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Phylogenetic interrelationships of the genus *Rubritalea* inferred from 16S rRNA and *gyrB* gene sequences

Jaewoo Yoon,[1,3]* Hiroaki Kasai[2,4] and Akira Yokota[1]

[1] Institute of Molecular and Cellular Biosciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
[2] Marine Biotechnology Institute Co. Ltd., 3-75-1, Heita, Kamaishi, Iwate 026-0001, Japan
[3] Current address: Department of Biotechnology, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[4] Current address: Marine Biosciences Kamaishi Research Laboratory, Kitasato University
3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan

The genus *Rubritalea* contains a number of environmental species and cultured species which are indistinguishable from one another by 16S rRNA gene sequence analysis. Therefore, the phylogenetic relationships of all known species and six new isolates of the genus *Rubritalea* were investigated by analyzing the sequence of *gyrB*, a gene that encodes the B subunit protein of DNA gyrase. Results from sequence analysis demonstrated that the interrelationships inferred from the *gyrB* gene-based phylogeny showed better resolution than those based on their 16S rRNA gene sequences for the differentiation of strains at the species level. Moreover, the genomic DNA-DNA relatedness values and differential chemotaxonomic data also supported the results of *gyrB* gene-based phylogeny. It is concluded that the *gyrB* could be used as a suitable gene marker for close relationships of the *Rubritalea* group within the phylum '*Verrucomicrobia*'.

Key words: '*Verrucomicrobia*', *Rubritalea, gyrB*, DNA-DNA relatedness value

The phylum '*Verrucomicrobia*' (Hedlund *et al*., 1997) represents a major lineage within the domain *Bacteria*. Many culture-independent studies based on 16S rRNA gene sequences revealed that this phylogenetic group is ubiquitous in nature (O'Farrell & Janssen, 1999; Joseph *et al*., 2003; Rappé & Giovannoni, 2003). At present, it has been informally classified into six subdivisions numbered 1 to 6 (Vandekerckhove *et al*., 2000). Members of the family *Verrucomicrobiaceae* (subdivision 1) within this phylum consist of four published genera. At the time of writing, just a few recognized genera such as *Prosthecobacter, Verrucomicrobium, Akkermansia* and *Rubritalea* (Staley *et al*., 1976; Schlesner, 1987; Derrien *et al*., 2004; Scheuermayer *et al*., 2006) are incorporated within this family. Among them, the genus *Rubritalea* was most recently proposed as a first representative of marine bacterium. Currently, it comprises *Rubritalea marina* (Scheuermayer *et al*., 2006), *R. squalenifaciens* (Kasai *et al*., 2007), *R. spongiae* (Yoon *et al*., 2007), *R. tangerina* (Yoon *et al*., 2007), and *R. sabuli* (Yoon *et al*. 2008) as cultivated and validated species. These members were isolated from marine invertebrates and marine sediments, and appeared as reddish-pink colored colonies by use of some kinds of seawater media containing polysaccharides, amino acids, vitamins, and several antibiotics. The 16S rRNA gene-based phylogenetic studies revealed that these strains were closely related, even though they showed a variety of phenotypic characteristics. In our previous study, comparative phylogenetic analysis of the 16S rRNA gene sequences revealed that the sequence of strain *R. sabuli* YM29-052^T had a high (>99%) similarity to that of a marine bacterium *R. squalenifaciens* HOact23^T (Yoon *et al*., 2008). However, the result of DNA-DNA hybridization test between these strains showed less than 70% relatedness value which is a common threshold for the phylogenetic definition of an independent bacterial species (Wayne *et al*., 1987). Moreover, chemotaxonomic and physiological analyses supported the DNA-DNA hybridization data (Yoon *et al*. 2008). These results suggested that phylogenetic analysis of the genus *Rubritalea* based on

*Corresponding author
E-mail: ayoon@mail.ecc.u-tokyo.ac.jp
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Table 1 Strains used in this study

Species	Strain	Source	Sampling site	Isolation medium	Reference
Rubritalea sp.	MN1-1030	Bryozoan	Onomichi, Japan	M1[a]+Nal[b]	
Rubritalea sp.	MN1-1042	Bryozoan	Onomichi, Japan	M1-Rif[c]+Nal+Fra[d]+AZT[e]	
Rubritalea sp.	MN1-1054	Sea squirt	Onomichi, Japan	M1+Nal	
Rubritalea sp.	MN1-1006	Sea squirt	Sado Island, Japan	M1-Rif+Nal	
Rubritalea sp.	shu-10-MA45-1	Eye of embiotocid	Kamaishi, Japan	1/5 strength MA[f]	
Rubritalea sp.	YM29-130	Feces of sea cucumber	Pohnpei, Micronesia	P[g]	
Rubritalea marina	(DSM 17716^T=Pol012^T)	Sponge	Banyuls-sur-Mer, France	M1	Scheuermayer *et al.* (2006)
Rubritalea squalenifaciens	(MBIC08254^T=HOact23^T)	Sponge	Kanagawa, Japan	M1	Kasai *et al.* (2007)
Rubritalea sabuli	(MBIC08323^T=YM29-052^T)	Sediments	Pohnpei, Micronesia	P	Yoon *et al.* (2008)
Rubritalea spongiae	(MBIC08281^T=YM21-132^T)	Sponge	Aomori, Japan	P	Yoon *et al.* (2007)
Rubritalea tangerina	(MBIC08282^T=YM27-005^T)	Sea hare	Sado Island, Japan	P	Yoon *et al.* (2007)

[a] Mincer et al. (2002), [b] Nalidixic acid 20 μg/ml, [c] Rifampin 5 μg/ml, [d] Fradiomycin 50 μg/ml, [e] Aztreonam 40 μg/ml, [f] 1/5 strength marine agar 2216 (Difco) with seawater, [g] Yoon *et al.* (2007)

the 16S rRNA gene (Woese 1987) sequences is not convenient for resolving interrelationships at the species level between closely related species because of its slow evolution (Fox *et al.*, 1992).

The *gyrB* gene encodes the B subunit protein of DNA gyrase, a type II DNA topoisomerase, which plays an essential role in DNA replication and is distributed universally among bacterial species (Watt & Hickson, 1994; Huang, 1996). In addition, it appears faster than the 16S ribosomal RNA genes in the evolutionary rate, which makes it especially useful for strain discrimination and identification of bacteria (Yamamoto & Harayama, 1995). In the present study, it has been shown that direct sequencing of the *gyrB* gene could be used for identification and phylogenetic analysis of species of the genus *Rubritalea* within the phylum '*Verrucomicrobia*'.

Strains investigated and source samples are listed in Table 1. Samples (0.5–1 cm^3) were homogenized with a glass rod in 5 m*l* sterile seawater. The homogenate (50 m*l*) was applied to each medium for isolation. Bacteria were purified on marine broth 2216 (Difco) containing 1.5% agar.

An approximately 1,500 bp fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1,492R (*Escherichia coli* numbering system; Weisburg *et al.*, 1991).

The *gyrB* gene fragment covering positions 274–1525 in the *E. coli gyrB* gene was amplified using the deoxyinosine nucleotides containing primers UP1Gi (5'- GAA GTC ATC ACC GTT CTG CAY GSI GGI GGI AAR TTY RG -3') and UP2ri (5'- AGC AGG GTA CGG ATG TGC GAG CCR TCI ACR TCI GCR TCI GTC AT -3'). PCR reaction was performed by using TaKaRa LA Taq polymerase (Takara, Japan) by the following conditions; one cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s and 58°C for 1 min, followed by 72°C for 1 min 30 s, followed by a final 5-min incubation at 72°C. Sequencing was performed using UP1s (5'-GAAGTCATCACCGTTCTGCA -3') and UP2rs (5'-AGCAGGGTACGGATGTGCGAGCC -3'), as previously described by Yamamoto & Harayama (1995).

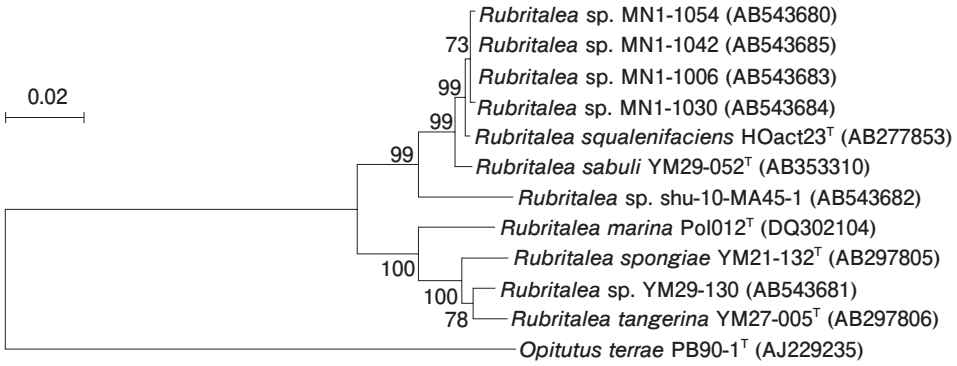
The GenBank/EMBL/DDBJ accession numbers for the *gyrB* gene sequences of 11 strains used in this study are AB543686–AB543696 and those of the 16S rRNA gene sequences are AB277853, AB297805–AB297806, AB353310, AB543680–AB543685 and DQ302104.

Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al*., 1997). Alignment gaps and ambiguous bases were not taken into consideration when both 1402 (16S rRNA gene) and 1061 (*gyrB* gene) bases of the gene nucleotides were compared respectively. Aligned sequences were analysed using MEGA3.1 software (Kumar *et al*., 2004). The evolutionary distances [distance options according to the Kimura two-parameter model (Kimura, 1983)] and clustering with the neighbor-joining (Saitou & Nei, 1987) method were determined by using bootstrap values based on 1000 replications (Felsenstein, 1985). The similarity values were calculated using the same software.

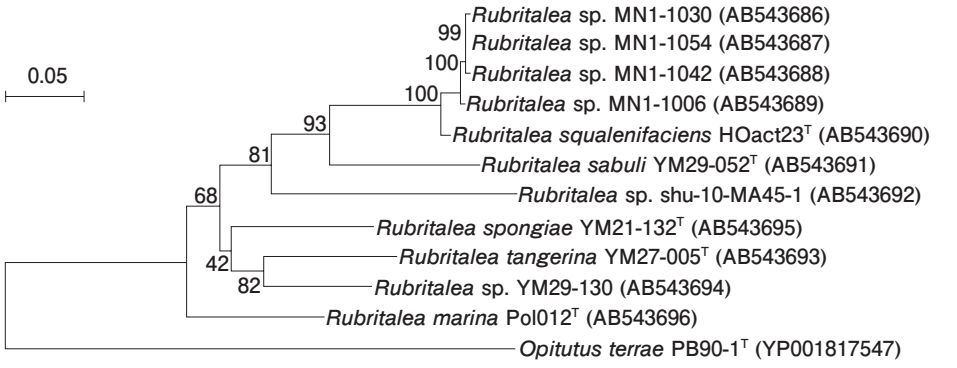
DNA-DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al*. (1989). Hybridization was performed using five replications for each. Of the values obtained, the highest and lowest for each sample were excluded and the means of the remaining three values were quoted as DNA-DNA relatedness values.

Determination of the respiratory quinone system and cellular fatty acid composition were carried out as described previously (Katsuta *et al*., 2005). DNA was prepared according to the method of Marmur (1961) and the DNA base composition was determined by using the HPLC method of Mesbah *et al*. (1989).

The phylogenetic relationships of all known members and six new isolates of the genus *Rubritalea* were investigated by analyzing the sequences of 16S



a: 16S rRNA



b: *gyrB*

Fig. 1 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 1,000 bootstrapped trees. The trees were created by aligning 1,402 (a) and 1,061 (b) bp. Bars, 0.02 (a) or 0.05 (b) substitutions per nucleotide position.

Table 2 Comparison of the 16S rRNA gene sequence similarity within the genus *Rubritalea*

Strain	1	2	3	4	5	6	7	8	9	10	11	12
1. *Rubritalea* sp. MN1-1006 (AB543683)												
2. *Rubritalea squalenifaciens* HOact23^T (AB277853)	99.9											
3. *Rubritalea* sp. MN1-1030 (AB543684)	100	99.9										
4. *Rubritalea* sp. MN1-1054 (AB543680)	100	99.9	100									
5. *Rubritalea* sp. MN1-1042 (AB543685)	99.9	99.9	99.9	99.9								
6. *Rubritalea sabuli* YM29-052^T (AB353310)	99.3	99.4	99.3	99.3	99.2							
7. *Rubritalea* sp. shu-10-MA45-1 (AB543682)	96.3	96.3	96.3	96.3	96.2	96.5						
8. *Rubritalea* sp. YM29-130 (AB543681)	93.6	93.5	93.6	93.6	93.5	93.4	92.9					
9. *Rubritalea tangerina* YM27-005^T (AB297806)	93.3	93.2	93.3	93.3	93.2	93.2	92.6	98.6				
10. *Rubritalea spongiae* YM21-132^T (AB297805)	93.3	93.2	93.3	93.3	93.2	93.2	92.6	98.1	97.5			
11. *Rubritalea marina* Pol012^T (DQ302104)	93.6	93.5	93.6	93.6	93.5	93.4	93.2	96.3	96	95.7		
12. *Opitutus terrae* PB90-1^T (AJ229235)	75.2	75.1	75.2	75.2	75.1	74.7	73.3	74.1	74.2	74	74.4	

Table 3 Comparison of the *gyrB* gene sequence similarity within the genus *Rubritalea*

Strain	1	2	3	4	5	6	7	8	9	10	11	12
1. *Rubritalea* sp. MN1-1006 (AB543689)												
2. *Rubritalea squalenifaciens* HOact23^T (AB543690)	97.7											
3. *Rubritalea* sp. MN1-1030 (AB543686)	99.4	97.5										
4. *Rubritalea* sp. MN1-1054 (AB543687)	99.5	97.6	99.9									
5. *Rubritalea* sp. MN1-1042 (AB543688)	99.5	97.6	99.9	100								
6. *Rubritalea sabuli* YM29-052^T (AB543691)	82.3	82.5	81.8	81.9	81.9							
7. *Rubritalea* sp. shu-10-MA45-1 (AB543692)	72.4	74.1	72.6	72.7	72.7	69.7						
8. *Rubritalea* sp. YM29-130 (AB543694)	74	74.8	73.9	74	74	76.5	73.4					
9. *Rubritalea tangerina* YM27-005^T (AB543693)	72	73.2	72.3	72.4	72.4	71.2	74.6	84.9				
10. *Rubritalea spongiae* YM21-132^T (AB543695)	74.1	75	73.8	73.9	73.9	72.8	75.6	81.5	81.2			
11. *Rubritalea marina* Pol012^T (AB543696)	72.9	73.6	72.6	72.7	72.7	73.2	72	79.8	78.7	81.7		
12. *Opitutus terrae* PB90-1^T (YP001817547)	39.1	38.9	39.2	39.4	39.4	43.2	25	47.5	40.4	40.4	47	

Table 4 DNA-DNA relatedness values (%) of the *Rubritalea* species

Strain	DNA-DNA relatedness values (%) with:		
	1	2	3
1. *Rubritalea squalenifaciens* HOact23^T	-	23.8	56.7
2. *Rubritalea sabuli* YM29-052^T	28.7	-	17.8
3. *Rubritalea* sp. MN1-1006	58.5	12.3	-

rRNA and *gyrB* gene. Analysis of the nearly complete 16S rRNA gene sequences from these strains indicated that the strains MN1-1006, MN1-1030, MN1-1042, MN1-1054, *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T formed a robust clade having only limited resolution (Fig. 1a). This group shared high sequence similarities in 16S rRNA gene (99.2–100%; Table 2). Difference of the 16S rRNA gene sequence between *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T were only 0.6% in 1402 nucleotides. However, the genomic DNA-DNA relatedness values between the strain MN1-1006, *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T were 12.3–58.5% (Table 4), indicating these strains are independent species of the genus *Rubritalea*. Moreover, the differential chemotaxonomic data (cellular fatty acid components: iso-$C_{16:0}$, anteiso-$C_{15:0}$ and $C_{15:1}\omega6c$, and menaquinones: MK-8 and MK-9) (Table 5) also supported the results of genomic DNA-DNA hybridization studies.

To resolve the micro-diversity in *Rubritalea* strains, the *gyrB* gene sequences were analyzed. The *gyrB* gene of the *Rubritalea* was able to be amplified by universal primers containing inosine

Table 5 Differntial chemotaxonomic values (%) of the *Rubritalea* strains

Characteristic	1	2	3	4	5	6
Fatty acid						
$C_{15:0}$	1	-	tr	tr	tr	1.9
$C_{16:1}$	1.1	-	-	-	-	-
$C_{15:1}\omega 6c$	2.1	-	tr	tr	tr	1
iso-$C_{14:0}$	49.4	54.7	62.4	60.8	60.5	43.1
iso-$C_{16:0}$	29.1	30.1	17	14.7	19.5	20.6
anteiso-$C_{15:0}$	5.1	9.3	6.3	6	6	18.1
Menaquinone						
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

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

nucleotides. No amplification was observed from the paralogous gene, *parE*. The *gyrB* gene sequence similarities among members within the clade including strains MN1-1030, MN1-1054, MN1-1042, MN1-1006, *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T were 81.8–100% (Table 3). Furthermore, the genomic DNA-DNA relatedness value agreed with the results of *gyrB* gene-based grouping at an independent species level (Table 4). Consequently, in view of these data, a yet-uncharacterized *Rubritalea* cluster comprising strains MN1-1006, MN1-1030, MN1-1042 and MN1-1054 should be officially proposed as a novel species. Also, strains YM29-130 and shu-10-MA45-1 are possible representatives of novel species of the genus *Rubritalea*.

This is the first report on the *gyrB* gene-based phylogenetic analysis of species in the genus *Rubritalea*. Based on the comparative phylogenetic analysis, it is clearly elucidated that the base substitution rate of the *gyrB* gene sequence was much faster than that of the 16S rRNA gene sequence. Sequence differences in the *gyrB* gene between the strain MN1-1006, or *R. squalenifaciens* HOact23^T and *R. sabuli* YM29-052^T were 82.3 or 82.5%, respectively. They had enough differences in the *gyrB* gene for the species differentiation comparing with other taxa (Kasai *et al*., 2000; Hatano *et al*., 2003). On the other hand, sequence difference in the *gyrB* between MN1-1006 and *R. squalenifaciens* HOact23^T was 97.7%. This value is relatively higher than that obtained in the previous studies. Hatano *et al*. (2003) had reported that the *gyrB* gene sequence similarity corresponding to DNA-DNA relatedness value of 70% was 96.5% in case of the genus *Streptomyces*. Moreover, a similar genomic DNA-DNA relatedness value had been reported in the genus *Micromonospora* (Kasai *et al*., 2000). These comparative studies between the DNA-DNA hybridization values and sequence similarities of the individual genes suggest that genome rearrangement occurs more frequently in the genus *Rubritalea* than that occurring in the other taxa.

Phylogenetic analysis based on the *gyrB* gene sequences also revealed that the *Rubritalea* members could be discriminated and grouped by different of sampling sites such as Onomichi (strains MN1-1030, MN1-1042 and MN1-1054) and Sado Island (MN1-1006) (Table 1). Thus, the *gyrB* gene might be an effective molecular ecological tool for the analysis of yet-uncultivated *Rubritalea* members in the marine environment. From these results, it is strongly suggested here that the *gyrB* gene is useful to resolve micro-diversity among the strains of the genus *Rubritalea* within the phylum '*Verrucomicrobia*'. In this study, we performed molecular phylogenetic analyses based on the 16S rRNA and the *gyrB* gene sequences to provide taxonomic information on the members of genus *Rubritalea*. Finally, it is concluded that the *gyrB* gene marker could be used for a phylogenetic identification of closely related strains of the genus *Rubritalea* within the phylum '*Verrucomicrobia*'.

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16S rRNA 遺伝子および *gyrB* 遺伝子に基づく *Rubritalea* 属細菌の系統解析

尹　載宇[1)]，笠井宏朗[2)]，横田　明[3)]

[1)] 東京大学分子細胞生物学研究所（現所属 東京大学大学院農学生命科学研究科）
[2)] 海洋バイオテクノロジー研究所（現所属 北里大学海洋バイオテクノロジー釜石研究所）
[3)] 東京大学分子細胞生物学研究所

様々な環境中に生息する *Rubritalea* 属細菌は，16S rRNA 遺伝子塩基配列では区別できない多数の種を含んでいる．本研究では，これまでに知られている *Rubritalea* 属の菌種および今回新たに分離した 6 株について *gyrB* 遺伝子による系統解析を行った．その結果，16S rRNA 遺伝子塩基配列より，*gyrB* 遺伝子塩基配列の方が系統解析上高い解像度を示した．さらに，ゲノム DNA 交雑実験や生化学実験の結果も *gyrB* 遺伝子塩基配列に基づく系統解析の結果を支持した．本研究の結果から *gyrB* 遺伝子は '*Verrucomicrobia*' 門に属する *Rubritalea* 属の系統解析に適していることが明らかになった．

（担当編集委員：田中尚人）