Phylogenetic position of the marine subdivision of \textit{Agrobacterium} species based on 16S rRNA sequence analysis

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In the genus \textit{Agrobacterium}, terrestrial and plant pathogenic species and marine star-shaped-aggregate-forming species were reported (Conn, 1942; Jordan, 1984; Ophel and Kerr, 1990; Rüger and Höfle, 1992; Starr and Weiss, 1943). The phylogenetic positions of the terrestrial and plant pathogenic species have already been made clear, but those of the marine species have not been published yet. Since Stapp and Knösel (1954) grouped a marine species as \textit{Agrobacterium stellulatum} based on their formation of star-shaped aggregation, many marine isolates resemble to \textit{A. stellulatum} as a species of the genus \textit{Agrobacterium} (Ahrens, 1968; Ahrens and Rheinheimer, 1967). However, intrageneric relationships between marine and terrestrial \textit{Agrobacterium} species remain uncertain. Rüger and Höfle (1992) performed a phenotypic analysis on these \textit{Agrobacterium} species in addition to their marine isolates, and they insisted that among these marine strains, five species could be recognized as a distinct taxon. They believe these five species form a group independently from the terrestrial \textit{Agrobacterium} species, but there were no distinctive phenotypic characteristics to separate the marine species from the genus \textit{Agrobacterium}. Therefore, they concluded that the genus \textit{Agrobacterium} must be divided into two subdivisions, accommodating the terrestrial and plant pathogenic species in subdivision 1 and the marine star-shaped-aggregate-forming five species \textit{Agrobacterium atlanticum, Agrobacterium ferrugineum, Agrobacterium gelatinovorum, Agrobacterium meteori} and \textit{A. stellulatum} in subdivision 2, until further taxonomic data are available (Rüger and Höfle, 1992).

In this communication, we determined 16S ribosomal RNA gene sequences of these marine subdivision species, including the non-validated species “\textit{Agrobacterium agile}” and “\textit{Agrobacterium kieliense},” and investigated their phylogenetic position to clarify the classification of marine \textit{Agrobacterium} species.

The bacterial strains used in this study were \textit{A. atlanticum} IAM 14463\textsuperscript{T}, \textit{A. ferrugineum} IAM 12616\textsuperscript{T}, \textit{A. gelatinovorum} IAM 12617\textsuperscript{T}, \textit{A. meteori} IAM 14464\textsuperscript{T}, \textit{A. stellulatum} IAM 12614, \textit{A. stellulatum} IAM 12621\textsuperscript{T}, “\textit{A. agile}” IAM 12615 and “\textit{A. kieliense}” IAM 12618. All the strains were grown aerobically on the plate of Difco marine agar 2216 for 2 days at 25°C. Cells grown in the late exponential phase were harvested, washed with sterile water and stored at −20°C until use. The 16S rRNA gene was amplified by PCR using Takara Taq (Takara Shuzo, Kyoto, Japan), the three forward primers 8F (5’-AGAGTTTGATCCTGGCTCAG-3’), 520F (5’-CAGCCCGCGGTAATAC-3’) and 926F (5’-AAACTCAAAGGAATTGACGG-3’), and three reverse primers 704R (5’-TCTACGCATTTCACC-3’), 1110R (5’-GGGTTGCCGCTGGTG-3’) and 1510R (5’-GGCTACCTTGTACGA-3’) [\textit{Escherichia coli} numbering system], by the combination of 8F-704R, 520F-1110R and 926F-1510R. Amplified fragments were cloned using an Invitrogen TA Cloning\textsuperscript{TM} Kit (Invitrogen, CA, U.S.A.). Extraction and purification of the plasmids were carried out using a QIAprep Spin Plasmid Kit (Qiagen, Hilden, Germany). The cloned plasmids were used for sequencing. Sequencing was carried out using an ABI PRISM\textsuperscript{TM} Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Co., CA, U.S.A.) and a model ABI 373S automatic DNA sequencer (Perkin-Elmer Co.). Previously published 16S rRNA gene sequences, which were already aligned,
were obtained from the Ribosomal Database Project (RDP). These were: Agrobacterium rhizogenes (accession number: D12788), Agrobacterium rubi (X67228), Agrobacterium tumefaciens (D14500), Agrobacterium vitis (X67225), Ancylobacter aquaticus (M27803), Aquabacter sp. strain SPL-1 (from RDP), Azorhizobium caulodinis (D11342), Bar- 

tonella bacilliformis (M65249), Blastobacter aggrega-

tus (X73041), Blastobacter denitrificans (X66025), Brevundimonas diminuta (M59064), Brucella suis 

(L26169), Escherichia coli (V00348), Hirschia ballica 

(X52909), Hyphomicrobium vulgare (X53182), Hy-

phomonas jannaschiana (M83806), Liberobacter sp. strain Poona (L22532), Methylobacterium radiotoler-

ans (D32227), Mycoplana dimorpha (D12786), Ochro-

bacterium anthropi (D12794), Paracoccus alk-

aliphilus (D32238), Paracoccus aminophilus (D32239), 

Paracoccus aminovorans (D32240), Paracoccus deni-

trificans (X69159), Paracoccus kocurii (D32241), 

Paracoccus thiocyanatus (D32242), Paracoccus ver-

satus (D32244), Phyllobacterium rubiacearum (D12790), Pseudomonas aeruginosa (X06684), Rhi-

zobium galegae (D11343), Rhizobium huakii (D12797), Rhizobium leguminosarum (X67227), Rhi-

zobium loti (X67229), Rhizobium tropici (X67234), 

Rhodobacter capsulatus (D16428), Rhodobacter sphaeroides (D16425), Rhodobacter veldkampii (D16421), Rhodobium mar-

inum (D30790), Rhodobium orientis (D30792), 

Rhodomicrobium vanniellii (M34127), Rhodoplanes elegans (D25311), Rhodopseudomonas acidophila (M34128), Rhodopseudomonas viridis (D25314), Rho-

dospirillum rubrum (D30778), Rhodovulum euryhalinum (D16426), Rhodovulum sp. strain MB253 (D32245), Rhodovulum sp. strain MB260 (D16420), Rhodovulum strictum (D16419), Rhodovulum sulfidophilum (D16423), Rochalimaea henselae (M73229), Roseobacter algi-

cola (X78315), Roseobacter denitrificans (M97467), 

Roseobacter litoralis (X78312), Sinorhizobium fredii (D14516), Sinorhizobium mellotii (X67222), Sinorhiz-

obium xinjiangensis (D12796), Sphingomonas pauci-

mobilis (D13725), Thiobacillus sp. strain THI 051 (D32248) and Zoogloea ramigera (D14255). Devosia 

ribolfavina (D49423), Paracoccus solventivorans (Y07705) and Rhodobacter azotoformans (D70846) 

were obtained from the DNA databank. The se-

quences determined in this study were deposited in 

the DNA database under accession numbers D88520 

(A. stellulatum IAM 12614), D88521 ("A. agile" IAM 

12615), D88522 (A. ferrugineum IAM 12616T), 

D88523 (A. gelatinovorum IAM 12617T), D88524 ("A. 

kieliense" IAM 12618), D88525 (A. stellulatum IAM 

12621T), D88526 (A. atlanticum IAM 14463T) and 

D88527 (A. meteori IAM 14464T). We aligned the se-

quences of these three organisms and the organisms 

that we sequenced with RDP’s aligned sequences 

using the command ALIGN_SEQUENCE on a WWW 

server. The calculation of nucleotide substitution rates 

(Kw) (Kimura, 1980) and construction of neighbor-

joining (NJ) phylogenetic trees (Saitou and Nei, 1987) 

were performed with the CLUSTAL W 1.6 program 

(Thompson et al., 1994). The variable regions of 16S 

rRNA gene sequences ranging in positions 69–100, 

181–219, 447–487, 1004–1036, 1133–1141 and 

1445–1456 [E.coli numbering system], and alignment 

positions that included gaps and unidentified bases 

were not taken into consideration for the calculations. 

The topology of the phylogenetic tree was evaluated 

by performing a bootstrap analysis (Felsenstein, 

1985) with 1,000 replications. To aid judgement, a par-

simony analysis was also performed by the computer 

package PAUP 3.1.1. (Swoford, 1993), and a boot-

strapped 50% majority consensus tree was con-

structed.

The nucleotides of the 16S rDNA of the five species 

of the marine subdivision of the genus Agrobacterium, 

in addition to "A. agile" and "A. kieliense" ranging from 

positions 28 to 1494 [E.coli numbering system] (those 

for "A. agile" ranging from 142 to 1494), were deter-

mined. The primary structures were aligned with those of 53 species of Proteobacteria α subdivision (Stacke-

brandt et al., 1988) and those of Escherichia coli and Pseudomonas aeruginosa as outgroups. The NJ tree 

inferred from 1,017 aligned sites is shown in Fig. 1. The NJ tree indicates that the marine subdivision 

species of the genus Agrobacterium (IAM 12616T, 

IAM 14463T, IAM 14464T, IAM 12617T, IAM 12621T 

and IAM 12614) and "A. kieliense" IAM 12618 are het-

erogenous and do not show a close relationship with members of the terrestrial subdivision of the genus 

Agrobacterium (A. rhizogenes, A. rubi, A. tumefaciens and A. vitis ), which are included in the Proteobacteria 

α-2 subgroup (Fig. 1). A. stellulatum IAM 12621T, IAM 

12614 and "A. kieliense" IAM 12618 grouped in a sin-

gle cluster and were located independently from other genera in the Proteobacteria α-2 subgroup. A. ferru-

gineum IAM 12616T, A. atlanticum IAM 14463T, A. meteori IAM 14464T and A. gelatinovorum IAM 12617T 

belonged to the Proteobacteria α-3 subgroup. A. ferru-

gineum IAM 12616T formed a cluster with species of the genus Rhodobacter, and A. atlanticum IAM 

14463T, A. meteori IAM 14464T, and A. gelatinovorum IAM 12617T formed a cluster with species of the genus Roseobacter.

The similarity value between the strains A. stellulatu-

um IAM 12621T and IAM 12614 was 97.5%. The indepen-

dence of the cluster is supported by a high
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Bootstrap value (100%) and high level of 16S rRNA sequence dissimilarity with over 5.4% to other genera in the Proteobacteria α-2 subgroup. "A. kieliense" IAM 12618 formed a cluster with A. stellulatum IAM 12621T and IAM 12614, but the bootstrap value of the cluster of these three strains was very low (27%), and the 16S rRNA sequence dissimilarity of "A. kieliense" IAM 12618 to those of A. stellulatum IAM 12621T and IAM 12614 was 6.0%. "A. kieliense" IAM 12618 is clearly separated from other genera of the α-2 subgroup of Proteobacteria, as was indicated by a 16S rRNA sequence similarity below 95.3%. In the parsimony analysis, the bootstrap value of the cluster of A. stellulatum IAM 12621T and IAM 12614 was 100%, and that of the cluster of A. stellulatum IAM 12621T, IAM 12614 and "A. kieliense" IAM 12618 was 10% (data not shown). We think that A. stellulatum and "A. kieliense" should be classified as species in new genera, respectively.

In order to analyze the relationships among A. ferrugineum IAM 12616T, A. atlanticum IAM14463T, A. meteori IAM 14464T and A. gelatinovorum IAM 12617T, another phylogenetic tree (Fig. 2) was constructed using a dataset of 32 species mainly belonging to the Proteobacteria α-3 subgroup, which consists of the genera Paracoccus, Rhodobacter, Rhodovulum, Roseobacter, Hirschia and Hyphomonas. Species of the genus Rhodobacter were
unified in one group, but the clustering was supported by a low bootstrap value of 63%. *A. ferrugineum* IAM 12616ᵀ was included in the group of the genus *Rhodobacter*, and its closest neighbors were *R. sphaeroides* and *R. azotoformans*. The branching of these three species was supported by a relatively high bootstrap value of 93%. The level of 16S rRNA sequence similarity with *R. sphaeroides* was 96.0% and that with *R. azotoformans* was 96.6%. In the parsimony analysis, the bootstrap value of the cluster of genus *Rhodobacter* and *A. ferrugineum* IAM 12616ᵀ was 30.9%, and that of *R. sphaeroides*, *R. azotoformans* and *A. ferrugineum* IAM 12616ᵀ was 56.7% (data not shown). The genus *Rhodobacter* is defined as the phototrophic genus, and *A. ferrugineum* IAM 12616ᵀ may be classified as a species of the genus *Rhodobacter* if the ability of photosynthesis is found in this strain. *A. ferrugineum* IAM 12616ᵀ has an insertion of 15 bases between positions 189 and 203 [E. coli numbering system] in the 16S rRNA gene, whereas species of the genus *Rhodobacter* did not have this insertion.

*A. atlanticum* IAM 14463ᵀ, *A. meteori* IAM 14464ᵀ and *A. gelatinovorum* IAM 12617ᵀ still formed a cluster with species of the genus *Roseobacter*, and the branching of these species was supported by a high bootstrap value (99%). These species were divided into three subgroups. The bootstrap value of the branching of the cluster of *A. atlanticum* IAM 14463ᵀ and *A. meteori* IAM 14464ᵀ was 100%, and that of *A.
gelatinovorum IAM 12617T and R. algicola was 39%. The 16S rRNA sequence similarity value between A. atlanticum and A. meteori was 100%, and that between A. gelatinovorum and R. algicola was 96.8%. In the parsimony analysis, the bootstrap value of the cluster of the genus Roseobacter was 100% (data not shown). We think that A. atlanticum, A. meteori and A. gelatinovorum could be classified in the genus Roseobacter based on this phylogenetic analysis.

“A. agile” was found to be closely related to Pseudomonas aeruginosa based on a phylogenetic analysis using 16S rRNA gene sequences (data not shown), which agrees with the early report by Rüger and Höfte (1992).

In conclusion, 16S rDNA sequence-based phylogeny revealed heterogeneity of the marine subdivision species of the genus Agrobacterium, and these species have no relation to the terrestrial Agrobacterium species. Thus, the taxonomic position of the marine subdivision of Agrobacterium should be re-examined, which is now being done in our laboratory. A. stellulatum IAM 12621T and IAM 12614, and “A. kieliense” IAM 12618 might belong to new genera, respectively. Furthermore, A. ferrugineum IAM 12616T might be a member of the genus Rhodobacter, and A. atlanticum IAM 14463T, A. meteori IAM 14464T and A. gelatinovorum IAM 12617T might be species of the genus Roseobacter.

References


