer pair.[29] These DNA fragments were cloned into the *rrn* operon-expression vector pRB103 and introduced into an *E. coli* strain KT101 ("Δ7 prrn strain"), in which all chromosomal *rrn* operons were inactivated and complemented by a counter-selectable plasmid pRB101 encoding the wild-type *rrnB* operon.[17,32-34] Using this system, we performed genetic selection of 16S rRNA genes that are functional in *E. coli*. From the 15,000 transformants, we could obtain 33 different clones (KT103) carrying the metagenomic 16S rRNA genes rather than the *E. coli* 16S rRNA gene. Through DNA sequencing, we found that most 16S rRNA genes were derived from Gammaproteobacteria, but some were derived from Betaproteobacteria with 80.9% identity to the *E. coli* sequence (note that the phylogenetic class of *E. coli* is Gammaproteobacteria; **Fig. 3**).[34] Doubling times (DT) of KT103 strains carrying foreign 16S rRNA genes (30–50 min) were comparable to those of KT101 carrying *E. coli* 16S rRNA (30 min). DTs of each KT103 strain increased only modestly with decreasing sequence identity; the average increase in DT was 4.6 sec per point mutation.[34] Because severe defects caused by mutation within the rRNA gene are known to be suppressed relatively easily through a spontaneous compensatory mutation (e.g., after continuous cultivation for two weeks),[21] smaller defects caused by HGT of 16S rRNA may also be circumvented by similar adaptation mechanisms. Our results indicate that the ribosome is an unexpectedly plastic complex and sufficiently robust to accommodate foreign 16S rRNA.

Conclusions and Perspectives

Recent progress in genomics and bioinformatics has shown that natural 16S rRNA genes are likely to experience HGT during the course of evolution. In our HGT experiment, we showed that *E. coli* can accommodate a foreign 16S rRNA gene with only 80.9% identity that belonged to different phylogenetic class. This evidence strongly suggests that the 16S rRNA gene is amenable to HGT, thereby questioning the theoretical background of microbial phylogeny (e.g., the complexity hypothesis); the 16S rRNA gene may well be represented in a phylogenetic network.[35,36] Results of the current method for rapid identification and classification of prokaryotes based on the 16S rRNA gene should thus be carefully analyzed and interpreted. Unexpectedly high horizontal transferability of the 16S rRNA gene suggests structural flexibility or plasticity of the ribosome because HGT introduces hundreds of mutations into the 16S rRNA at once. This finding accelerates the study of ribosome engineering mediated by 16S rRNA replacement.[21,33,34] Because it has been known that subtle alterations in ribosomal components can induce large phenotypic changes in bacteria,[37] the 16S rRNA gene may be a promising target for molecular engineering to manipulate bacterial phenotypes.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

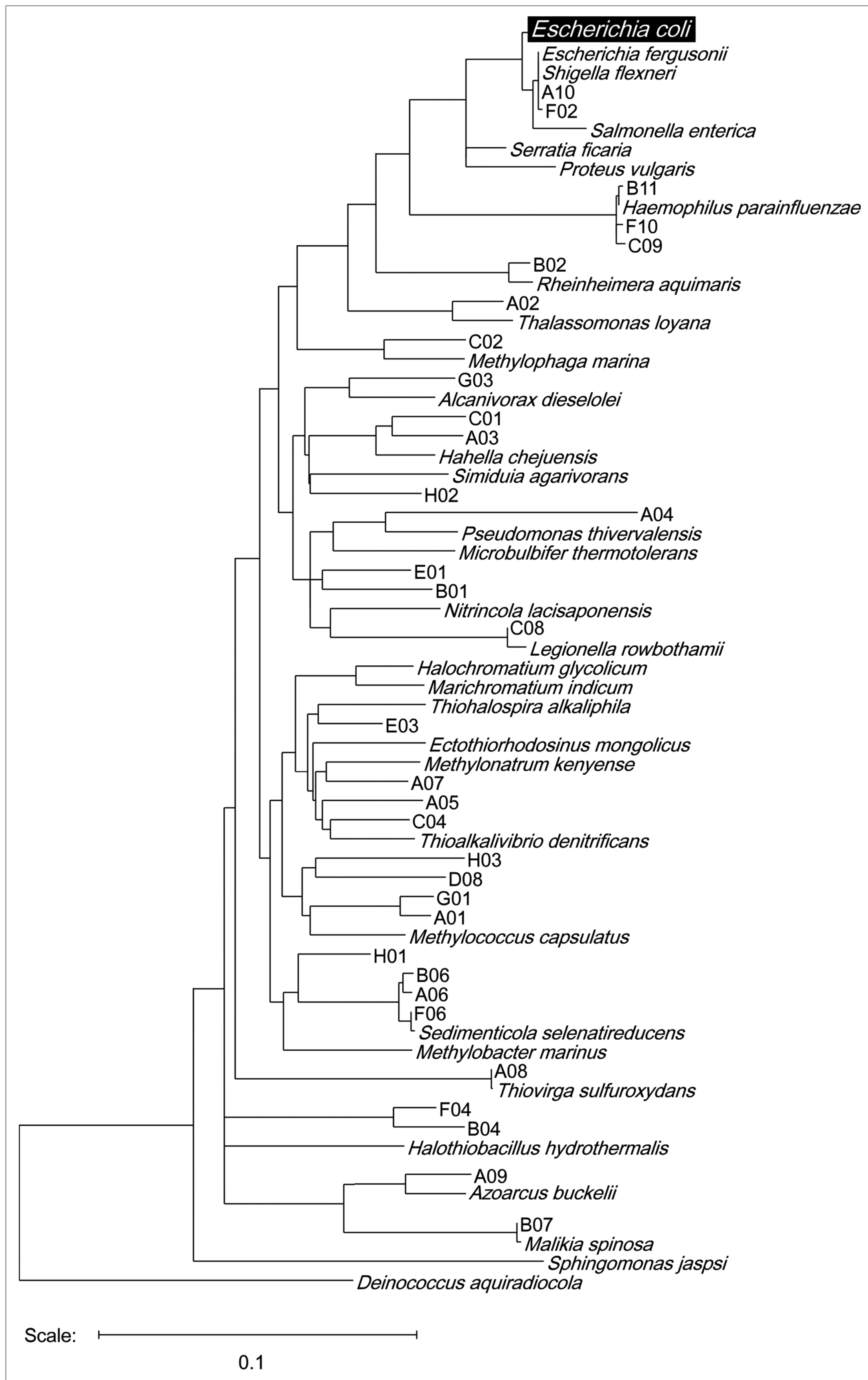

Figure 3. Neighbor-joining phylogenetic tree of 16S rRNA genes. Environmental 16S rRNA genes that were functional in *E. coli* are shown as the clone ID (A01–H03) with their closest relatives (nomenclature). Several other relevant strains are also shown.

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