**Genome sequence and overview of Oligoflexus tunisiensis Shr3<sup>T</sup> in the eighth class Oligoflexia of the phylum Proteobacteria**

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**Abstract**

*Oligoflexus tunisiensis* Shr3<sup>T</sup> is the first strain described in the newest (eighth) class *Oligoflexia* of the phylum *Proteobacteria*. This strain was isolated from the 0.2-μm filtrate of a suspension of sand gravels collected in the Sahara Desert in the Republic of Tunisia. The genome of *O. tunisiensis* Shr3<sup>T</sup> is 7,569,109 bp long and consists of one scaffold with a 54.3% G + C content. A total of 6,463 genes were predicted, comprising 6,406 protein-coding and 57 RNA genes. Genome sequence analysis suggested that strain Shr3<sup>T</sup> had multiple terminal oxidases for aerobic respiration and various transporters, including the resistance-nodulation-cell division-type efflux pumps. Additionally, gene sequences related to the incomplete denitrification pathway lacking the final step to reduce nitrous oxide (N<sub>2</sub>O) to nitrogen gas (N<sub>2</sub>) were found in the *O. tunisiensis* Shr3<sup>T</sup> genome. The results presented herein provide insight into the metabolic versatility and N<sub>2</sub>O-producing activity of *Oligoflexus* species.

**Keywords:** Oligoflexia, Proteobacteria, RND-type efflux pump, Denitrification, Nitrous oxide (N<sub>2</sub>O)

**Introductions**

The phylum *Proteobacteria* traditionally comprises five classes of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* [1, 2], with two additional classes ‘Zetaproteobacteria’ and *Acidithiobacillia* proposed by Emerson et al. [3] and Williams and Kelly [4], respectively. *Proteobacteria* hosts the greatest number of isolates and sequenced genomes among the prokaryotic phyla [5] and contains members exhibiting extremely diversified metabolisms relevant to global carbon, nitrogen, and sulfur cycles [2]. This phylum recently gained the eighth (or seventh if yet-to-be-validated ‘Zetaproteobacteria’ is excluded) class *Oligoflexia* with the cultured species *Oligoflexus tunisiensis* type strain Shr3<sup>T</sup> [6]. The class *Oligoflexia* includes environmentally-derived 16S rRNA gene sequences, otherwise known as environmental clones or phylotypes, recovered from a variety of habitats including soils, the Taklamakan Desert, glacial ice, lake water, seawater, human skin, and the guts of earthworms [6]. In contrast to their wide distribution, *Oligoflexia*-affiliated clones have rarely been found in clone libraries [7]; accordingly, it has been suggested that the *Oligoflexia* members show a small population size, belonging to the so-called rare biosphere [8].

At the time of writing, *O. tunisiensis* Shr3<sup>T</sup> was the only cultured species within the class *Oligoflexia*. Physiological and biochemical features of strain Shr3<sup>T</sup> could not be fully characterized because of restrictive culture conditions owing to the slow-growing nature of this strain [6]. The phenotypic information is essential for understanding its ecological role and biotechnological potentials. Here, we compensated for the limited knowledge regarding *Oligoflexia* members by conducting genomic analysis of strain Shr3<sup>T</sup>.

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During a study of ultramicro-sized bacteria that could pass through 0.2-μm pore-size filters, which are generally used for sterile filtration to remove microorganisms, we isolated the bacterium designated isolate Shr3 [9]. The isolation source of this bacterium was a 0.2-μm filtrate of the suspension of sand gravels collected in December 2008 in Matmata (33°31’ N 9°57’ E) on the eastern margin of the Sahara Desert in the Republic of Tunisia. Isolate Shr3 was thereafter described as the type strain of *Oligoflexus tunisiensis*, the first cultured representative of the novel class *Oligoflexia* [6]. Figure 1 shows the phylogenetic position of *O. tunisiensis* and related environmental clones in a 16S rRNA-based evolutionary tree. The sequence of the three 16S rRNA gene copies in the genome was 100% identical to the previously published 16S rRNA gene sequence (DDBJ/EMBL/GenBank accession no. AB540021 [6]). The database search showed that seven environmental clones had a >97% high similarity with the *O. tunisiensis* 16S rRNA gene sequence [7]. The seven clones were from rice paddy soil, cyanobacterial blooms in a hypereutrophic lake, a microalgal photobioreactor, a bio-filter, and human skin [7]. Strain Shr3T has been deposited in the Japan
Collection of Microorganisms and the National Collection of Industrial, food and Marine Bacteria under accession numbers JCM 16864<sup>T</sup> and NCIMB 14846<sup>T</sup>, respectively. The general features of strain Shr3<sup>T</sup> are reported in Table 1.

*O. tunisiensis* Shr3<sup>T</sup> is a Gram-negative, aerobic, non-motile, filamentous bacterium of 0.4–0.8 μm in width when cultivated under the experimental culture conditions [6]. Some cells exhibited a spiral, spherical (or curled), or curved rod morphology [7]. Although the factors controlling the cell shapes are still unclear, the morphological flexibility is likely associated with their ability to pass through 0.2-μm filters. Strain Shr3<sup>T</sup> grows in the R2A medium [6]. The cells showed slow growth, with 3–5 days required before colonies could be seen by the naked eye [6]. The growth occurs at NaCl concentrations <1.0% (w/v), 20–37 °C (optimum 25–30 °C), and pH 7.0–9.5 (optimum pH 7.0–8.0) [6]. Enzyme activities of esterase lipase, leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and α-mannosidase are positive [6]. Transmission electron microscopy revealed that cells contained many low electron-dense particles (Fig. 2). Some, but not all, particles were stained by Sudan black B upon staining PHB or lipophilic particles. Because cells swollen by accumulated PHB were not observed when grown on PHB-containing medium [6], the particles stained with Sudan black B are likely lipophilic granules.

**Chemotaxonomy**

The major respiratory quinone was menaquinone-7 (MK-7) [6]. The dominant cellular fatty acids were C<sub>16:1ω5c</sub> (65.7%) and C<sub>16:0</sub> (27.5%), the major hydroxy fatty acid was C<sub>12:0 3-OH</sub> (1.3%), and the minor fatty acids included C<sub>10:0</sub>, C<sub>12:0</sub>, C<sub>15:0</sub>, C<sub>17:0</sub>, C<sub>18:0</sub> and C<sub>18:1ω5c</sub> [6]. The fatty acid, C<sub>16:1ω5c</sub>, was also detected in

| Table 1 Classification and general features of Oligoflexus tunisiensis type strain Shr3<sup>T</sup> according to MIGS standards [30] |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **MIGS ID** | **Property** | **Term** | **Evidence code** |
|--------------|--------------|----------|-------------------|
| Classification | Domain Bacteria | TAS [31] |
| Phylum Proteobacteria | TAS [32] |
| Class Oligoflexia | TAS [6] |
| Order Oligoflexales | TAS [6] |
| Family Oligoflexaceae | TAS [6] |
| Genus Oligoflexus | TAS [6] |
| Species Oligoflexus tunisiensis | TAS [6] |
| Type strain: Shr3<sup>T</sup> | TAS [6] |
| Gram stain | negative | TAS [6] |
| Cell shape | filamentous-shaped | TAS [6, 7] |
| Motility | non-motile | TAS [6] |
| Sporulation | none | TAS [6] |
| Temperature range | 20–37 °C | TAS [6] |
| Optimum temperature | 25–30 °C | TAS [6] |
| pH range; Optimum | 7.0–9.5; 7.0–8.0 | TAS [6] |
| Carbon source | heterotrophic | TAS [6] |
| Habitat | desert | TAS [6] |
| Salinity | 0–0.5% (w/v) NaCl | TAS [6] |
| Oxygen requirement | aerobic | TAS [6] |
| Biotic relationship | free-living | TAS [6] |
| Pathogenicity | not reported | TAS [6] |
| Geographic location | Matmata, Republic of Tunisia | TAS [6] |
| Sample collection | December 2008 | TAS [6] |
| Latitude | 33.53 | TAS [6] |
| Longitude | 9.96 | TAS [6] |
| Altitude | not determined | TAS [6] |

*Evidence codes – IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [33]*
myxobacteria of Cystobacterineae in the class Deltaproteobacteria, but at only 15–39% [10].

**Genome sequencing information**

**Genome project history**

Phenotypic features of strain Shr3<sup>T</sup> are described above, but could not be fully tested because of restrictive culture conditions [6]. Therefore, this organism was selected for genome sequencing to investigate the basis of its ecological role and biotechnological potentials. The genome project is deposited in the Genomes OnLine Database [11] under the accession number Gp0139475. The information genome sequence is available from the DDBJ/EMBL/GenBank database. A summary of this genome project is shown in Table 2.

**Growth conditions and genomic DNA preparation**

A culture of *O. tunisiensis* Shr3<sup>T</sup> grown aerobically in R2A broth (DAIGO; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) at 30 °C was used to prepare genomic DNA. The genomic DNA was extracted using Qiagen Genomic-Tip 500/G columns according to the manufacturer’s instructions. The quantity and purity of the extracted DNA was checked by spectrophotometric measurement at 260 nm and agarose gel electrophoresis.

**Genome sequencing and assembly**

The genome sequence was generated using paired-end sequencing (2 × 90 bp) on an Illumina HiSeq 2000 platform at the BGI with the pair-end library and mate-pair library of two different insert sizes, 456 to 496 bp and 6310 to 6350 bp. After trimming of low quality reads, 1130 Mb was obtained and assembled into 19 contigs in one scaffold using SOAPdenovo version 2.04 [12]. The assembly result was locally optimized according to the paired-end and overlap relationship via mapping reads to obtained contigs. A summary of this genome sequence is shown in Table 3.

**Genome annotation**

Gene sequences were identified via the Prodigal V2.6.3 [13] as part of the DOE-JGI genome annotation pipeline in the Integrated Microbial Genomes—Expert Review (IMG-ER) system [14]. Gene functional annotation as well as data visualization was conducted within the IMG-ER [15]. The predicted coding sequences were translated and used to search the National Center for Biotechnology Information non-redundant, UniProt, TIGR-Fam, Pfam, KEGG, COG, and InterPro databases. Identification of RNA gene sequences and miscellaneous features were carried out using HMMER 3.1b2 [16] and INFERNAL 1.0.2 and 1.1.1 [17]. Additional functional prediction was performed with the RAST server [18] under accession

| Table 2 Project information |
|-----------------------------|
| **MIGS ID** | **Property** | **Term** |
| MIGS 31 | Finishing quality | High-quality draft |
| MIGS-28 | Libraries used | Pair-end library and mate-pair library |
| MIGS 29 | Sequencing platforms | Illumina HiSeq 2000 |
| MIGS 31.2 | Fold coverage | 149 x |
| MIGS 30 | Assemblers | SOAPdenovo version 2.04 |
| MIGS 32 | Gene calling method | Prodigal |
| Locus Tag | Ga0118670 (IMG-ER) |
| GenBank ID | BDFO01000001 |
| GenBank Date of Release | 30 June 2016 |
| GOLD ID | Gp0139475 |
| BIOPROJECT | PRJDB4872 |
| MIGS 13 | Source Material Identifier | JCM 16864, NCIMB 14846 |
| Project relevance | ecology, biotechnology |
Candidate CRISPR regions were detected using the CRISPRFinder program [19].

**Genome Properties**

The genome of *O. tunisiensis* Shr3T consists of a 7,569,109 bp long chromosome with a 54.3% G + C content (Table 3). Of the 6463 predicted genes, 6406 were protein-coding genes and 57 were RNA genes (three rRNA operons, 46 tRNAs, and two miscRNAs). The majority of the protein-coding genes (62.7%) were assigned to a putative function. The remaining ones were annotated as hypothetical proteins. The distribution of genes classified into COGs functional categories is shown in Table 4 and Fig. 3.

**Insights from the genome sequence**

The genome of *O. tunisiensis* Shr3T encoded genes for ABC transporters of amino acid, oligopeptide/dipeptide, and phosphonate, ammonium and nitrate/nitrite transporters, as well as RND-type efflux pumps. One of the amino acid sequences (Ga0118670_114686) classified as an RND pump showed a high similarity (67% identity and 99% coverage) to sequences of the pathogenic

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**Table 3** Genome statistics

| Attribute                  | Value       | % of Total |
|----------------------------|-------------|------------|
| Genomic size (bp)          | 7,569,109   | 100.00     |
| DNA coding (bp)            | 6,849,121   | 90.49      |
| DNA G + C (bp)             | 4,113,347   | 54.34      |
| DNA scaffolds              | 1           | 100.00     |
| Total genes                | 6,463       | 100.00     |
| Protein coding genes       | 6,406       | 99.12      |
| RNA genes                  | 57          | 0.88       |
| Pseudogenes                | not determined | not determined |
| Genes in internal clusters | 1,494       | 23.12      |
| Genes with function prediction | 4,051     | 62.68      |
| Genes assigned to COGs     | 2,938       | 45.46      |
| Genes with Pfam domains    | 4,268       | 66.04      |
| Genes with signal peptides | 1,084       | 16.77      |
| Genes with transmembrane helices | 1,393 | 21.55      |
| CRISPR repeats             | 8           |            |

**Table 4** Number of genes associated with general COG functional categories

| Code | Value | % of Total | Description                                                                 |
|------|-------|------------|------------------------------------------------------------------------------|
| J    | 228   | 6.91       | Translation, ribosomal structure and biogenesis                              |
| A    | 1     | 0.03       | RNA processing and modification                                              |
| K    | 154   | 4.67       | Transcription                                                                |
| L    | 103   | 3.12       | Replication, recombination and repair                                         |
| B    | 1     | 0.03       | Chromatin structure and dynamics                                             |
| D    | 31    | 0.94       | Cell cycle control, Cell division, chromosome partitioning                   |
| V    | 87    | 2.64       | Defense mechanisms                                                           |
| T    | 314   | 9.52       | Signal transduction mechanisms                                               |
| M    | 229   | 6.94       | Cell wall/membrane biogenesis                                                |
| N    | 125   | 3.79       | Cell motility                                                                |
| U    | 44    | 1.33       | Intracellular trafficking and secretion                                      |
| O    | 159   | 4.82       | Posttranslational modification, protein turnover, chaperones                  |
| C    | 172   | 5.21       | Energy production and conversion                                             |
| G    | 142   | 4.30       | Carbohydrate transport and metabolism                                        |
| E    | 264   | 8.00       | Amino acid transport and metabolism                                          |
| F    | 73    | 2.21       | Nucleotide transport and metabolism                                          |
| H    | 170   | 5.15       | Coenzyme transport and metabolism                                            |
| I    | 193   | 5.85       | Lipid transport and metabolism                                               |
| P    | 156   | 4.73       | Inorganic ion transport and metabolism                                       |
| Q    | 100   | 3.03       | Secondary metabolites biosynthesis, transport and catabolism                |
| R    | 337   | 10.22      | General function prediction only                                             |
| S    | 153   | 4.64       | Function unknown                                                             |
| -    | 3,525 | 54.54      | Not in COGs                                                                  |

The total is based on the total number of protein coding genes in the genome.
bacteria \textit{Achromobacter xylosoxidans} and \textit{Pseudomonas aeruginosa}. The RND-type efflux system is widely distributed in Gram-negative bacteria and known to promote resistance to various kinds of antimicrobial substances, termed as multidrug resistance [20].

In support of its aerobic growth, gene sequences assigned to different terminal oxidases including \textit{aa}_{3}- and \textit{cbb}_{3}\textit{-type cytochrome }c\textit{ oxidases (COG0843 and COG3278) and cytochrome }\textit{bd}-\textit{type quinol oxidase (COG1271 and COG1294) were found in the Shr3T genome.}

The Shr3T genome contained a \textit{nirK} gene coding for a copper-dependent nitrite reductase (Nir) (Ga0118670_114712) involved in denitrification, a major component of the nitrogen cycle [21]. Denitrification is the dissimilatory reduction of nitrate or nitrite to nitrogen gas (\(\text{NO}_{3}^{-} \rightarrow \text{NO}_{2}^{-} \rightarrow \text{NO} \rightarrow \text{N}_{2}O \rightarrow \text{N}_{2}\)) [22] that usually occurs under oxygen-limiting conditions [21]. The key steps releasing gaseous products NO, N\(_2\)O, and N\(_2\) are catalyzed by Nir, nitric-oxide reductase (Nor) and nitrous oxide reductase (Nos), respectively [23, 24]. There are two structurally different nitrite reductases among denitrifiers: a copper-containing type (Cu-Nir) encoded by the \textit{nirK} gene and a cytochrome \(\text{cd}_{1}\)-containing one (\(\text{cd}_{1}\)-Nir) encoded by the \textit{nirS} gene [24]. The \textit{nirS} gene was absent from the \textit{O. tunisiensis} Shr3T genome.

The NirK deduced amino acid sequence of \textit{O. tunisiensis} Shr3T was most closely related to that of \textit{Bdellovibrio bacteriovorus} of the class \textit{Deltaproteobacteria}, with 70% identity and 96% coverage. \textit{B. bacteriovorus} has an incomplete denitrifying pathway with a Cu-Nir, a cytochrome \(\text{c}\)-dependent Nor (cNor), and no Nos [25, 26]. \textit{O. tunisiensis} Shr3T also had a partial pathway containing the Cu-Nir described above, a quinol-dependent Nor (qNor), and no Nos inferred from the genome data. Strain Shr3T also had two copies of the gene encoding qNor (Ga0118670_112818 and Ga0118670_114769). NorR protein is known to regulate Nor expression in response to NO [27, 28]. The transcription regulator \textit{norR} gene (Ga0118670_114771) was nearly adjacent to one of two copies of the qNor-encoding gene in the genome.

Our results suggest that the \textit{Oligoflexus} species has the capability to produce N\(_2\)O as a final product of the incomplete denitrification lacking the last step (reduction}
of N₂O to N₂). N₂O is known as a strong greenhouse gas, as well as an ozone-depleting substance [29]. Accordingly, future studies should examine the N₂O-producing phenotype of strain Shr³T.

Conclusions
In this study, we characterized the genome of O. tunisensis Shr³T, the first cultured representative of the novel proteobacterial class Oligoflexia. The genome sequence gives us insight into the metabolic versatility and incomplete denitrification pathway of Oligoflexus species. The genome information will facilitate future systematics and comparative genomics studies within the phylum Proteobacteria.

Abbreviations
PDB: Polyhydroxybutyrate; RND: Resistance-nodulation-cell division

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Authors’ contributions
RN coordinated the study, annotated the genome and drafted the manuscript. RN, FK, SS, HI, and TN maintained and cultured the strain. RN conducted the wet-lab work, MN performed the electron microscopy, RN, TF, YN, TB, and HN discussed the bioinformatics analysis. RN, TB, MN, TN, and HN discussed the data. All authors read and approved the final manuscript.

Competing interests
None of the authors has any competing interests.

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