Reassessment of the Phylogenetic Position of *Caulobacter subvibrioides*

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Determination of the 16S rRNA gene sequence of *Caulobacter subvibrioides* ATCC 15264\(^T\) (\(T = \) type strain) confirmed that this species is a member of the alpha subclass of the *Proteobacteria* and showed that it is phylogenetically most closely related to the *Caulobacter* group comprising the species *Caulobacter bacteroides*, *Caulobacter crescentus*, and *Brevundimonas* (*Pseudomonas*) *diminuta*, for which 16S rRNA sequences of the type strains are currently available. The closest known relative of strain ATCC 15264\(^T\) among these species is *B. diminuta* (level of direct pairwise sequence similarity, 95%). On the basis of its previously determined 16S rRNA sequence (accession number M83797), *C. subvibrioides* is most closely related to *Sphingomonas adhaesiva* in the alpha-4 subgroup (level of similarity, 97.7%). Analysis of the hydroxy fatty acids of *C. subvibrioides* ATCC 15264\(^T\) showed that the 2-hydroxymyristic acid which is characteristic of the genus *Sphingomonas* was absent.

In 1992 Stahl et al. (20) reported on the phylogeny of the genus *Caulobacter*. These authors noted that most of the caulobacters, which belong to the alpha subclass of the *Proteobacteria* (20, 23), made up a diverse but coherent phylogenetic assemblage based on a comparison of their 16S rRNA sequences; the only exception was *Caulobacter subvibrioides*, which was only peripherally related to the main *Caulobacter* assemblage. However, insufficient outgroup reference sequences were used for accurate phylogenetic positioning of *C. subvibrioides*, and the relationship of this organism with other members of the alpha subclass of the *Proteobacteria* remained unclear.

Later, Hugenholtz et al. (4) showed that the published 16S rRNA sequence of *C. subvibrioides* CB81 (nucleotide sequence accession number M83797) was most closely related to the sequences of members of the alpha-4 subgroup of the *Proteobacteria* and that *C. subvibrioides* grouped with *Blastobacter natatorius* (19), *Erythrobacter longus* (15), and *Porphyrobacter neustonensis* (5). Subsequently, it was found that the genera *Sphingomonas* and *Rhizomonas* were also members of the expanding alpha-4 subgroup (21, 22). Strain CB81 was designated the type strain of the species by Poindexter (12) and was deposited in the American Type Culture Collection as strain ATCC 15264. The culture used by Stahl et al. (20) also originated as strain CB81 but has had a different history since.

In our experience the designated type strain of *C. subvibrioides*, strain ATCC 15264 (17), is not a typical member of the alpha-4 subgroup and appears to be typical of the caulobacters. Consequently, we decided to examine the hydroxy fatty acid profile of the type strain of *C. subvibrioides* to see if it contained the signature 2-hydroxymyristic acid characteristic of the fatty acids of other members of the alpha-4 subgroup, including *Sphingomonas* spp. (24), *Rhizomonas* spp. (21, 22), and *B. natatorius* (16).

*C. subvibrioides* ATCC 15264\(^T\) (\(T = \) type strain) was received by us from the American Type Culture Collection in 1982 and was deposited in the Australian Collection of Microorganisms as strain ACM 2483\(^T\). Cells from our original freeze-dried ampoules were grown in peptone-yeast extract medium (18) with agitation in an environmental incubator at 28°C, harvested by centrifugation, washed twice in distilled water, and freeze-dried. Hydroxy fatty acids were extracted and methylated by direct acid methanolysis (2 ml of 2 M methanolic HCl, 85°C, 18 h). Fatty acid esters (FAMES) were extracted three times in 1 ml of hexane-chloroform (4:1) after 1 ml deionized water was added to the extract to improve phase separation. The combined extract (3 ml) was dried under nitrogen, 50 μl of chloroform was added, and the hydroxy acids were converted to their corresponding trimethylsilyl (TMS)-ether derivatives with bis(trimethylsilyl)trifluoroacetamide–1% trimethylchlorosilane (50 μl) during incubation for 2 h at 70°C (6). Following cooling to room temperature, the samples were evaporated to dryness with nitrogen gas and then redissolved in chloroform (200 μl). TMS-hydroxy FAMES were analyzed by gas chromatography–mass spectrometry by using a Varian model 3300 gas chromatograph linked directly to a Hewlett-Packard model 5970 mass selective detector. Samples (1 μl) were injected in splitless (1-min) mode. The analytical column used was an Alltech BP5 column (25 m by 0.22 mm [inside diameter]; phase thickness, 0.33 μm), the oven temperature was programmed to increase at a rate of 4°C/min from 150 to 270°C with 0- and 7.5-min hold times, respectively, and the injection temperature was 260°C. The helium carrier gas flow rate was 8 ml/min. The mass spectrometer was run in total-ion chromatograph mode (50 to 500 amu). Constituent FAMES were identified by comparing retention times and mass spectra with the retention times and mass spectra of TMS-derivatized fatty acid standards (4). The results of the analysis showed that no hydroxy fatty acids were present in *C. subvibrioides* ATCC 15264\(^T\). As 2-hydroxymyristic acid was absent in *C. subvibrioides* but present in *Blastobacter natatorius* (16; this study), an organism with which the available 16S rRNA sequence of *C. subvibrioides* (accession number M83797) shows 93.4% sequence similarity (4), the relationship between these two organisms was placed in doubt.

We therefore decided to sequence the 16S ribosomal DNA of *C. subvibrioides* ATCC 15264\(^T\) in order to confirm or clarify the phylogenetic position of this organism.

Extraction of genomic DNA and amplification of the 16S rRNA gene were performed as described by Dorsch and Stackebrandt (1). The PCR products were purified by using a Micro-Spin S-300 purification column (Pharmacia Biotech) as...
described by the manufacturer. A PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) was used to directly sequence the PCR products by following the protocol provided by the manufacturer, and the reaction mixtures were sequenced automatically with an Applied Biosystems model 373A DNA sequencer. The 16S ribosomal DNA sequences were aligned manually with representative bacterial 16S rRNA sequences contained from the Ribosomal RNA Database Project database (8). Positions where length and sequence variations made alignment uncertain were omitted from the analysis, and the final analysis included 1,102 bases. Pairwise evolutionary similarities and distances (7) were computed by using the DNA-DIST program in the PHYLIP 3.4 software package (2). Phylogenetic trees were constructed by using the neighbor-joining method of Saitou and Nei (13). A bootstrap analysis with 1,000 replicates was performed before any definite conclusions about the phylogenetic analysis of the genus Caulobacter on the basis of the fatty acid compositions of their cellular membranes. The current data also confirm the close relationship between Brevundimonas diminuta and the Caulobacter group observed by Stahl et al. (20). The genus Caulobacter currently contains 11 species (9, 17), and sequences for the remaining majority of species will have to be determined before any definite conclusions about the phylogenetic coherence of this group can be made, but the indications from this and previous research (10, 20) support the hypothesis that this is a coherent taxon.

Nucleotide sequence accession number. The nucleotide sequence determined in this study for C. subvibrioides ATCC 15264T (= ACMB 24835) has been deposited in the EMBL Data Library, Cambridge, United Kingdom, under accession number X94470.

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