

Cultivation of Uncultured Bacteria of the Class *Ktedonobacteria* in the Phylum *Chloroflexi*

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Abstract

The class *Ktedonobacteria* in the phylum *Chloroflexi* is known to contain a large number of uncultured, environmental 16S rRNA gene clones, and cultured representatives are a limited number. In this review, recent findings on the taxonomical and ecological significance of the class *Ktedonobacteria* in the phylum *Chloroflexi* are discussed based on the findings from both the characteristics of the cultured *Ktedonobacteria* and molecular-based analysis. The microorganisms characterized so far include four species in three genera, *Ktedonobacter*, *Thermosporothrix* and *Thermogemmatispora*, and were proposed to represent three families, *Ktedonobacteraceae*, *Thermosporotricaceae*, and *Thermogemmatisporaceae*, and two orders, *Ktedonobacterales* and *Thermogemmatisporales*. *Ktedonobacteria* strains showed a common property of gram-positive, aerobic organisms that produce branched vegetative mycelia and form spores by budding.

Abstrak

Kultivasi Bakteri yang tidak dapat Dikultur dari Kelas *Ktedonobacteria* pada Filum *Chloroflexi*. Kelas *Ktedonobacteria* dari filum *Chloroflexi* terdiri atas sejumlah besar taksa yang tidak dapat dikultur, klon-klona gen 16S rRNA yang berasal dari lingkungan, dan sejumlah kecil taksa yang dapat dikultur. Tulisan ini mengulas temuan terakhir mengenai taksonomi dan ekologi kelas *Ktedonobacteria* dari filum *Chloroflexi* berdasarkan karakteristik yang ditemukan pada biakan *Ktedonobacteria* dan analisis molekuler. Sejauh ini, mikroorganisme yang telah dikarakterisasi mencakup empat spesies dari tiga marga, yaitu *Ktedonobacter*, *Thermosporothrix*, dan *Thermogemmatispora*. Ketiga marga tersebut diusulkan untuk mewakili tiga famili, yaitu *Ktedonobacteraceae*, *Thermosporotricaceae*, dan *Thermogemmatisporaceae*, dan dua bangsa, *Ktedonobacterales* dan *Thermogemmatisporales*. Strain-strain *Ktedonobacteria* memiliki ciri-ciri umum gram-positif, bersifat aerob, menghasilkan hifa vegetatif yang bercabang, dan membentuk spora dengan cara pertunasan.

Keywords: blastospore, Chloroflexi, Ktedonobacteria, Thermosporothrix, Thermogemmatispora

1. Introduction

The phylum *Chloroflexi*, formerly known as the green non-sulfur bacteria, has been recognized as bacterial taxa containing a number of environmental 16S rRNA gene clones with a limited number of cultured representatives [1-5]. The phylum *Chloroflexi* is generally considered to be a deep-branching lineage of the domain bacteria [6-8]. The phylum has formerly been divided into four subphyla (class-level taxa) on the basis of 16S rRNA gene sequences [1]; subphylum I (now the class *Anaerolinea* and the class *Caldilinea* [9]), subphylum II

(the class "*Dehalococcoidetes*"), subphylum III (the class *Chloroflexi*) and subphylum IV (clone cluster). Later, the cluster of genus *Thermomicrobium* in the subphylum III has been reclassified as an additional subphylum, the class *Thermomicrobia* [10], and more recently, Yabe *et al.* [11] proposed to include the class *Ktedonobacteria* [12] in the phylum *Chloroflexi*. In addition to these major six subphyla (the classes), other uncultured lineages at the subphylum (class) level, "E05 lineage" and "G04 lineage", are also known in the phylum *Chloroflexi* [2,13].

The class *Ktedonobacteria*, which is now belonging to the phylum *Chloroflexi*, is also known to contains many uncultured environmental clones by the cultivation-independent molecular studies. The finding of the presence of uncultured bacteria in the environments prompted the efforts of cultivation and isolation of such bacterial strains by the use of various methods. The class *Ktedonobacteria* is such bacterial group which has recently been cultured and characterized.

2. Present taxonomy of the phylum *Chloroflexi*

As shown in Table 1, taxonomy of the phylum *Chloroflexi* at present are summarized. Phylogenetic relationships among all the genera in the phylum *Chloroflexi* is shown in Figure 1.

The class *Chloroflexi* has been represented by cultured organisms of the genera *Chloroflexus*, *Oscillochloris*, *Chloronema*, *Roseiflexus*, *Heliothrix*, *Herpetosiphon*, and "*Kouleothrix*". These organisms are mostly filamentous, phototrophic and/or chemolithoheterotrophic growth under mesophilic or moderately thermophilic conditions.

The class *Thermomicrobia* also involves cultured organisms of the genera *Thermomicrobium*, *Sphaerobacter* and "*Thermobaculum*", which are rod-shaped, moderately thermophilic or hyperthermophilic, chemolithoheterotrophic and aerobic. The class *Anaerolinea* is most frequently detected in the various environment and involves cultured organisms of the genera *Anaerolinea*, *Bellilinea*, *Leptolinea*, *Levilinea* and *Longilinea*. A new class *Caldilineae* was proposed by Yamada *et al.* [9] for the genus *Caldilinea*. The class

Table 1. Taxonomy of the Pylum *Chloroflexi*

Phylum	Class	Order	Family	Genus	Species					
<i>Chloroflexi</i>	<i>Chloroflexi</i>	<i>Chloroflexales</i>	<i>Chloroflexaceae</i>	<i>Chloroflexus</i>	<i>Chloroflexus aggregans</i> , <i>C. aurantiacus</i>					
				<i>Roseiflexus</i>	<i>Roseiflexus castenholzii</i>					
				<i>Heliothrix</i>	<i>Heliothrix oregonensis</i>					
				" <i>Kouleothrix</i> "	" <i>Kouleothrix aurantiaca</i> "					
			<i>Oscillochloridaceae</i>	<i>Oscillochloris</i>	<i>Oscillochloris crysea</i> , <i>O. trichoides</i>					
				<i>Chloronema</i>	<i>Chloronema giganteum</i>					
				<i>Herpetosiphonales</i>	<i>Herpetosiphonaceae</i>	<i>Herpetosiphon</i>	<i>Herpetosiphon aurantiacus</i> , <i>H. geysericola</i>			
						<i>Thermomicrobia</i>	<i>Thermomicrobiales</i>	<i>Thermomicrobiaceae</i>	<i>Thermomicrobium</i>	<i>Thermomicrobium roseum</i>
						<i>Sphaerobacterales</i>	<i>Sphaerobacteraceae</i>	<i>Sphaerobacter</i> " <i>Thermobaculum</i> "	<i>Sphaerobacter thermophilus</i> " <i>Thermobaculum terrenum</i> "	
		<i>Anaerolinea</i>	<i>Anaerolineales</i>	<i>Anaerolineaceae</i>	<i>Anaerolinea</i>	<i>Anaerolinea thermolimosa</i>				
					<i>Bellilinea</i>	<i>Bellilinea caldifistulae</i>				
					<i>Leptolinea</i>	<i>Leptolinea tardivitalis</i>				
					<i>Levilinea</i>	<i>Levilinea saccharolytica</i>				
<i>Longilinea</i>	<i>Longilinea arvoryzae</i>									
<i>Caldilineae</i>	<i>Caldilineales</i>	<i>Caldilineaceae</i>	<i>Caldilinea</i>	<i>Caldilinea aerophila</i>						
<i>Dehalococcoidetes</i>	<i>Dehalococcoidetales</i>	<i>Dehalococcoidetaceae</i>	<i>Dehalogenimonas</i>	<i>Dehalogenimonas</i> <i>lykanthroporepellens</i>						
			" <i>Dehalococcoides</i> "	" <i>Dehalococcoides</i> <i>ethenogenes</i> "						
			<i>Ktedonobacteria</i>	<i>Ktedonobacteriales</i>	<i>Ktedonobacteraceae</i>	<i>Ktedonobacter</i>	<i>Ktedonobacter racemifer</i>			
<i>Thermosporotrichaceae</i>	<i>Thermosporothrix</i>	<i>Thermosporothrix hazakensis</i>								
	<i>Thermogemmatissporales</i>	<i>Thermogemmatissporaceae</i>	<i>Thermogemmatisspora</i>	<i>Thermogemmatisspora</i> <i>onikobensis</i> , <i>T. foliorum</i>						
	"Ellin 7237 lineage"									
	"Subphylum IV "									
	"E05 lineage"									
	"G04 lineage"									

The Table was created based on the report by Euzéby (14), and according to the reports by Costello *et al.* [13] and Davis *et al.* [15], the table was modified.

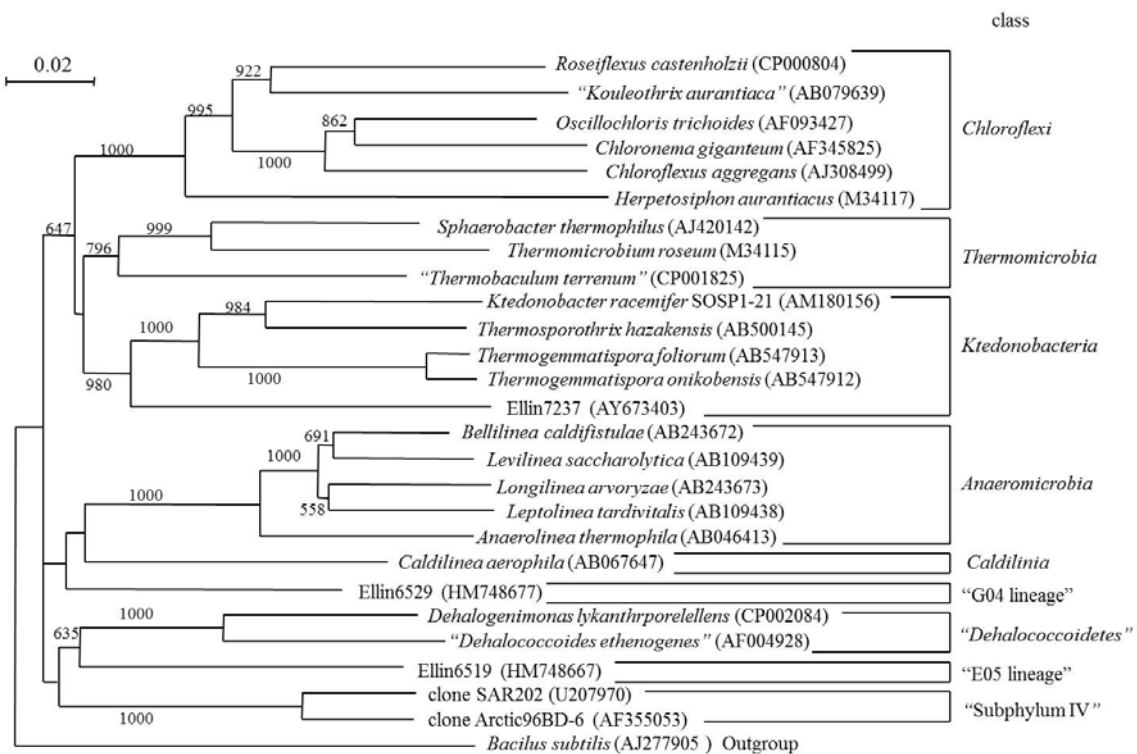


Figure 1. Neighbour-Joining Phylogenetic Tree of the Phylum *Chloroflexi* Derived from Comparative Analysis of 16S rRNA Gene Sequences

"*Dehalococcoidetes*" include the cultured organisms of the genera *Dehalogenimonas* and "*Dehalococcoides*". The class *Ktedonobacteria* contains the cultured organisms of the genera *Ktedonobacter*, *Thermosporothrix* and *Thermogemmatisspora*. In this class, additional cluster at a new order level, "Ellin 7237 cluster", is included. At class level, Subdphylum IV (clone cluster), "E05 lineage" and "G04 lineage", are also known, but not yet cultured.

The morphology, physiology and genetic properties of cultivated genera of the phylum *Chloroflexi* are shown in Table 2. These strains are obligately aerobic, mesophilic or moderately thermophilic, multicellular filamentous or rod-formed or cocci, chemolithohetero-trophic organisms degrading carbohydrates. Thus, the phylum *Chloroflexi* is highly heterogeneous with respect to phylogenetic, morphological and physiological characteristics.

Thus, the phylum contains a large number of yet-to-be cultured organisms from natural and artificial environments. Further studies to isolate and cultivate these organisms are required.

3. Cultivation of uncultured *Ktedonobacteria* strains

Among the classes in the phylum *Chloroflexi*, present paper mainly deals with the cultured strains in the class *Ktedonobacteria*. Phylotypes affiliated with the

Ktedonobacteria are detected in a wide range of environments, and the group now contains quite diverse rRNA gene sequences among known orders in the class *Ktedonobacteria* (Figure 2). To reveal their physiological and biochemical role, attempts were made to cultivate them. The first pure culture representing the group, i.e., *Ktedonobacter racemifer* was obtained in 2006 from Italian soil [12]. The bacterial strains obtained were filamentous, aerobic and mesophilic, but not thermophilic. Later, the second tangible organism of the group, *Thermosporothrix hazakensis* was isolated in 2010 from thermophilic atmosphere, hot compost, in Japan [11]. Simultaneously, a new thermophilic genus *Thermogemmatisspora* with two new species were successfully cultivated and characterized, which was isolated in 2010 from geothermal soils in Japan [22]. To our knowledge, these four species are the only named bacteria of cultivated organisms of the class *Ktedonobacteria*. However, in addition to these species, other isolates, which has not been taxonomically characterized, are known [12,24-25] (Figure 2): SOSP strains were isolated from Italian soil [12]. Strains SOSP1-9, SOSP1-79 and SOSP1-142 probably belong to a new genus; SOSP1-63 probably belong to a new genus; strains SOSP1-0, SOSP1-30, SOSP1-52 and SOSP1-85 probably belong to a new species of the genus *Ktedonobacter*; a strain Hsw-67 probably belong to *Thermogemmatisspora hazakensis*; strains SOSP1-0 and SOSP1-165 probably belong to a new genus; strains P-352, T104 and T81 isolated from geothermal soils by

Table 2. Phenotypic Characteristics of Valid Genera Affiliated with the Phylum Chloroflexi

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Morphology	M-cell: F	M-cell: F	M-cell: F	M-cell: F	M-cell: F	M-cell: F	M-cell: F	M-cell: F	Irregular rods	Pleomorphic	Irregular cocci	M-cell: F	M-cell: F	M-cell: F
Cell diameter (mm)	0.2-0.3	0.7-0.8	0.7-1.5	1.0-5.5	0.8-1.0	1,5	2.0-2.5	0.5-1.5	1.0-3.0	1.3-1.8	0.3-0.6	ND	0.7-1.0	0.5-1.5
Gliding	-	-	+	+	ND	+	+	+	ND	-	ND	-	-	-
Color	Colorless	Orange-pinkish	Green, orange	Green, yellow-green	Red, reddish-green	Bright orange	Yellow green	Yellow	ND	Pink	ND	White	Orange or white	White
Bacteriochlorophyll	-	-	a,c	a,c	a	a	a,c,d	-	-	-	-	-	-	-
Spore formation	-	-	-	-	-	-	-	-	-	-	-	Sphere, wrinkle	Fig-shaped, smooth-surfaced	Egg-shaped, rough-surfaced
Sheath	-	-	+/-	+/-	-	ND	ND	+	-	-	-	-	-	-
Gram-stain	-	-	-	+	-	ND	ND	-	+	-	-	+	+	+
Optimum growth temp. (C)	55	55	20-25, 55	28-30	50	40-55	4.0-15.0	25-30	55	70-75	28-34	28-33	50	60-65
Metabolism:														
Photoheterotroph	-	-	+	+	+	+	+	-	ND	ND	ND	-	-	-
Photoautotroph	-	-	+/-	+/-	-	-	-	-	ND	ND	ND	-	-	-
O ₂ respiration	-	-	+	+	+	+	+	+	+	+	-	+	+	+
Fermentation	+	+	+/-	ND	-	-	-	-	-	ND	ND	-	-	-
Cell-wall amino acids	ND	ND	ND	ND	Orn	ND	ND	ND	ND	ND	ND	Glu, Ser, Gly, Ala, Orn	Glu, Ser, Gly, Ala, Orn	Glu, Ser, Gly, His, Ala, Orn
Major cellular fatty acids	C16:0, C15:0, C14:0, C18:0	C18:0, C16:0, C18:1	C18:0, C16:0, C18:1	C18:1, C16:0, C16:1	ND	ND	ND	ND	ND	ND	ND	C18:1, C16:1, C16:0	iso-C16:0, anteiso-C17:0, iso-C17:0	iso-C17:0, iso-C19:0, 12,17-diMe-C18:0
Polar lipids	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	PL, PG, DPG	PI, PIM, PG, DPG	PI, PG
Major menaquinone	-	MK-10	MK-10	MK-10	MK-11	ND	ND	MK-6	MK-8	ND	ND	MK-9(H ₂)	MK-9(H ₂)	MK-9(H ₂)
DNA G+C content (mol%)	54,5	54,5	62.8-63.9	60.4-61.3	62	ND	ND	48.1-48.5	66,3	64,3	53-54	53,9	54	58.1-60.2

Table was created based on a review by Hanada and Pierson [16]. Data for *Anaerolinea* and *Caldilinea* were obtained from Sekiguchi *et al.* [17], data for *Chloronema* were from Overmann [5], data for *Sphaerobacter* were from Hensel *et al.* [18], data for *Thermomicrobium* were from Jackson *et al.* [19], data for *Dehalogenimonas* were from Moe *et al.* [20], data for *Ktedonobacterium* were from Cavaletti *et al.* [12], data for *Thermosporothrix* and *Thermogemmatipora* were from Yabe *et al.* [11,21-22].

Genera: 1. *Anaerolinea*; 2. *Caldilinea*; 3. *Chloroflexus*; 4. *Oscillochloris*; 5. *Roseiflexus*; 6. *Heliothrix*; 7. *Chloronema*; 8. *Herpetosiphon*; 9. *Sphaerobacter*; 10. *Thermomicrobium*; 11. *Dehalogenimonas*; 12. *Ktedonobacter*; 13. *Thermosporothrix*; 14. *Thermogemmatipora*.

Abbreviations: Glu= glutamic acid; Ser= serine; Gly= glycine; Ala= alanine; His= histidine; Orn= ornithine; PL= phospholipid; PG= phosphatidylglycerol; DPG= diphosphatidylglycerol; PI= phosphatidylinositol; PIM= phosphatidylinositol mannoside; M-cell= multi cellular; F= filaments

Stott *et al.* [25] probably belong to a new species of the genus *Thermogemmatipora*, and a strain P359 probably belong to *Thermogemmatipora foliorum*. A strain Ellin 7237 isolated from soil in Australia [24] probably belong to a new species of a new genus of a new order in the class *Ktedonobacteria*. As these strains has not yet been taxonomically characterized, taxonomic studies for these strains are waited.

Stott *et al.* [25] used media solidified with gellan for the isolation of SOSP strains, and Davis *et al.* [24] also used media solidified with gellan for the isolation of Ellin7237. Although many improved methods, such as mini-colony formation [26], micromanipulation method [27], diluted nutrient broth method [23], and flow cytometry method [28] are reported for cultivating the uncultured bacteria, the approach by gellan gum media

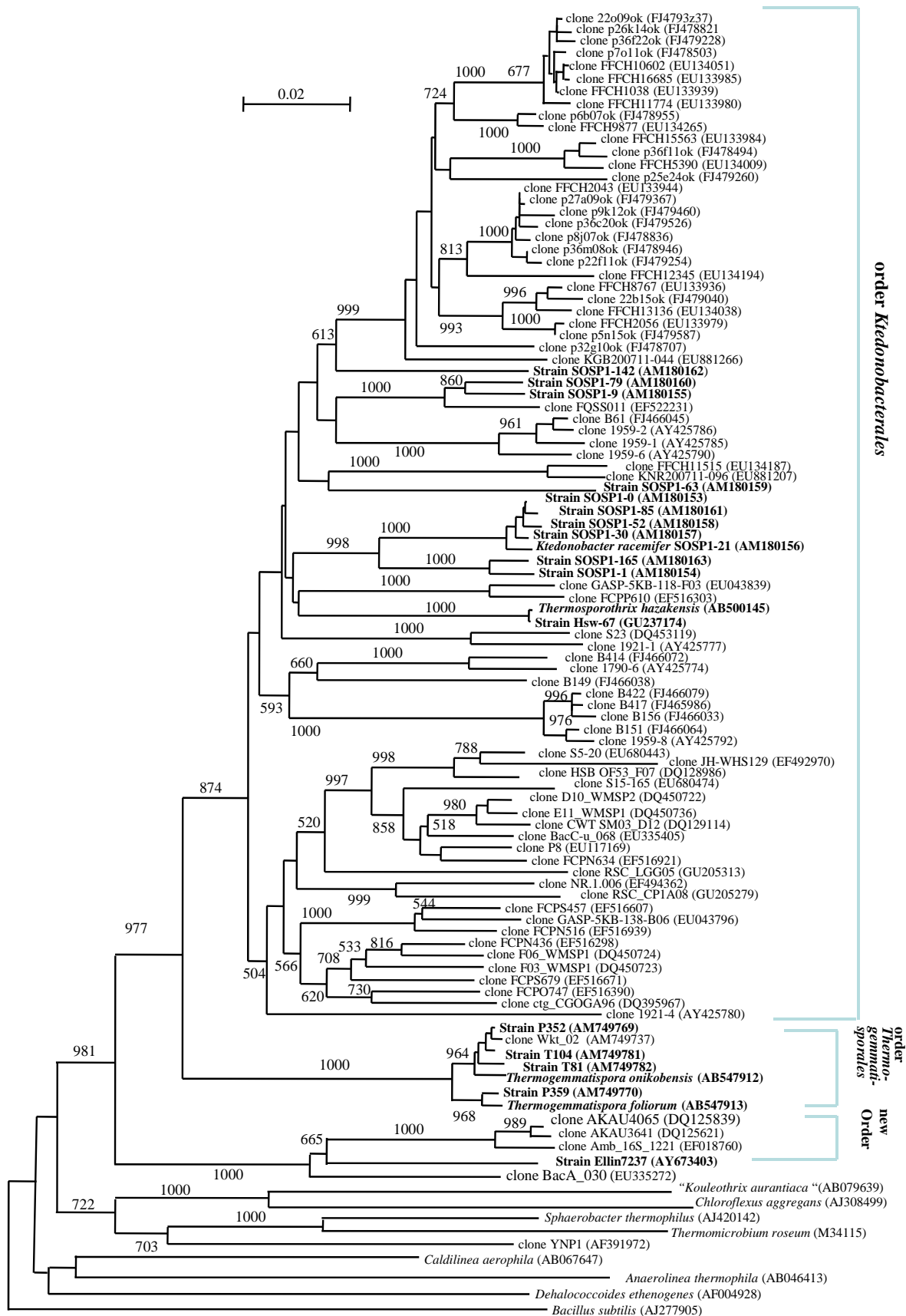


Figure 2. Neighbour-Joining Phylogenetic Tree of the Class Ktedonobacteria Derived from Comparative Analysis of 16S rRNA Gene Sequences. Sequences of Clones were Obtained from Greengenes Databases [23]

may be useful for further isolating uncultured *Ktedonobacteria*.

As chemotaxonomic characteristics of *Ktedonobacteria* strains, they contained menaquinone MK-9(H₂) as respiratory quinone, iso-C16:0 and/or iso-C17:0 as major cellular fatty acid. A quite rare fatty acid, 12,17-dimethyl-C18:0, was found in the cellular fatty acids of *Thermogemmatipora* species, and the presence of this fatty acid is taxonomic significance [29]. *Ktedonobacteria* strains contained DNA of 54-60 mol% DNA G+C content, and ornithine, glutamic acid, serine, glycine, and alanine in the cell wall. In *Thermogemmatipora* species, histidine was additionally included in the cell wall, which indicates the presence of histidine is also taxonomically significant.

4. Morphological characteristics of the *Ktedonobacteria*

The morphological properties of cultivated strains in the orders *Ktedonobacterales* and *Thermogemmatiporales* in the *Ktedonobacteria* are shown in Figure 3 [11,22]. The strains are gram-positive, aerobic, mesophilic or thermophilic, chemolithoorganoheterotrophic organisms degrading polysaccharides and peptides. Morphological characteristic features of these strains are the formations of branched multicellular filaments and spores. The spores are formed by budding, therefore, the spores

formed could be called "blastospores". As far as known, sporulation modes in prokaryotes include formation of endospores exemplified by *Firmicutes* bacteria, myxospores by myxobacteria and arthrospores by actinomycetes. Microscopic observations showed that these strains formed multiple exospores per mother cells by budding in branched aerial mycella. Although branched aerial mycella is a characteristic of actinomycetes, multiple budding sporulation has not been previously described in prokaryotes [30]. Life cycle of the strain of *Thermosporothrix hazakensis* is shown in Figure 4 [31].

5. Genome sequence of the *Ktedonobacteria*

Very recently, genome sequence of *Ktedonobacter racemifer* SOSP1-21^T was reported by Chang *et al.* [32]. It was revealed that the strain contained DNA with a length of 13.7 Mbp, which is the largest of all completely sequenced so far. *K. racemifer* stands out because of its enormous genome size of more than 13 Mbp. The genomes of neighboring taxa, *Sphaerobacter thermophilus* and *Thermomicrobium roseum* are significantly smaller, 3.9 Mbp and 2.9 Mbp, respectively. Our preliminary data on the draft genome sequence of *Thermosporothrix hazakensis* SK20-1^T, the closest relative of *K. racemifer*, suggested that it contained 7.3 Mbp genome size, which is significantly lower than that of *K. racemifer* (Yabe *et al.*, unpublished results).

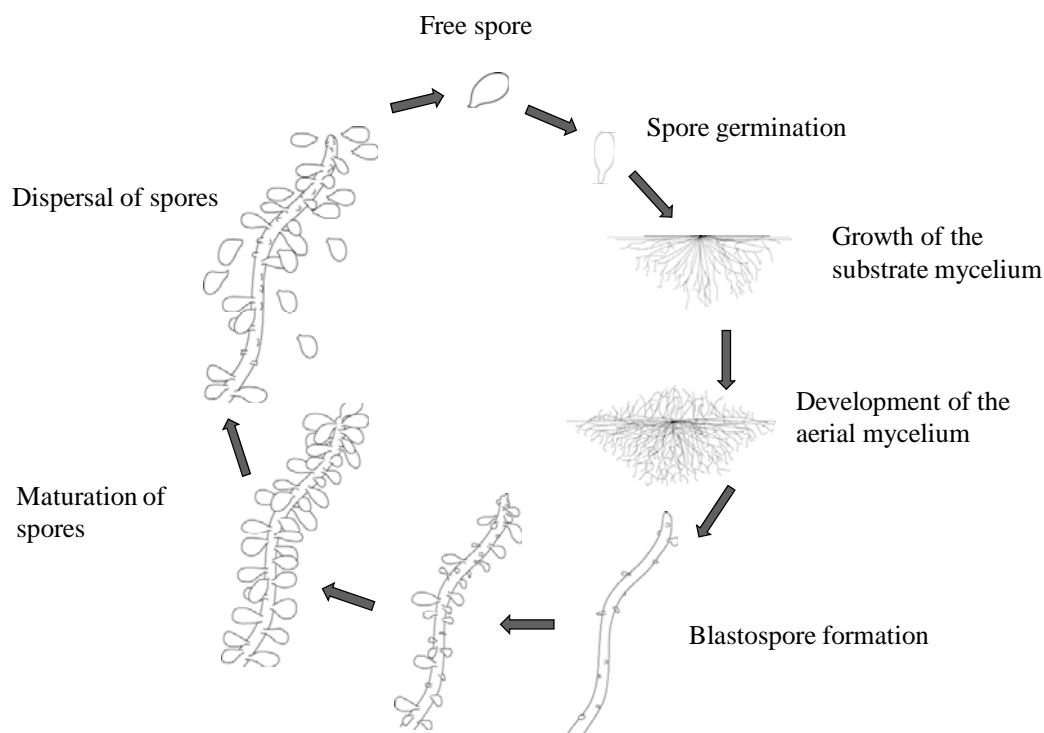


Figure 3. Life Cycle of *Thermosporothrix hazakensis* SK20-1^T

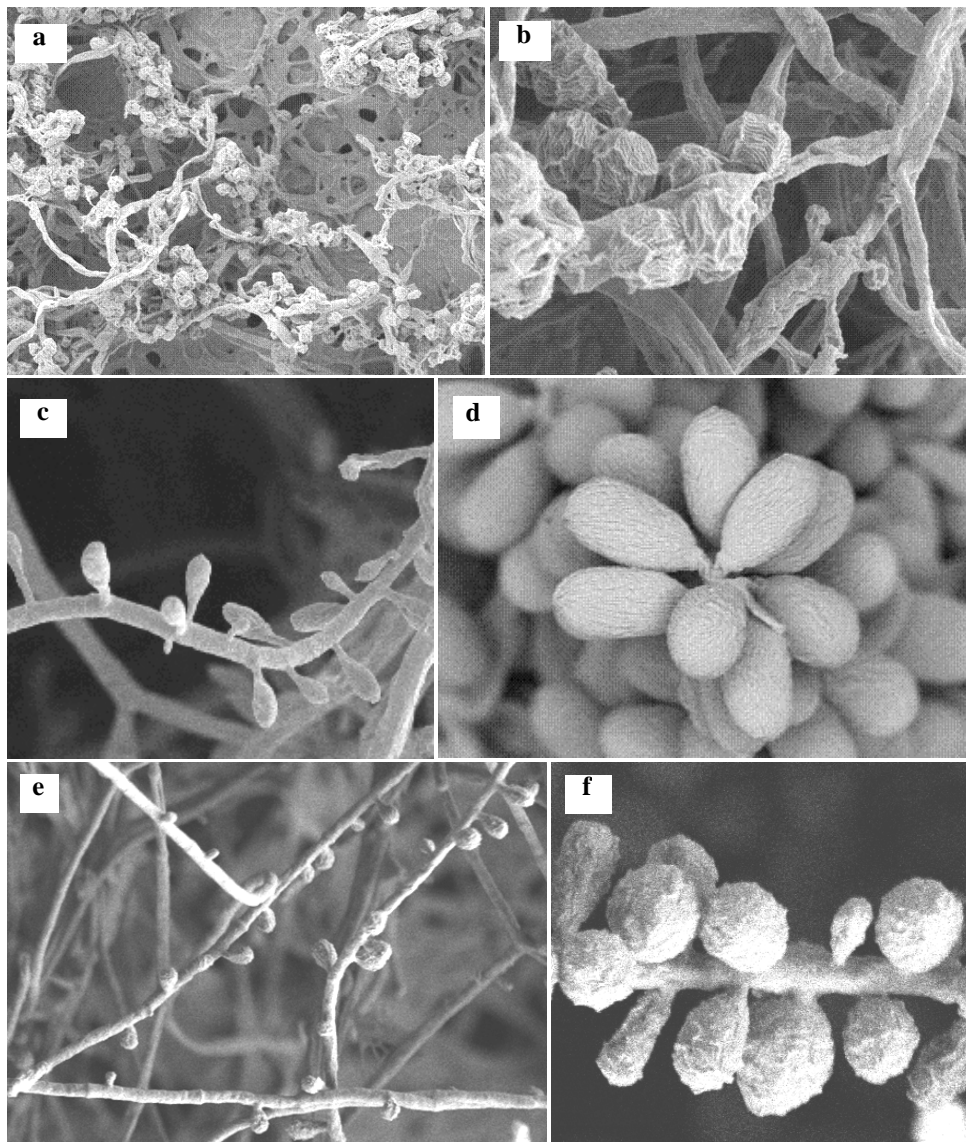


Figure 4. Scanning Electron Micrographs of *Ktedonobacteria* Strains. (a,b) *Ktedonobacter racemifer* SOSPI-21^T; (b,c) *Thermosporothrix hazakensis* SK20-1^T; (d,e) *Thermogemmatispora onikobensis* ONI-1^T

6. Conclusions

The class *Ktedonobacteria* contains a number of uncultured, environmental 16S rRNA gene clones, and cultured representatives are a limited number. By improving isolation methods and sources of isolation, further cultured strains should be obtained to reveal the taxonomy and their physiological and environmental role of *Ktedonobacteria* strains.

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