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Oligoflexia, the Newest Class of the Phylum *Proteobacteria*, Consisting of only One Cultured Species and Uncultured Bacterial Phylotypes from Diverse Habitats

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Abstract

The phylum *Proteobacteria* has recently gained a new taxon *Oligoflexia* that represents the seventh or eighth (if yet-to-be-validated "*Zetaproteobacteria*" is included) class, described by the only cultured species (as of December 2014), *Oligoflexus tunisiensis*, as the type species. This bacterium exhibits cellular polymorphism and presence of the uncommon fatty acid C16:1ω5c as high as >65% of total fatty acids, besides its unique 16S rRNA gene sequence. The class *Oligoflexia* is characterized by the distinct phylogenetic cluster within the greater proteobacterial cluster, and certain environmentally-derived 16S rRNA gene sequences, *a.k.a.* environmental clones or phylotypes, of uncultured bacteria are now grouped into the *Oligoflexia* cluster; however, the content and extent of the cluster has not been clearly depicted. This mini-review illustrates that the *Oligoflexia* cluster hosts a variety of environmental clones from diverse sources. Currently 20 phylotypes (or clones) are affiliated with the *Oligoflexia* cluster, and the sources were ranging from soils to cyanobacterial mat, bio-filter, human skin, ant colony, desert, glacial ice, earthworm intestine, and seawater. However, their frequencies in respective clone libraries were generally as low as <1%, which indicates their corresponding species are only minor in respective microbial communities. Moreover, 61 environmental metagenome libraries yielded only 1198 partial sequences having >85% similarities (class-level affiliation) to the 16S rRNA gene sequence of *O. tunisiensis*, which accounts for merely 0.04% of those registered in Meta-Metagenomic Data Base (MetaMeta DB). On the other hand, >97%-similarities sequences were found in rhizosphere, and <95%-similar sequences were found from hydrocarbon-rich habitats such as petroleum reservoir. Thus, it is suggested that members of *Oligoflexia* may display cosmopolite distribution in general as well as endemism in certain geochemical settings.

Keywords: Bacteria; *Proteobacteria*; *Oligoflexia*; Phylogeny; 16S rRNA gene; Metagenome

Introduction

The largest eubacterial phylum *Proteobacteria* accommodates about 1600 bacterial species exhibiting extremely diversified morphologies (as expressed in its name as "Proteus"), phylogenies, and metabolisms relevant to geochemical cycling of carbon, nitrogen, sulphur, etc. [1]. The phylum *Proteobacteria* hosts the greatest number of "culturable" described and deposited species among the prokaryotic phyla [1,2], while the phylum harbors numerous environmentally-derived 16S rRNA gene sequences, *a.k.a.* environmental clones or phylotypes, of "uncultured" bacteria whose physiological properties are largely unknown [3,4].

The proteobacterial taxa were formerly grouped as "purple bacteria" [5], then re-organized phylogenetically based on 16S rRNA gene sequences into five classes of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* , *Deltaproteobacteria* and *Epsilonproteobacteria* [6] and, two more classes were proposed thereafter; they are yet-to-be-validated '*Zetaproteobacteria*' [7] and already-validated *Acidithiobacillia* [8] which is a revision of part of the species formerly affiliated with *Gammaproteobacteria* [8].

We have proposed the seventh or eighth class, if '*Zetaproteobacteria*' is excluded or included, respectively, in the phylum *Proteobacteria*, with the description of the type species *Oligoflexus tunisiensis* [9] that is the only "cultured" species of the class currently. In proposing the new class, we have constructed a phylogenetic tree of the *Oligoflexia* cluster consisting of a number of environmental clones of uncultured bacteria from various sources. Thus, this mini-review discusses their environmental and geographical features as well as some unique properties of *O. tunisiensis*.

Unique morphological and phenotypic traits of *O. tunisiensis*

Sterile filtration has been a common technique to remove microorganisms from fluids in pharmaceutical and food industries as well as in many areas of biology. For sterilization purposes, membrane filters having a pore size of 0.2 μm are generally used. Nevertheless, not all bacteria are trapped with the filters; certain novel taxa have been found in the 0.2 μm-filtrates [10,11]. Likewise, *O. tunisiensis* was first obtained from the 0.2 μm-filtrate of liquid suspended with Saharan sand and pebbles [9]. While the cells of *O. tunisiensis* indeed pass the 0.2 μm-filters, they also show polymorphism with filamentous, spiral, spherical, or curved rod shapes (Figure 1). Though factors controlling the cell shapes have been unclear, their morphological flexibility and versatility would be associated with the nature to pass through 0.2 μm-filters. Therefore, it is considered that our 0.2 μm-filtration was effective to remove fast-growers and preferably selective for slow-growing species. In addition, *O. tunisiensis* resumes growth when transferred to fresh agar plates from senile colonies as old as half a year (M. Nishijima, personal communication).

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Physiological and biochemical properties of *O. tunisiensis* are listed in Table 1. This bacterium shows mesophilic and neutrophilic growth characteristics with optimum temperature and pH of 25-30°C and 7.0-8.0, respectively, as seen in many other bacterial species. In contrast, this bacterium has unique cellular fatty acid contents. More than 90% of total fatty acids is composed only of two fatty acids, C16: 1ω5c (65.7%) and C16: 0 (27.5%), the former being rarely seen in other species [12,13]. It is unclear whether high content of C16: 1ω5c would be general feature of the whole class *Oligoflexia*; this rare fatty acid could be taken as a biomarker at least of the species *O. tunisiensis*.

The C16: 1ω5c fatty acid has also been detected in myxobacteria belonging to *Deltaproteobacteria* but to a lesser content of 15-39% (of total fatty acids) at most [13]. It was proposed to be a possible biomarker of microbial consortia responsible for anaerobic methane oxidation, or ANME, in methane-seeping marine sediments [14]. The ANME consortia are composed of archaea capable of reverse methanogenesis as anaerobic methane oxidizers and sulfate-reducing bacteria belonging to *Deltaproteobacteria* as potential hydrogen-donors in the sediments [15]. About half of the sediment fatty acids is dominated by various C16: 1 acids, most of which is due to C16: 1ω5c probably from bacterial part of the consortia [14]. In *O. tunisiensis*, C16: 1ω5c solely accounts for 65.7% of total fatty acids, and thus should serve as a useful biomarker.

Not-yet-cultured bacteria within the class *Oligoflexia*

In the phylogenetic tree of the phylum *Proteobacteria* depicted in Nakai *et al.* [9], the class *Oligoflexia* was shown to harbour environmental clones or phylotypes of uncultured or "not-yet-cultured" bacteria from various sources. Table 2 shows a set of sequence information of 20 phylotypes, which are selected from clone libraries consisting of PCR-amplified near-full-length 16S rRNA gene sequences registered in DDBJ/EMBL/GenBank databases (partly published). Their isolation sources are ranging from soils to cyanobacterial mat, bio-filter, human skin, ant colony, desert, glacial ice, earthworm intestine and seawater (Table 2). This implicates that the class *Oligoflexia* hosts a variety of not-yet-cultured bacterial species occurring in wide-ranging habitats.

So far, the closest phylotype to the *O. tunisiensis* 16S rRNA gene sequence has been TSBAR001_G23 acession no. AB486128; (Table 2) with a similarity of 98.3%. The phylotype was found in a clone library constructed from a Japanese paddy field microflora, and its occurrence frequency was as low as 1/1036, i.e., only one out of total 1036 clones [16]. The paddy field soil was added experimentally with nitrate (in reference to nitrate reduction) and yielded the emerging clone TSNIR001_J18 (AB487112; 16) that is only distantly related to *O. tunisiensis* at 84.6% similarity but within the *Oligoflexia* cluster. The emerging clone may have been associated with nitrate reduction. On the other hand, *O. tunisiensis* shows no nitrate reduction activity with a phenotypic test using API 20NE kit (bioMérieux) [9]. Nonetheless, involvement of the *Oligoflexia* members in nitrate cycling should be surveyed.

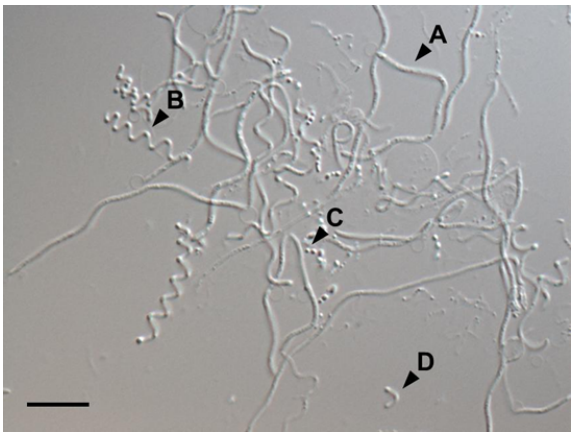

Figure 1: Micrograph of *Oligoflexus tunisiensis* cells
The cells exhibited a (A) slender filamentous, (B) spiral, (C) spherical (or curled), or (D) curved rod morphology. Scale bar, 10 µm.

In contrast to their wide-ranging sources, *Oligoflexia*-affiliated clones are rarely found in many clone libraries. For example, no *Oligoflexia* clones are seen in a soil clone library (consisting of 1700 clones of PCR-amplified near-full-length 16S rRNA gene sequences), 32% of which are affiliated with the phylum *Proteobacteria* [17]. Another soil clone library (consisting of 13,001 clones) focusing on "rare members of the soil biosphere" yielded no *Oligoflexia* clones, either [18]. Thus, *Oligoflexia*-affiliated bacteria are likely very minor in soils, and their biogeochemical importance may be low accordingly. Yet, quantitative techniques such as real-time PCR and cell counting with fluorescence in situ hybridization are needed to quantify the occurrences of *Oligoflexia*-affiliated bacteria in environments.

There have been six phylotypes so far that show >97% similarities with the *O. tunisiensis* 16S rRNA gene sequence (Table 2). They are second to seventh closest to and presumably regarded as the same species as *O. tunisiensis*. The second to fifth closest phylotypes are from a lacustrine cyanobacterial mat in China and a microalgal photo-bioreactor in Germany; the latter yielded an occurrence frequency of 3/171 [19], which suggests co-occurrence relationship between *O. tunisiensis* and phototrophs. The sixth and seventh closest phylotypes were obtained from a bio-filter in China and from American children's knee-pit skin, respectively. Combining with the phylotypes to the tenth closest, occurrence of the *O. tunisiensis* kins extend to wider habitats (Table 2).

Previous affiliations of certain clones were revised to be members of the class *Oligoflexia*. The clones' glb351c (EU978839) and glb342c (EU978830) were obtained from glacial ice if the Northern Schneeferner in Germany, and originally affiliated with the classes *Deltaproteobacteria* and *Betaproteobacteria*, respectively [20]: the glacial phylotypes are now re-affiliated with the class *Oligoflexia* [9]. Although their similarities are as only 89.8% and 87.1%, respectively, to the *O. tunisiensis* 16S rRNA gene sequence (Table 2), but they are still placed within the *Oligoflexia* cluster.

The original clone library of the glacial clones consisted of 338 near-full-length clones, dominated by *Proteobacteria* phylotypes at a frequency of 190/338, most of which were affiliated with the class *Betaproteobacteria* [20]. The *Betaproteobacteria* dominance has commonly been evident in other microflorae of cold habitats such as glacial ice [21], alpine snow [22] and sub-glacial sediment [23]. In contrast, no phylotypes were affiliated to *Oligoflexia* from these cold habitats (Table 2), and only the few clones were seen at a frequency as low as 4/338 of the German glacier clones [20].Additionally, the glacier phylotype glb351c and the earthworm intestine clone A02-05D (FJ542822) [24] show a 97.0% similarity and form a small but monophyletic cluster [9]. This close relationship leads to an idea that the phylotypes may not be specific to cold habitats.

Recent development of high-throughput sequencing has enabled extensive metagenomic analyses of environmental microorganisms [25,26], as well as massive analyses of 16S rRNA gene sequences

Table 1: Morphological and phenotypic traits of *Oligoflexus tunisiensis.*

Characteristic	***O. tunisiensis***
Cell morphology	Mainly slender filamentous, but some exhibited a spiral, spherical (or curled), or curved rod form (see, Figure 1)
Cell width (μm)	0.4-0.8
Growth Conditions	
Temperature (°C)	20–37 (optimal range, 25-30)
NaCl concentration (%)	<1.0
pH	7.0–9.5 (optimal range, 7.0-8.0)
Dominant cellular fatty acid	$C_{16:1\omega5c}$, $C_{16:0}$
Major respiratory quinone	menaquinone-7 (MK-7)
DNA G+C content (mol%)	54.0
Positive enzyme activities	
Esterase lipase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase,	
protease (gelatinase), trypsin, α-mannosidase	
Data from Nakai *et al.* (2014)	

Table 2: Isolation source of environmentally-derived 16S rRNA gene sequences, *a.k.a.* environmental clones or phylotypes, within the class *Oligoflexia*

Phylotype	**Accession no.**	**Identity (%)**[a]	**Alignment length (bp)**[a]	**Isolation source**	**Detection frequency in clone library**	**Reference**
clone TSBAR001_G23	AB486128	98.3	1374	rice paddy soil	1/1036	[16]
clone E21	HQ827927	97.7	1413	cyanobacterial blooms in a hypereutrophic lake	-[b]	unpublished
clone BF 006	KC994686	97.5	1458	microalgae photobioreactor	1/171	[19]
clone BF 004	KC994684	97.5	1458	microalgae photobioreactor	1/171	[19]
clone BF 014	KC994694	97.5	1458	microalgae photobioreactor	1/171	[19]
clone V201-58	HQ114073	97.4	1455	biofilm in a vermifilter	-	unpublished
clone ncd2130c10c1	JF183716	97.2	1354	skin (popliteal fossa)	ND[c]	[31]
clone H-169	HM565023	96.6	1460	concrete	-	unpublished
clone SINZ1495_ N11D4_16S_B	LN563658	96.2	1365	refuse dumps of leafcutter ant	-	unpublished
clone ncd2100g03c1	JF181808	95.9	1356	skin (volar forearm)	ND	[31]
clone T5CLN43	AB696523	90.8	1461	Taklamakan desert soil	-	unpublished
clone glb351c	EU978839	89.8	1463	glacier ice	3/338	[20]
clone Elev_16S_1354	EF019970	89.7	1369	trembling aspen rhizosphere	<1%	[32]
clone Dok52	FJ710772	89.6	1457	anaerobic ammonium oxidation reactor	-	unpublished
clone A02-05D	FJ542822	89.5	1461	earthworm gut	1/105	[24]
clone glb342c	EU978830	87.1	1461	glacier ice	1/338	[20]
clone SHWH_night1_16S_626	FJ744863	86.3	1374	surface seawater	ND	[33]
clone UA24	DQ269039	85.6	1413	surface of marine macro-alga	-	unpublished
clone FCPS531	EF516682	85.5	1443	grassland soil	ND	[30]
clone TSNIR001_J18	AB487112	84.6	1382	rice paddy soil	1/1064	[16]

Data are from the phylogenetic tree for the phylum *Proteobacteria* described in Nakai *et al.* (2014) and a BLASTN search dated October 2014

[a] The sequence identity and its alignment length to 16S rRNA gene sequence of *Oligoflexus tunisiensis* (acession no. AB540021)

[b] –: unknown

[c] ND: no description

derived from environmental samples [27]. Then, we have conducted an extensive database search on Meta-Metagenomic DataBase (MetaMetaDB; http://mmdb.aori.u-tokyo.ac.jp/) [28], which contained 2,737,833 sequences (shorter than 300-500 bp generated by 454-pyrosequencing) of 16S rRNA genes from 61 environments (as of October 2014).

The database search showed that the *O. tunisiensis* 16S rRNA gene sequence matches with 5, 11, 106 and 1198 sequences at 97%, 95%, 90% and 85% similarities, respectively. If 85%-similarity outlines the *Oligoflexia* cluster, the number of matched sequences (1198) accounts for ca. 0.04% of total MetaMetaDB sequences (2,737,833).

The MetaMetaDB also provides a "habitability index" (Figure 2) to infer possible habitats of a queried sequence or a "query" and its related sequences [28] based on the BLASTN search [29]. The result suggested that the >97%-similar sequences (to the *O. tunisiensis* 16S rRNA gene sequence) were likely from rhizosphere, while <95%-similar sequences were mostly from underground habitats such as groundwater and hydrocarbon (e.g. petroleum and gas) reservoir. Figure 2 illustrates "habitability indices" as percentages for source habitats of the matched sequences. Certain geographical tendencies of the source habitats are shown, despite phylogenetic limitation due to short lengths (less than 300-500 bp) of the sequences generated by 454-pyrosequencing.

In summary, general features of the phylotypes (clones) and partial sequences affiliated with the class *Oligoflexia* are: 1) that they are derived from a variety of source habitats, suggestive of cosmopolite distribution; and 2) that they are minor in occurrence frequencies, possibly associated with the slow-growing nature of the type species, *O. tunisiensis*. An emerging character is "cosmopolite but minor" or "minor cosmopolitan", which should be tested with other *Oligoflexia* species to be cultured in future studies.

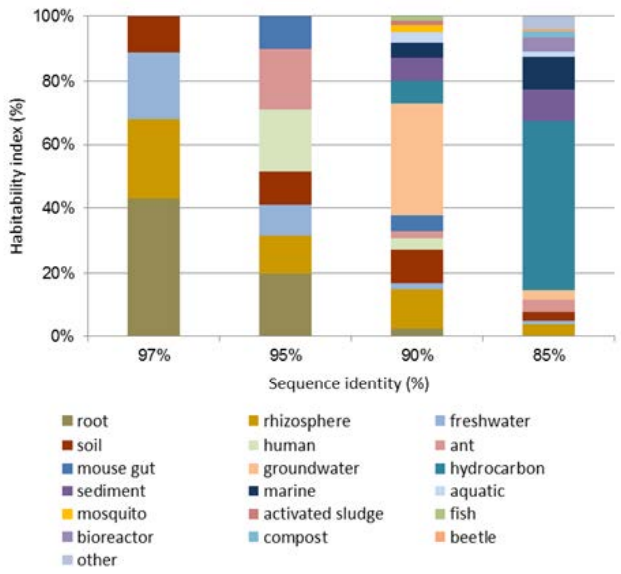

Figure 2: Inferred source habitats for partial 16S rRNA gene sequences of not-yet-cultured bacteria potentially affiliated with the class *Oligoflexia* The microbial habitability indices (*y*-axis) calculated using MetaMetaDB were based on a set of normalized ratios of environmental categories among the significant hits exhibiting greater than 97%, 95%, 90%, and 85% identity from the BLASTN search with the 16S rRNA gene sequence of *Oligoflexus tunisiensis* (accession no. AB540021) against curated metagenomic sequences in MetaMetaDB.

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