

## Phylogenetic Relationships of the Hard-to-Culture Bacterial Phylum '*Verrucomicrobia*'

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### Abstract

The phylum '*Verrucomicrobia*' represents a primary lineage within the bacterial phylum and contains a number of environmental clones as well as a small number of cultured species. Despite wide ecological distribution of representatives of the phylum '*Verrucomicrobia*' in nature, owing to relatively few pure cultivated and characterized species, the classification of this phylum is still ambiguous and informal. For this reason, for formal classification of the phylum '*Verrucomicrobia*', it is recommended that many verrucomicrobia that thrives in a wide range of terrestrial, aquatic and marine habitats should be isolated and taxonomically investigated. In this study, we attempted to elucidate the phylogenetic relationships of the thirty novel isolates using polyphasic taxonomic approaches including 16S rRNA and *gyrB* gene sequence analysis, together with molecular, physiological, biochemical and chemotaxonomic analyses to characterize the novel strains. Based on these data, it is proposed that these isolates represent seven novel genera and twenty-two new species within the phylum '*Verrucomicrobia*'. Furthermore, with these novel strains, the formal classification of the phylum '*Verrucomicrobia*' is established.

**Key Words :** Phylogenetic analysis, Phylum '*Verrucomicrobia*', 16S rRNA gene, *gyrB* gene

### Introduction

The phylum '*Verrucomicrobia*' [1] is one of the primary lineages within the domain *Bacteria*. They represent a distinct lineage within the phylogenetic trees and contain a

number of environmental species as well as a small number of cultured species. A number of molecular phylogenetic approaches and culture-independent studies based on 16S rRNA gene sequences [2-4] revealed that the members of this phylogenetic group have

been detected in a very wide range of quite different habitats within the global ecosystem. Especially, recent results of a phylogenetic analysis of soil metagenomic libraries [5] indicated that many rRNA genes from the bacterial artificial chromosome (BAC) library had substantially affiliated with the bacterial phylum of '*Verrucomicrobia*'; all of the sequences were affiliated with subdivisions that lack cultured representatives. At present, these lineages have been informally classified into five subdivisions numbered 1 to 5 [2] and have also been classified into six monophyletic subdivisions numbered 1 to 6 [6] of which three are recognized in the second edition of *Bergey's Manual of Systematic Bacteriology* [7] as the families *Verrucomicrobiaceae* (subdivision 1), *Opitutaceae* (subdivision 4) and '*Xiphinematobacteriaceae*' (subdivision 2). Since the six informal monophyletic subdivisions of this phylogenetic group were first proposed, the names of only a few genera such as *Akkermansia*, *Alterococcus*, *Opitutus*, *Prostheco bacter*, *Rubritalea* and *Verrucomicrobium* [1,8-14] belonging to subdivisions 1 and 4 have been validly published. Originally, this phylum was known from two genera and five species of prosthecate, aerobic heterotrophs isolated from freshwater environments: *Verrucomicrobium spinosum*, *Prostheco bacter fusiformis*, *P. debontii*, *P. vanneervanii* and *P. dejongeii*, although an obligately anaerobic, heterotrophic genus (*Opitutus terrae*) from rice paddy field soil has recently been isolated and characterized. In addition to the named microorganisms, three taxonomically uncharacterized isolates of "ultramicrobacteria" from rice paddy field soil with 16S rRNA gene sequences closely related to *O. terrae* have also been described. *Alterococcus agarolyticus* ADT3<sup>T</sup>, formerly misclassified as a member of the class

*Gammaproteobacteria*, has been proposed to be included in the family *Opitutaceae*. The class *Opitutae*, comprising two orders: *Puniceicoccales* containing the family *Puniceicoccaceae* and *Opitutaes* containing the family *Opitutaceae*, was formally proposed recently for the classification of subdivision 4 [15]. However, in spite of wide ecological distribution of representatives of the phylum '*Verrucomicrobia*' in nature, owing to relatively few pure cultivated and characterized species, the classification of this phylum is still ambiguous and informal. Also, little is known about the detailed ecophysiology of the phylum. For this reason, for formal classification of the phylum '*Verrucomicrobia*', it is recommended that many verrucomicrobia that thrives in a wide range of terrestrial, aquatic and marine habitats should be isolated and taxonomically investigated. Moreover, the remaining subdivisions must be proposed formally by the rank of class and this phylum should be also formally proposed.

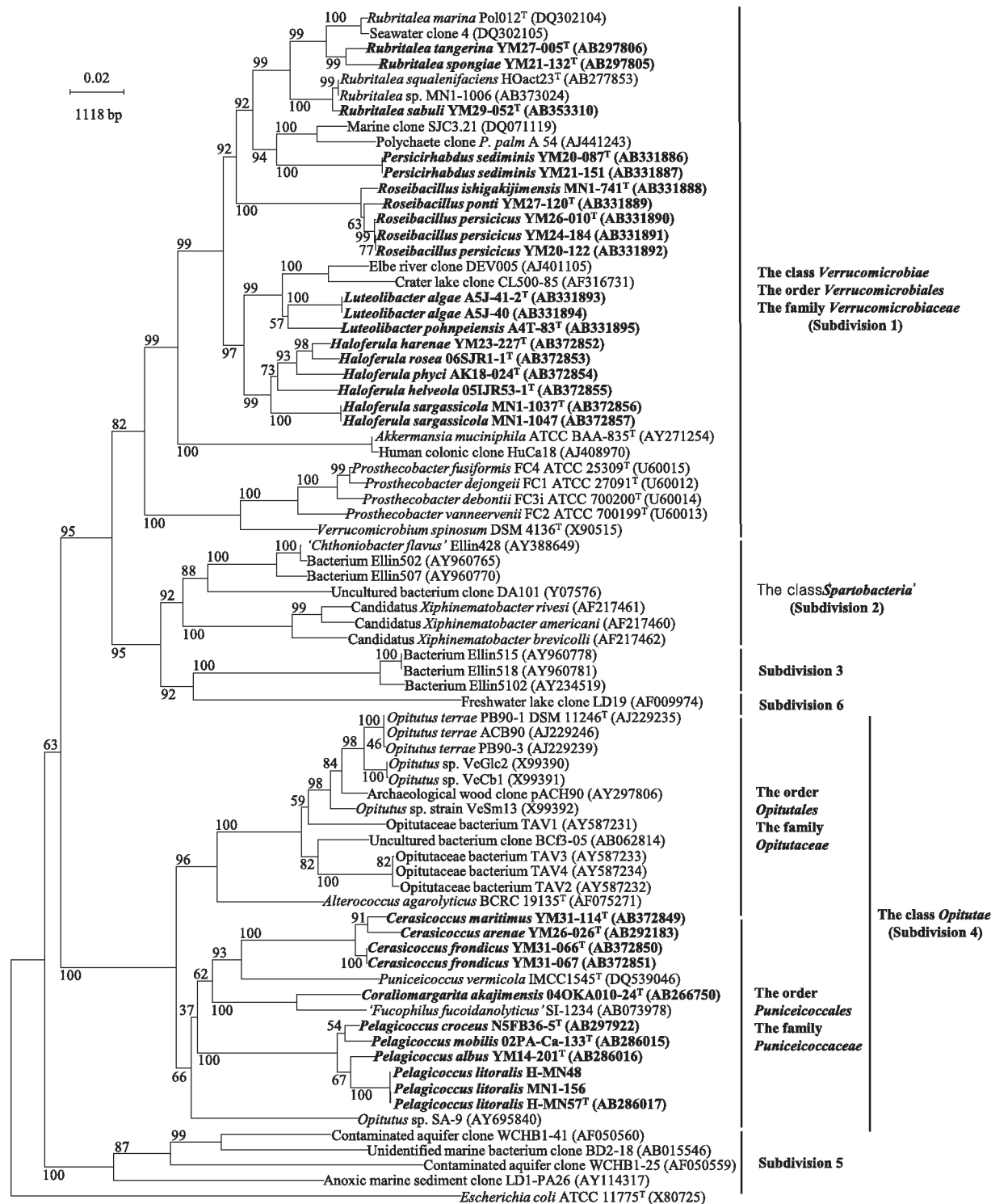
### Phylogenetic analyses on the strains within the class *Opitutae* (subdivision 4)

Phylogenetic studies were performed using polyphasic taxonomic approaches on eleven chemoheterotrophic bacteria which were isolated from various marine environments. The phylogenetic tree based on the neighbour-joining (NJ) generated comparisons of the 16S rRNA gene sequences revealed that these isolates were clustered within the family *Puniceicoccaceae* of the class *Opitutae* (subdivision 4). Eleven novel strains represented three independent monophyletic lineages that were distinct from species with validly

published genera with high bootstrap confidence values (Fig. 1). Furthermore, molecular (DNA–DNA hybridization test and determination of the genomic DNA G+C content), physiological (acid production from carbohydrates, enzyme activities and basic physiological tests) and biochemical and chemotaxonomic analyses (determination of respiratory quinone system and cellular fatty acid content and pigments analysis) were investigated on the novel isolates. In addition, increasing concentrations of the  $\beta$ -lactam antibiotic susceptibility tests and amino acid analysis of the cell wall hydrolysates indicated the absence of muramic acid and *meso*-diaminopimelic acid (Table 1) and the presence of relatively low molar ratios of alanine and glutamate (data not shown), which suggested that these strains lack an ordinary Gram-negative type of peptidoglycan in the cell wall. Based on the results of phylogenetic analysis and its polyphasic taxonomic evidences, the eleven isolates were considered to represent three novel genera and eight new species belonging to the family *Puniceicoccaceae* within the class *Opitutae* (subdivision 4), for which the names *Coralimargarita akajimensis* gen. nov., sp. nov. (strain 04OKA010–24<sup>T</sup>; [16]), *Pelagicoccus mobilis* gen. nov., sp. nov. (strain 02PA–Ca–133<sup>T</sup>; [17]), *Pelagicoccus albus* sp. nov. (strain YM14–201<sup>T</sup>; [17]), *Pelagicoccus litoralis* sp. nov. (strains H–MN57<sup>T</sup>, H–MN48 and MN1–156; [17]), *Pelagicoccus croceus* sp. nov. (strain N5FB36–5<sup>T</sup>; [18]), *Cerasicoccus arenae* gen. nov., sp. nov. (strain YM26–026<sup>T</sup>; [19]), *Cerasicoccus maritimus* sp. nov. (strain YM31–114<sup>T</sup>; [20]) and *Cerasicoccus frondicus* sp. nov. (strains YM31–066<sup>T</sup> and YM31–067; [20]) are proposed.

### Phylogenetic analyses on the strains within the family *Verrucomicrobiaceae* (subdivision 1)

Nineteen chemoheterotrophic bacterial strains were isolated from various marine environments and organisms, and were subjected to phylogenetic investigations using polyphasic taxonomic methods. Phylogenetic analysis based on the neighbour-joining (NJ) generated comparisons of the 16S rRNA gene sequences indicated that these isolates were affiliated to the family *Verrucomicrobiaceae* (subdivision 1) within the phylum 'Verrucomicrobia'. Among them, three novel strains had sequences that were related to the genus *Rubritalea*. Sixteen isolates studied form four different monophyletic clades that were distinct from species with validly published genera with high bootstrap confidence values (Fig. 1). Furthermore, molecular (DNA–DNA hybridization test and determination of the genomic DNA G+C content), physiological (acid production from carbohydrates, enzyme activities and basic physiological tests) and biochemical and chemotaxonomic analyses (determination of respiratory quinone system and cellular fatty acid content and pigments analysis) were investigated on the novel isolates. A novel acyl glyco-carotenoid, diapolycopenedioic acid xylosyl ester, possessing antioxidative activities was discovered in *R. squalenifaciens* HOact23<sup>T</sup> [21]. Analysis of the nearly complete 16S rRNA gene sequences from these strains indicated that the *Rubritalea* strains formed a robust clade having only limited resolution for this very tight group of species. However, the interrelationships deduced from the *gyrB* gene (a gene that encodes the B subunit protein of



**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relative positions of the novel strains described in this study among the currently known and cultivated species of the phylum 'Verrucomicrobia'. Numbers at the nodes indicate the bootstrap value derived from 1000 replications. Sequences determined in this study are shown in bold. Bar, 2% sequence divergence.

**Table 1.** Analysis of cell wall hydrolysates and  $\beta$ -lactam antibiotic susceptibility tests

+, Detected; -, not detected; SD, subdivision											
Characteristic	YM31 -114 <sup>T</sup>	YM26 -026 <sup>T</sup>	04OKA 010-24 <sup>T</sup>	02PA- Ca-133 <sup>T</sup>	YM14 -201 <sup>T</sup>	H-MN 57 <sup>T</sup>	N5FB 36-5 <sup>T</sup>	IMCC 1545 <sup>T</sup>	BCRC 19135 <sup>T</sup>	DSM 11246 <sup>T</sup>	YM20-122 (SD 1)
Cell wall component of:											
Muramic acid	—	—	—	—	—	—	—	—	—	—	+
<i>meso</i> -Diamino-pimelic acid	—	—	—	—	—	—	—	—	—	—	+
Antibiotic resistance ( $\mu\text{g ml}^{-1}$ ) to:											
Ampicillin (1-1000)	+	+	+	+	+	+	+	+	+	ND	—
Penicillin G (1-1000)	+	+	+	+	+	+	+	+	+		—
Carbenicillin (1-500)	+	+	+	+	+	+	+	+	+		—
Oxacillin (1-500)	+	+	+	+	+	+	+	+	+		—
Cephalothin (1-500)	+	+	+	+	+	+	+	+	+		—

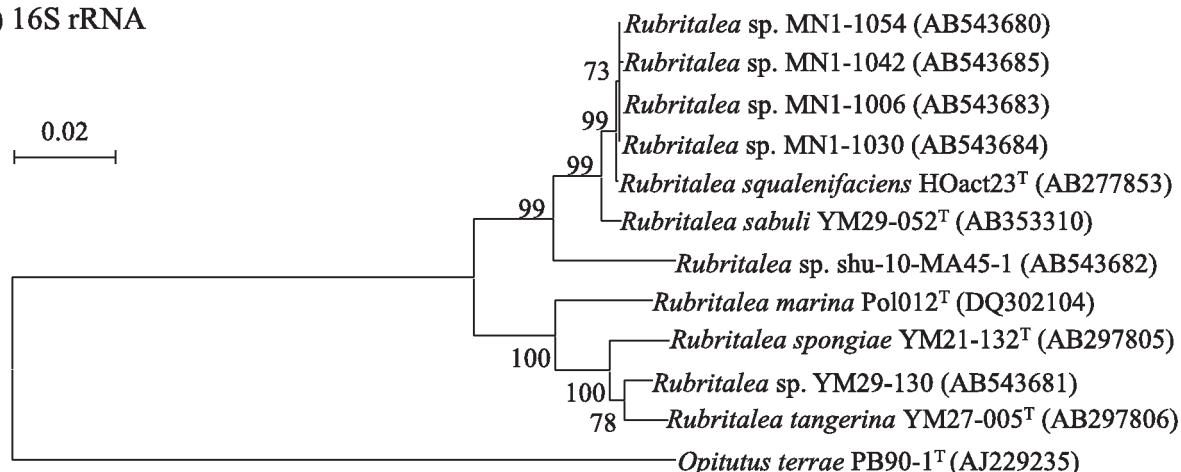
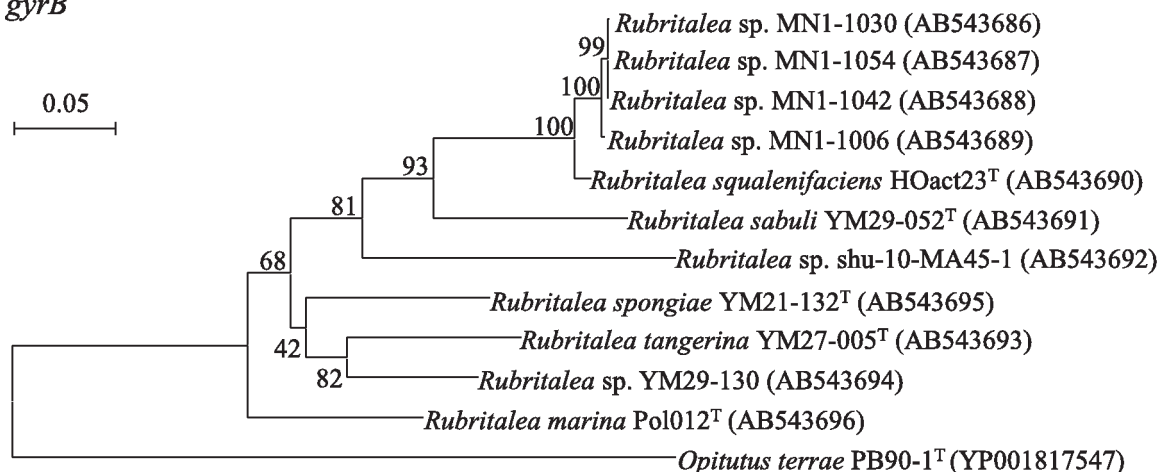
+, Resistant; -, susceptible; ND, no data

DNA gyrase)-based phylogeny showed better resolution than those based on their 16S rRNA gene sequences for the differentiation of strains at the species level (Fig. 2a, b) [22]. The genomic DNA-DNA relatedness values (<70%), biochemical and chemotaxonomic data (proportions of cellular fatty acids and menaquinones) also supported the results (Table 2). On the basis of polyphasic evidences and phylogenetic characteristics, it was concluded that these strains should be classified as four novel genera and fourteen new species of the family *Verrucomicrobiaceae* within the class *Verrucomicrobiae* (subdivision 1), for which the names *Rubritalea spongiae* sp. nov. (strain

YM21-132<sup>T</sup>; [23]), *Rubritalea tangerina* sp. nov. (strain YM27-005<sup>T</sup>; [23]), *Rubritalea sabuli* sp. nov. (strain YM29-052<sup>T</sup>; [24]), *Persicirhabdus sediminis* gen. nov., sp. nov. (strains YM20-087<sup>T</sup> and YM21-151; [25]), *Roseibacillus ishigakijimensis* gen. nov., sp. nov. (strain MN1-741<sup>T</sup>; [25]), *Roseibacillus ponti* sp. nov. (strain YM27-120<sup>T</sup>; [25]), *Roseibacillus persicus* sp. nov. (strains YM26-010<sup>T</sup>, YM24-184 and YM20-122; [25]), *Luteolibacter pohnpeiensis* gen. nov., sp. nov. (strain A4T-83<sup>T</sup>; [25]), *Luteolibacter algae* sp. nov. (strains A5J-41-2<sup>T</sup> and A5J-40; [25]), *Haloferula harenae* gen. nov., sp. nov. (strain YM23-227<sup>T</sup>; [26]), *Haloferula rosea* sp. nov. (strain 06SJR1-1<sup>T</sup>; Yoon *et al.*,



## (a) 16S rRNA

(b) *gyrB*

**Fig. 2.** Phylogenetic trees of 11 *Rubritalea* strains based on the 16S rRNA (a) and *gyrB* (b) gene sequences. The trees were constructed with the neighbor-joining method and genetic distances were computed by Kimura's two-parameter model. Numbers at nodes indicate the percentage occurrence in 1000 bootstrapped trees. The trees were created by aligning 1402 (a) and 1061 (b) bp. Bars, 0.02 (a) or 0.05 (b) substitutions per nucleotide position.

2008c), *Haloferula phyci* sp. nov. (strain AK18-024<sup>T</sup>; [26]), *Haloferula helveola* sp. nov. (strain 05IJR53-1<sup>T</sup>; [26]) and *Haloferula sargassicola* sp. nov. (strains MN1-1037<sup>T</sup> and MN1-1047; [26]) are proposed. With these isolates, emended descriptions of the class

*Verrucomicrobiae*, the order *Verrucomicrobiales* and the family *Verrucomicrobiaceae* are presented. Moreover, to establish the formal classification of the phylum 'Verrucomicrobia', we formally proposed *Verrucomicrobia* phyl. nov., nom. Rev [20].

**Table 2.** Differential chemotaxonomic values (%) of the *Rubritalea* strains

Taxa: 1, *Rubritalea sabuli* YM29-052<sup>T</sup>; 2, *Rubritalea* sp. MN1-1006; 3, *Rubritalea* sp. MN1-1030; 4, *Rubritalea* sp. MN1-1042; 5, *Rubritalea* sp. MN1-1054; 6, *Rubritalea squalenifaciens* HOact23<sup>T</sup> (Kasai *et al.*, 2007). ND, Not described; -, not detected; tr, trace.

Characteristic	1	2	3	4	5	6
<b>Fatty acid</b>						
C <sub>15:0</sub>	1	-	tr	tr	tr	1.9
C <sub>16:1</sub>	1.1	-	-	-	-	-
C <sub>15:1</sub> ω6c	2.1	-	tr	tr	tr	1
iso-C <sub>14:0</sub>	49.4	54.7	62.4	60.8	60.5	43.1
iso-C <sub>16:0</sub>	29.1	30.1	17	14.7	19.5	20.6
anteiso-C <sub>15:0</sub>	5.1	9.3	6.3	6	6	18.1
<b>Menaquinone</b>						
MK-7	1.5	tr	tr	tr	tr	tr
MK-8	30.2	4.2	5.1	8.6	7.2	6.9
MK-9	65.4	92.5	91.7	89.3	90.7	90.8
MK-10	2.9	3	2.9	1.5	1.5	2.3

## Conclusion

In this study, we attempted to elucidate the phylogenetic relationships of the thirty novel isolates using polyphasic taxonomic approaches including 16S rRNA and *gyrB* gene sequence analysis, together with molecular, physiological, biochemical and chemotaxonomic analyses to characterize the novel strains. Based on these data, it is proposed that these isolates represent seven novel genera and twenty-two new species within the phylum 'Verrucomicrobia'. Furthermore, with these novel strains, the formal classification of the phylum 'Verrucomicrobia' is established.

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