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Tepidibacter aestuarii sp. nov., isolated from the Bach Dang Estuary, Haiphong, Vietnam

Nathalie Pradel^{1,*}, Sandrine Chifflet¹, Angelina Beavogui², Manon Bartoli¹, Thuoc Chu Van³, Aicha Aouane⁴, Vuong Bui Van³, Xavier Mari^{1,5}, Thu Pham The³, Pedro H. Oliveira² and Jean-Luc Cayol¹

Abstract

A mesophilic, anaerobic, endospore-forming, fermentative bacterium designated strain 8C15b^T was isolated from bank sediment of the Bach Dang Estuary, Haiphong, Vietnam. The Bach Dang Estuary, where Haiphong harbour is located, is subject to strong anthropogenic influence, resulting in high concentrations of black carbon and heavy metals. Strain 8C15b^T grew optimally at 30°C, pH 7.5 and with 2.5% (w/v) NaCl. The main cellular fatty acids consisted of iso-C_{15:0} (51%), iso-C_{15:1} ω7c (32%) and iso-C_{13:0} (5%). Genomic considerations of strain 8C15b^T and comparisons with the phylogenetically closest strains of the genus *Tepidibacter* provide evidence that *Tepidibacter thalassicus* SC562^T (=DSM 15285^T), *Tepidibacter formicigenes* DV1184^T (=DSM 15518^T), *Tepidibacter mesophilus* B1^T (=JCM 16806^T) and strain 8C15b^T could be differentiated at the species level. We propose the name *Tepidibacter aestuarii* sp. nov. for the type strain 8C15b^T (=JCM 35983^T=KCTC 25692^T). Finally, the nickel-tolerance properties of strain 8C15b^T are highlighted in this study.

INTRODUCTION

Micro-organisms are highly sensitive to environmental changes and are therefore considered indicators of local environmental conditions [1, 2]. It has been hypothesized that microbial communities adapted to long-term heavy metal contamination play a key role in the remediation of heavy-metal pollutants [3]. Estuaries and coastal areas are highly sensitive to local and global anthropogenic disturbances. In the Gulf of Tonkin, Northern Vietnam, anthropogenic sources of contamination are numerous, such as mining activities, metallurgical industries, waste incineration, agricultural practices, transport, fossil fuel combustion and runoff drained by the Red River Delta [4, 5]. A recent work has shown that the Red River Delta is recurrently contaminated by trace metals such as nickel [6]. However, the microbial communities of this area have been poorly studied and no strain has been previously characterized nor shown to have heavy metal resistance.

Among the genus *Tepidibacter*, only three species have been characterized so far, namely *T. thalassicus* SC562^T (the type species of the genus), *T. formicigenes* DV1184^T and *T. mesophilus* B1^T. The first two strains are both thermophilic micro-organisms: *T. thalassicus* SC562^T was isolated from hydrothermal vents at 13° N on the East-Pacific Rise at a depth of 2650 m [7]; *T. formicigenes* DV1184^T was isolated from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge [8]; and *T. mesophilus* B1^T was isolated from soil polluted by crude oil in the Karamay Oil Field, China, and is a mesophilic micro-organism [9]. In this study, we report the physiological and genetic characterization of strain 8C15b^T, isolated about 50 km north of the Red River Delta, in the Bach Dang Estuary, Haiphong, Vietnam [10]. We found this strain to be phylogenetically related to *T. mesophilus* B1^T. Comparison of their genomes allowed us to classify strain 8C15b^T in a new species of the genus *Tepidibacter*, *Tepidibacter aestuarii* sp. nov., represented by the type strain 8C15b^T. Moreover, we studied the tolerance capacity of strain 8C15b^T in regards to nickel.

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Keywords: *Peptostreptococcaceae*; Haiphong harbour; metal resistance; anti-phage defensible.

The GenBank accession number for the 16S rRNA gene is OQ970588.

Two supplementary figures and four supplementary tables are available with the online version of this article.

METHODS

Enrichment and isolation of strain 8C15b^T

Enrichment and isolation of strain 8C15b^T were performed by inoculating in a rich medium 200 mg of the first 5 cm sediments of the Bach Dang Estuary, Haiphong, collected in September 2019 and chilled in a cooler box with dry ice for transportation to the laboratory [10]. The rich medium contained: 0.02% (w/v) KH₂PO₄, 0.02% (w/v) K₂HPO₄, 0.005% (w/v) NH₄Cl, 2.4% (w/v) NaCl, 0.6% (w/v) MgCl₂·6H₂O, 0.4% (w/v) MgSO₄·7H₂O, 0.1% (w/v) KCl, 0.1% CaCl₂·2H₂O, 0.04% (w/v) cysteine-HCl, 0.1% (w/v) yeast extract (Difco Laboratories), 0.2% (w/v) biotrypcase (Difco Laboratories), 0.2% (w/v) MES (2-(*N*-morpholino) ethane sulphonic acid), 0.1% (v/v) Widdel's trace mineral element solution [11], 0.1% (v/v) selenite-tungstate solution (see medium DSMZ 385) and 0.0001% (w/v) resazurin (Sigma-Aldrich). The pH was adjusted to pH 6.5 with a 1 N HCl solution. The medium was boiled under a stream of O₂-free N₂ gas and cooled at room temperature. Aliquots of 20 and 5 ml were dispensed respectively into 25 ml penicillin flasks or Hungate tubes, under N₂-CO₂ (80/20, v/v) and subsequently sterilized by autoclaving at 120 °C for 20 min. The aliquot of sediments (200 mg) was inoculated into a 25 ml penicillin flask and incubated at 30 °C. The Hungate technique was used throughout the study [12]. After 7 days of incubation, a 0.5 ml aliquot of the enrichment culture was inoculated into Hungate tubes that were subsequently incubated at 30 °C. To obtain pure cultures, the enrichment was sub-cultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in roll tubes containing the rich medium supplemented with 2% agar (w/v) [13]. Colonies developed after 7 days of incubation at 30 °C and were picked up separately. The process of serial dilution was repeated several times until the isolate was deemed to be axenic.

Determination of the optimal temperature, NaCl and pH conditions for growth

For the characterization experiments of strain 8C15b^T, we used the medium previously described for the same purposes in *T. mesophilus* B1^T [9]. The pH was adjusted to pH 6.5 with 1 M KOH solution. The medium was conditioned in Hungate tubes. Prior to inoculation, 0.2% (w/v) NaHCO₃, from a 10% (w/v) sterile anaerobic stock solution, was injected into the tubes. The temperature, NaCl and pH ranges for growth were determined with the addition of glucose as substrate at 20 mM final concentration. The temperature was tested from 15 to 70 °C. To study NaCl tolerance, the latter was added to the tubes at final concentrations ranging from 0–9% (w/v). The pH of the medium was adjusted by injecting aliquots of anaerobic sterile stock solutions of 10% NaHCO₃ (w/v) or 8% (w/v) Na₂CO₃ (to test pH between 5 and 10), in Hungate tubes, after autoclaving. The strain was sub-cultured at least twice under the same experimental conditions before growth rates were determined.

Substrate utilization assays

Various substrates were added at 20 mM final concentration to the characterization medium containing 0.01% (w/v) yeast extract. Complex organic substrates (casamino acids, yeast extract, beef extract, tryptone, peptone) were tested at a final concentration of 0.2% (w/v). To test for electron acceptors, sodium sulphate (20 mM final concentration), sodium thiosulphate (20 mM), sulphite (2 mM), elemental sulphur (1%, w/v), nitrate (20 mM), or nitrite (2 mM) were added to the culture medium, using peptone or casamino acids as substrate. Growth was determined by OD 580 nm measurements and microscopy observations, after 72 h of culture at 30 °C. The strain was sub-cultured at least twice with the same substrate. Experiments were performed in duplicates. Substrate consumption and organic acid production were measured by HPLC (UltiMate 3000, Thermo Scientific). Sulphate, thiosulphate, sulphite, nitrate and nitrite consumption/production were measured by ionic chromatography, using the Metrohm 761 Compact device. H₂S production was measured using the Cord-Ruwisch method [14]. The gases produced were analysed by gas chromatography (Shimadzu GC-8A, 70 mA; WiniLabIII software). The oxidase test was performed using the Bio-Rad Oxidase-Streifen strips (Bio-Rad).

Light and scanning electron microscopy

The Gram reaction was determined using Difco kit reagents, according to the manufacturer's instructions (BD Difco Gram Stain Kit Components, Becton Dickinson and Co). The presence of spores was analysed by phase contrast microscopic observations of young and old cultures. Cell morphology was observed by phase contrast microscopy (Nikon Eclipse E600) and by transmission electron microscopy. Cells for electron microscopy observations were adsorbed on glow discharged copper grids, negatively stained with 0.5% (w/v) uranyl acetate for 30 s and examined with a G2 FEI Technai transmission electron microscope operating at 200 kV. Micrographs were performed with a 2k Veleta camera. For ultra-thin sections, cells were fixed in glutaraldehyde 2.5% (w/v), post-fixed in OsO₄ 2% (w/v), dehydrated in ethanol and embedded in Epon. Ultrathin sections of 75 nm have been performed for wall observation and vesicles formation after 72 h of growth at 30 °C with glucose as substrate. The preparations were contrasted with 2% (w/v) uranyl acetate and lead citrate.

The presence of flagella was observed both under transmission electron microscopy after negative staining, and by using the BD BBL Flagella Stain Droppers, according to the manufacturer's instructions (Becton Dickinson and Co).

Cellular fatty acid analysis

Analysis of the cellular fatty acids were carried out by DSMZ services, Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Lipids were extracted from 300 mg wet biomass after 72 h growth in the medium containing 0.2% (w/v) yeast extract as substrate. The preparation and identification of fatty acids were performed according to standard protocols of the Microbial Identification System, Sherlock version 6.4 (MIDI Inc.). Compounds were identified against the TSBA6 peak naming table database. The results for fatty acids were confirmed by GC/MS, which also enabled to resolve summed features.

Nickel susceptibility experiments

The growth of strain 8C15b^T in a modified medium containing nickel ions was monitored over 5 days using OD 580 nm measurements. The modified medium contained 0.02% (w/v) KH₂PO₄, 0.02% (w/v) K₂HPO₄, 0.02% (w/v) NH₄Cl, 2% (w/v) NaCl, 0.2% (w/v) MgCl₂·6H₂O, 0.4% (w/v) MgSO₄·7H₂O, 0.1% (w/v) KCl, 0.1% (w/v) CaCl₂·2H₂O, 0.04% (w/v) cysteine-HCl, 0.01% (w/v) yeast extract (Difco Laboratories), 0.5% (v/v) Balch's trace mineral elements solution [15], and 0.1% (v/v) selenite-tungstate solution (see medium DSMZ 385). Glucose was added as sole carbon and energy source, at 20 mM final concentration. Aqueous solutions containing Ni as NiCl₂·6H₂O were added to the medium, from 1 M or 100 mM stock solutions. Final concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 4, 10, 20, 30, 40, 50 and 100 mM NiCl₂ were tested. The minimal inhibitory concentration and the LD50 to test the sensitivity to the metal were carried out in triplicate at 30°C. Controls consisted of strain 8C15b^T cultures grown in the medium without nickel ions. The medium was prepared 2 h before inoculation to let the equilibrium of the conditions to occur. Growth monitoring was performed by optical density measurements at OD=580 nm, under an Agilent Technologies Cary 60 UV-Vis spectrophotometer, and by direct cell counts under an optical microscope Nikon Eclipse E600, using a 1 mm Thoma cell (Hawksley).

Phylogeny and comparison to the closest *Tepidibacter* species

The genome sequence of strain 8C15b^T has been previously deposited in the GenBank database under accession numbers OW235315 (chromosome) and OW235316 (plasmid) [10]. The 16S rRNA gene sequence was deposited in GenBank under accession number OQ970588, edited in the sequence alignment editor software BioEdit version 7.2.5 [16], and manually corrected. The sequence was aligned with closest relative sequences from the NCBI nr database, using the MUSCLE tool implemented in MEGA7 package [17]. A phylogenetic tree based on 16S rRNA gene sequences was reconstructed using the neighbour-joining method [18].

Calculations were limited to a selection of sequences comprising type strains affiliated with the family *Peptostreptococcaceae* with a significant relationship to strain 8C15b^T. Evolutionary distances were computed using the maximum composite likelihood method [19]. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. The final dataset consisted of 1275 positions. The average nucleotide identity (ANI) calculations were obtained with GTDB-Tk 2.3.2 (<https://ecogenomics.github.io/GTDBTk/>) and EzBioCloud (www.ezbiocloud.net/tools/ani). Average amino acid identity (AAI) was computed using EzAAI version 1.2.2 (<https://link.springer.com/article/10.1007/s12275-021-1154-0>) and digital DNA-DNA hybridization (dDDH) using the Type Strain Genome Server (<https://tygs.dsmz.de/>).

Structural and functional genome analyses

Functional classes were classified in COGs (Clusters of Orthologous Groups) of proteins by the COGnitor software imbedded into the MicroScope system (<https://mage.genoscope.cns.fr/microscope/>). Metabolic pathways were analysed with the KEGG database [20]. Research of heavy metal-associated genes was performed using the MicroScope system with manual inspection of the surrounding significant search hits. The genome tools and comparative tools of the MicroScope system were used to analyse the genome of strain 8C15b^T and for comparative analyses with other sequenced genomes. The RefSeq/PkGDB Synteny Statistics tool provides statistics about the similarity results between the selected organism and all the bacterial genomes available in RefSeq/WGS NCBI sections and in the PkGDB database. The orthologous relations between two genomes were defined as gene couples satisfying the bi-directional best hit (BBH) criterion or a BLASTP alignment threshold (> 35% sequence identity on 80% protein length). These relations were subsequently used to search for conserved gene clusters, e.g. synteny groups (syntons) among several bacterial genomes (inversions and indels allowed). A gap parameter, representing the maximum number of consecutive genes which are not involved in a synteny group, was set to five genes. Regarding non-B features, we proceeded as follows: G-quadruplexes were detected with pqsfinder version 2.14.1 (option grun=2) [21], and positive hits were confirmed with G4Hunter (options w=25, s=1.2) [22]; triplexes were computed with the R tool Triplex version 1.0.10 (default settings) [23]; Z-DNA was computed with Z-Hunt-II (options: windowsize=8, minsize=6, maxsize=8) [24]; DNA repeats were computed with Repseek version 6.6 [25], with a minimal seed length given by the Karlin and Ost extreme statistics [26], at a *P*-value<0.001. The output of Repseek was further clustered into two categories (direct and inverted). G+C content and G+C skew were computed using in-house scripts using window and step values of 1 kb (chromosome) and 0.1 kb (plasmid). All non-B-associated data are

shown in Tables S1-S3, available in the online version of this article. Defence genes and systems were computed with DefenseFinder version 1.08, using default settings [27] (Table S4).

RESULTS AND DISCUSSION

Morphological characteristics of strain 8C15b^T

The isolation procedure allowed us to get a pure culture of an obligatory anaerobic rod-shaped bacterium, named strain 8C15b^T. Morphologic characteristics of the novel isolate were observed by light and transmission electron microscopy (Figs 1 and 2). The cells were motile (peritrichous flagella), endospore-forming, rod-shaped, about $5(\pm 1)$ μm long and $1(\pm 0.2)$ μm in diameter and they stained Gram-positive. Notably, 61 genes involved in spore formation and germination were evidenced in the genome of the strain (genome accession numbers OW235315 and OW235316). In old cultures, cells formed filamentous rods. Cellular fatty acids (CFAs) composition analysis of strain 8C15b^T grown at 30°C for 48 h indicated that the main CFAs were iso-C_{15:0} (51% of the total CFAs), iso-C_{15:1} $\omega 7c$ (32%) and iso-C_{13:0} (5%).

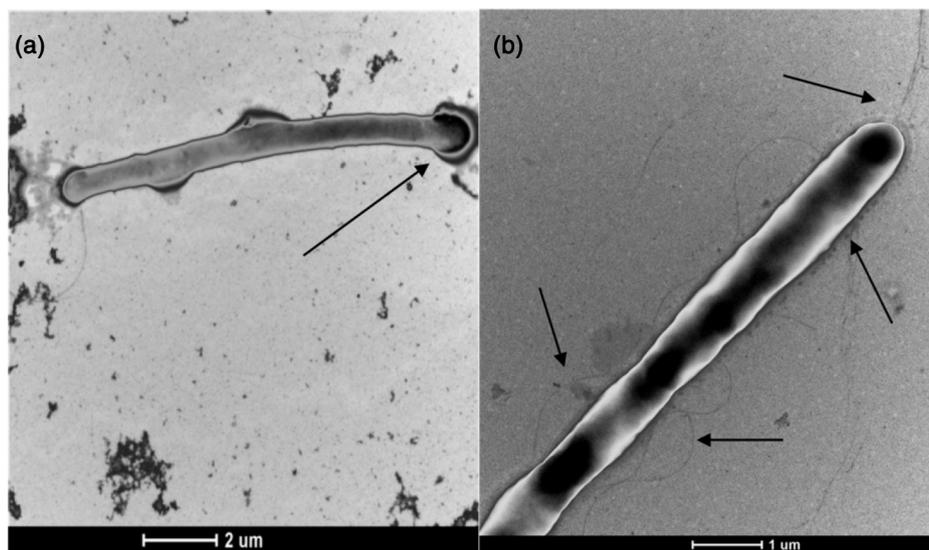


Fig. 1. Negatively stained cells. (a) Arrow, spore formation. (b) Arrows, flagella.

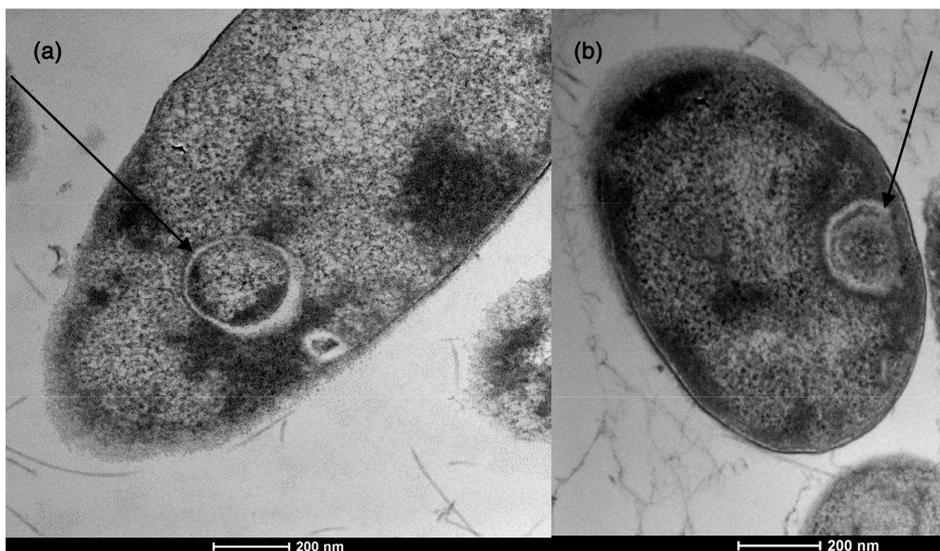


Fig. 2. Ultrathin sections. Arrows, endospore formation.

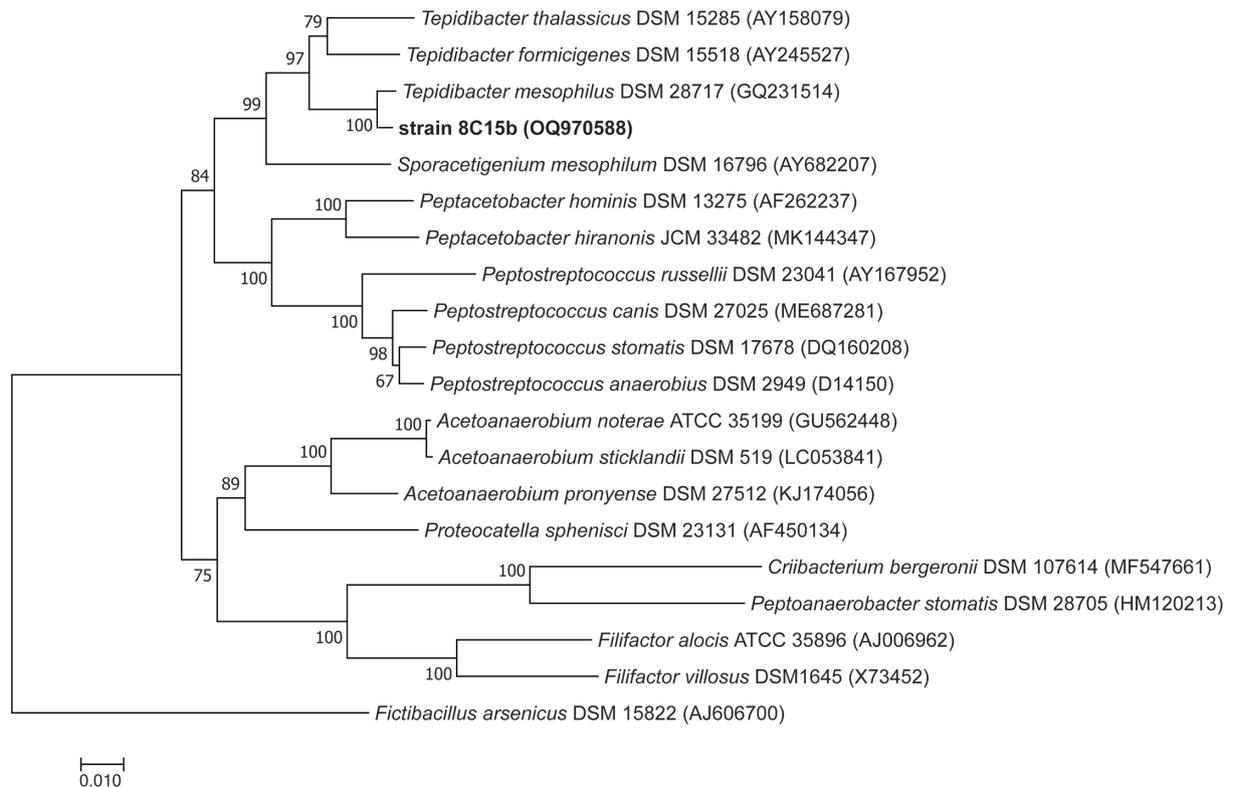


Fig. 3. Evolutionary relationships of strain 8C15b^T and related taxa based on the 16S rRNA gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The bootstrap test was performed for 1000 replicates [38]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. Bar, 1 substitution per 100 nt positions.

Phylogenetic position of strain 8C15b^T

A BLAST search in the nt database revealed that the closest relatives of strain 8C15b^T were *T. mesophilus* B1^T, *T. formicigenes* DV1184^T and *T. thalassicus* SC562^T. Phylogenetic analysis based on 16S rRNA genes sequences indicated that strain 8C15b^T clustered with *T. mesophilus* B1^T (99.2% of 16S rRNA genes sequence similarity), and both strains clustered separately of the two other recognized members of the genus *Tepidibacter*, showing 96.0% of 16S rRNA gene sequences similarity to *T. formicigenes* DV1184^T and 95.3% to *T. thalassicus* SC562^T (Fig. 3). ANI and AAI genome sequence comparisons between strains 8C15b^T and *T. mesophilus* B1^T indicated values of only 88.6 and 89.3% respectively, which is below the level of species differentiation [10]. Moreover, a dDDH value of 38% was obtained between the two strains. This provides evidence that the two strains should be considered as distinct species. In addition, we observed an ANI of only 78.4% between 8C15b^T and *T. formicigenes* DV1184^T, and only 77.6% between 8C15b^T and *T. thalassicus* SC562^T. We thus propose the species *Tepidibacter aestuarii* sp. nov. for the type strain 8C15b^T.

Physiological properties of strain 8C15b^T

Physiological characteristics of strain 8C15b^T are given in Table 1 and compared to those of the phylogenetically close *Tepidibacter* species. Strain 8C15b^T has a strictly anaerobic heterotrophic metabolism mainly based on the fermentation of sugars, and it is oxidase and catalase negative. Yeast extract (at 0.01% (w/v)) was indispensable as a growth supplement. It was also unable to grow autotrophically under H₂/CO₂ (80% (v/v)/20% (v/v)) atmosphere with or without acetate (2 mM). The sugars used as sole carbon and energy sources were fructose, glucose, maltose, rhamnose, ribose, sucrose, trehalose and, to a lesser extent, arabinose, cellulose, galactose, mannose and xylose. Lactose was not metabolized. Complex substrates beef extract, casamino acids, peptone, tryptone and yeast extract were fermented. The amino acids alanine+proline (Stickland reaction), arginine, serine and valine, were metabolized and, to a lesser extent, alanine+glycine (Stickland reaction). Interestingly, the strain was able to grow with the organic acids lactate, malate and pyruvate as substrates, and weakly with formate and fumarate. No growth was detected with alcohols as substrates (except weakly on ethanol). The capacity of strain 8C15b^T to use lactate can be explained by the presence of a lactate operon on the plasmid found in the former [10]. The main fermentation product formed from the multiple substrates was acetate (up to 6 mM). In addition, formate was found in small amounts (around 1 mM) with sugars as substrate. Some isovalerate

Table 1. Characteristics of strain 8C15b^T and of the species of the genus *Tepidibacter*

| Characteristics | Strain 8C15b ^T (this study) | <i>T. mesophilus</i> B1 ^T [9] | <i>T. formicigenes</i> DV1184 ^T (=DSM15518 ^T) [8] | <i>T. thalassicus</i> SC562 ^T (=DSM15285 ^T) [7] |
|------------------------------|--|---|---|---|
| Cell shape (size) | Rod (0.8–1.2×4–6 μm) | Rod (1.1–1.6×2.3–4.7 μm) | Rod (0.75–0.85×3.8–4.2 μm) | Rod (ND) |
| Origin | Marine sediments of Bach Dang Estuary, Haiphong, Vietnam | Crude-oil polluted soil, Karamay oil field, China | Mid-Atlantic Ridge hydrothermal vent, Lucky Strike | Hydrothermal vent chimney, 13°N East-Pacific rise, 2650 m, AMISTAD cruise |
| Spore shape | Round | Round | Void refractile | ND |
| Spore location | Terminal | Terminal | Sub-terminal | Terminal |
| Motility | Motile (peritrichous flagella) | Motile (peritrichous flagella) | Motile (peritrichous flagella) | Motile (peritrichous flagella) |
| Temperature for growth (°C): | | | | |
| Range | 20–40 | 10–40 | 35–55 | 28–60 |
| Optimum | 30 | 28–32 | 45 | 50 |
| pH for growth: | | | | |
| Range | 6.0–9.5 | 6.0–8.9 | 5.0–8.0 | 5.0–8.5 |
| Optimum | 7.5 | 7.3 | 6.0 | 6.5–7.0 |
| NaCl or growth (%): | | | | |
| Range | 1–4.5 | 0–9 | 1.5–4.6 | 1.5–6 |
| Optimum | 2.5 | 0.5 | 2.3 | 2 |
| Electron acceptors | None | S° | None | S° |
| Utilization of: | | | | |
| Arabinose | +/- | + | + | - |
| Cellulose | +/- | + | + | - |
| Fructose | + | + | + | - |
| Galactose | +/- | + | - | - |
| Glucose | + | + | + | + |
| Lactose | - | - | - | - |
| Maltose | + | + | + | + |
| Mannose | +/- | - | +/- | - |
| Rhamnose | + | + | - | - |
| Ribose | + | + | - | - |
| Starch | +/- | + | + | + |
| Sucrose | + | - | + | - |
| Trehalose | + | + | - | - |
| Xylose | +/- | + | - | - |
| Beef extract | + | + | + | + |
| Peptone | + | + | + | + |
| Tryptone | + | + | + | + |
| Yeast extract | + | + | + | + |
| Albumin | - | - | - | + |
| Gelatin | - | - | + | - |

Continued

Table 1. Continued

| Characteristics | Strain 8C15b ^T (this study) | <i>T. mesophilus</i> B1 ^T [9] | <i>T. formicigenes</i> DV1184 ^T (=DSM15518 ^T) [8] | <i>T. thalassicus</i> SC562 ^T (=DSM15285 ^T) [7] |
|-----------------------|---|--|---|---|
| Casamino acids | + | + | + | + |
| Alanine | - | - | - | - |
| Alanine+glycine | +/- | + | + | - |
| Alanine+proline | + | + | + | + |
| Arginine | + | - | + | + |
| Betaine | - | - | - | - |
| Glycine | - | - | - | - |
| Proline | - | ND | + | - |
| Serine | + | ND | + | ND |
| Valine | + | + | + | + |
| Ethanol | +/- | - | +/- | - |
| Glycerol | - | - | - | - |
| Mannitol | - | - | - | - |
| Methanol | - | - | - | - |
| Acetate | - | - | - | - |
| Butyrate | - | - | - | - |
| Formate | +/- | - | - | - |
| Fumarate | +/- | - | - | - |
| Lactate | + | - | - | - |
| Malate | + | ND | ND | ND |
| Pyruvate | + | - | + | + |
| Succinate | - | - | - | - |
| Fermentation products | Acetate, formate, and some isobutyrate and isovalerate (with complex substrates), CO ₂ and some H ₂ | Acetate, ethanol | Formate, acetate, ethanol | Acetate, ethanol, H ₂ , CO ₂ |

+, Growth; -, no growth; ND, not determined.

and isobutyrate were also produced with casamino acids and peptone as substrate. The produced acetate/consumed glucose ratio was 2.0 (\pm 0.5). Fermentative growth was also accompanied by the production of CO₂ and small amounts of H₂. No requirement for an external electron acceptor and no significant production of H₂S were observed.

The temperature range for growth was 20 °C to 40 °C with an optimum at 30 °C (Table 1). No growth was observed below 20 °C or above 40 °C, suggesting that the strain is mesophilic and psychro-sensitive (Fig. S1A). The growth rate was maximum with NaCl concentration at 25 g l⁻¹. No growth was observed below 10 g l⁻¹ or above 45 g l⁻¹ (Fig. S1B). The optimum pH for growth was pH 7.5 and no growth was apparent at pH 5.5 or 10 (Fig. S1C).

Genomic features and comparative genomics of strain 8C15b^T

Circos representations of both chromosome and plasmid of strain 8C15b^T are depicted in Fig. 4a. Its genome properties and those of its closest related cultivated species are summarized in Table 2. The genome size of strain 8C15b^T (chromosome, 3628320 bp; plasmid, 38213 bp) was bigger than the currently available sequenced *Tepidibacter* genomes (~1 Mb larger than those of *T. formicigenes* DV1184^T and *T. thalassicus* SC562^T, and ~307 kb larger than that of *T. mesophilus* B1^T). The G+C content of the *Tepidibacter* representatives is low (around 27–29 mol%), which agrees with the value of 29.79 mol% of strain 8C15b^T. A total

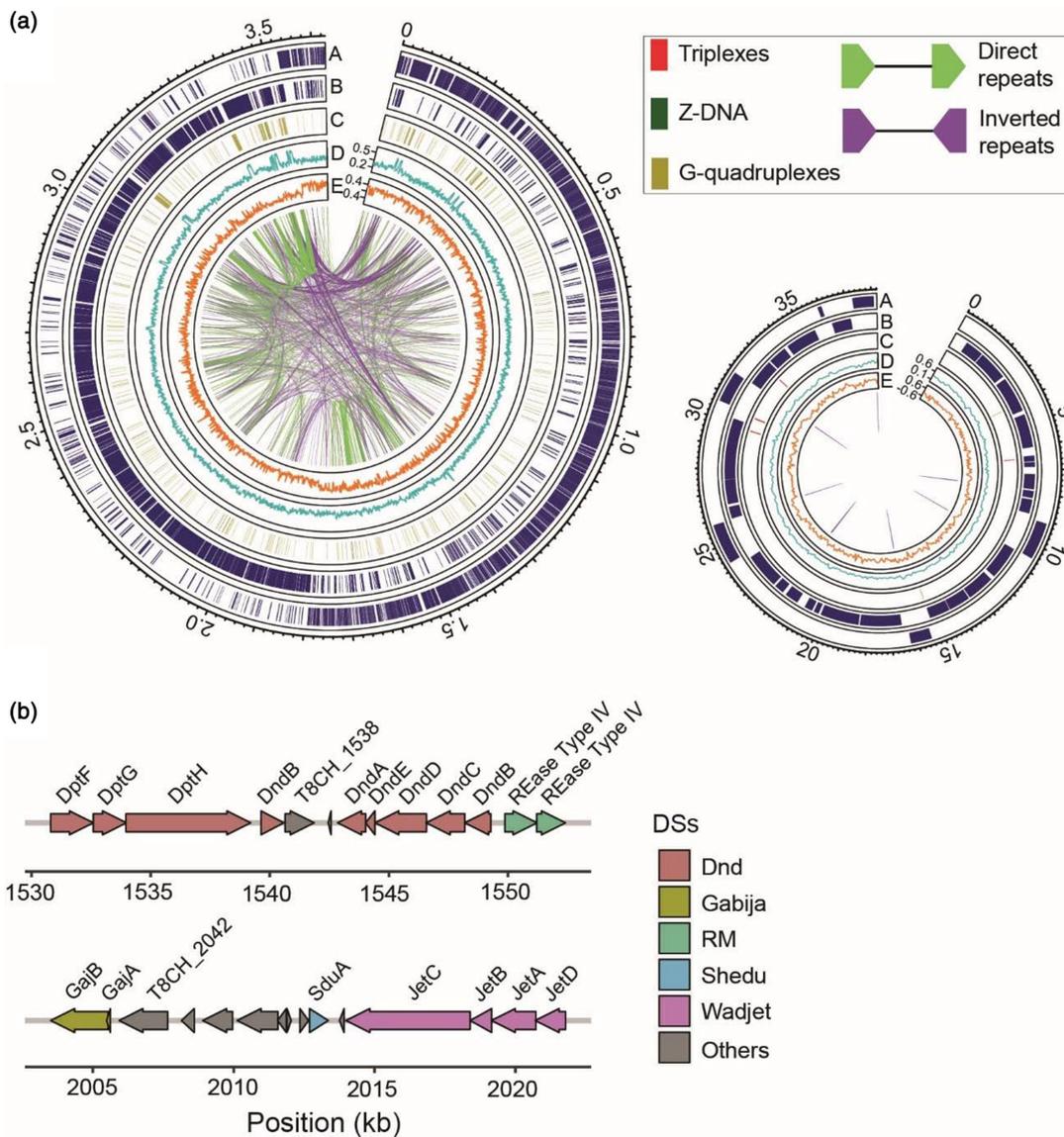


Fig. 4. Genomic organization of strain 8C15b^T and zoom into its non-B features and anti-phage defensible. (a) Circos representation of the chromosome (left) and plasmid (right) highlighting CDS distribution for sense and antisense strands (A, B respectively), triplexes, Z-DNA, and G-quadruplexes (C), G+C content (D), G+C skew (E), and repeat pairs (inner circle). (b) Schematic representation of two defence islands with high clustering density of defence systems (DSs). No defence systems or solitary defence genes were found in the plasmid.

of 3516 coding DNA sequences (CDSs) were predicted for the chromosome, as well as 22 pseudogenes, 84 miscellaneous RNA genes (misc-RNA), 51 rRNA genes (17 for 16S, 19 for 5S and 15 for 23S) and 124 tRNA genes (Fig. 4a).

A total of 36 CDSs were predicted for the plasmid (Fig. 4a). Among them, two operons encoded proteins of unknown function (T8CH_1824–1828; T8CH_1837–1838). Other CDSs encoded for membrane transporters, a transposase (T8CH_1852), an integrase (T8CH_1853) and a phage-associated protein (T8CH_1837). Interestingly, an operon encoding for lactate metabolism functions (TMC_1820–1822), was found on the plasmid sequence. The presence of this operon could explain the capacity of strain 8C15b^T to metabolize lactate, while *T. mesophilus* B1^T was not able to metabolize it (Table 1).

Among the NCBI Genomic Reference sequences, the most similar genome to that of 8C15b^T was that of *T. mesophilus* B1^T (=JCM16806^T; accession number GCA_002724055.1) [28]. Reciprocal best BLAST hit analysis indicated that strain 8C15b^T shares 2704 orthologous proteins with *T. mesophilus* B1^T. A total of 46 regions of genomic plasticity have been found along the genome, using the RGP finder tool of the MicroScope system, by comparison to the genome of *T. mesophilus* B1^T (Table 2). In addition, we observed a profuse landscape of genetic features associated with an elevated propensity for non-B DNA formation (a hallmark

Table 2. Genome features of strain 8C15b^T and closely related species

| Attribute | Strain 8C15b ^T (this study and [10]) | <i>T. mesophilus</i> B1 ^T (=JCM16806 ^T) [†] | <i>T. formicigenes</i> DV1184 ^T (=DSM15518 ^T) [†] | <i>T. thalassicus</i> SC562 ^T (=DSM15285 ^T) [†] |
|---------------------------|--|---|---|---|
| Genome size (bp) | 3628320 | 3321172 | 2654625 | 2239970 |
| G+C content (mol%) | 29.79 | 28.85 | 27.73 | 27.86 |
| Protein coding sequences | 3516 | 3248 | 2709 | 2310 |
| Number of predicted RGP*s | 46/ <i>T. mesophilus</i> B1 ^T 59/ <i>T. formicigenes</i> | 41/8C15b ^T 60/ <i>T. thalassicus</i> | 41/ <i>T. thalassicus</i> 51/ <i>T. mesophilus</i> B1 ^T | 16/ <i>T. formicigenes</i> 30/8C15b ^T |
| Total rRNA genes | 51 | 47 | 30 | 28 |
| 5S RNA genes | 19 | 15 | 11 | 9 |
| 16S RNA genes | 17 | 20 | 12 | 10 |
| 23S RNA genes | 15 | 12 | 7 | 9 |
| Total tRNA genes | 124 | 70 | 25 | 27 |
| Different tRNA genes | 22 | 21 | 14 | 14 |
| Pseudo-genes | 22 | 14 | 22 | 10 |
| misc-RNA genes | 84 | 81 | 84 | 51 |
| Number of defence genes | 128 | 132 | 153 | 130 |

*Regions of genomic plasticity compared to their closest relative.

†Data from [28].

for ssDNA formation and genetic instability) (Fig. 4a, b; Tables S1–S3). The former, found in both chromosome and plasmid, include regions prone to form G-quadruplexes, triplexes, Z-DNA and hairpins/cruciforms. We anticipate that a subset of these may effectively play a key role in regulating multiple cellular processes, alter the read-out of genetic information and ultimately drive the evolution of this species.

Regarding the gene families in the genome of 8C15b^T and across the three *Tepidibacter* strains for which the genome had been sequenced, the Venn diagram of Fig. 5 indicated that the four strains had only 1303 gene families in common using the ‘30% aa identity / 80% align’ coverage MICFAM parameter. Strain 8C15b^T presented 879 gene families independent of the three other strains. Most of specific genes are often acquired by horizontal gene transfer (HGT) and assumed to encode adaptive functions [29]. Eight genes have been annotated as transposases and two as integrases on the chromosome sequence.

The functional annotation of the genome of strain 8C15b^T indicated that 85% of the proteins were assigned to a COG category, mostly into the ‘amino acid transport and metabolism’ (E; 9%) and ‘energy production and conversion’ (C; 7%) classes. The major differences regarding the COG repartition of the two genomes concerned the ‘energy production and conversion’, ‘amino acids transport and metabolism’ and ‘replication, recombination and repair’ categories (Fig. 6). It suggests that these functions are likely important for the adaptation of this species to its environment. To our knowledge, the genome of strain 8C15b^T possesses the highest number of CDSs and is the only one for which a plasmid has been evidenced among the *Tepidibacter* species, so far.

In addition, using the RefSeq Synteny Statistics tool of the MicroScope system, it was interesting to note that the best score was obtained with the genome of *Alkaliphilus metalliredigens* QYMF (NC_009633), with 41% of CDSs found in syntons and 51% of genes in BBH. Using the PkGDB Synteny Statistics tool, the best score was obtained with the genome of *T. mesophilus* B1^T, with 71% of CDSs in syntons and 69% of genes in BBH.

Specificity in the genome of strain 8C15b^T

A profuse arsenal of 128 defence genes pertaining to well-known defence systems such as restriction-modification, and other recently described ones, such as Gabija, Sheddu and Wadjet, were found in the genome of strain 8C15b^T (Table S4). This suggests that gene flux in 8C15b^T occurs at high levels and is countered by a complex and diverse layer of defence systems. Defence gene abundance in 8C15b^T was of the same order of magnitude than that observed in *T. mesophilus* B1^T (=JCM16806^T), *T. formicigenes* DV1184^T (=DSM15518^T) and *T. thalassicus* SC562^T (=DSM15285^T), possessing 132, 153 and 130 defence genes, respectively (Table 2). Two defence islands spanning ~20 kb each were found in the chromosome of 8C15b^T (Fig. 4b). They concentrate a higher-than-expected frequency of defence-related genes from five different families and are known to typically co-localize with sinks for HGT [30]. The functional roles of the few genes classified as non-defensive found inside these islands are unclear at this

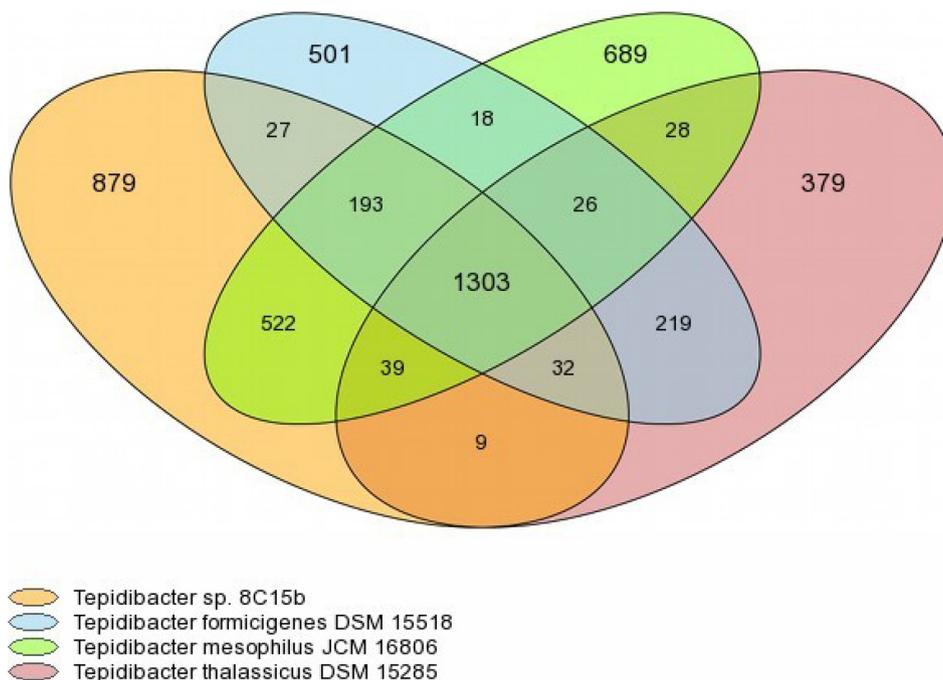


Fig. 5. Venn diagram of the gene families of the genomes of strain 8C15b^T, *T. mesophilus* B1^T, *T. formicigenes* DV1184^T and *T. thalassicus* SC562^T. The analysis of the pan-genome and its components (core-genome, variable-genome) for this organism set was performed using the MicroScope gene families (MICFAM) which are computed with the SiLiX software (ultra-fast sequence clustering from similarity networks with SiLiX) [39]. The '30% aa identity / 80% align' coverage MICFAM parameter was used.

point (Fig. 4b). Some possibilities include functions linked to mobility, unappreciated defensive roles or resistance/adaptation (<https://www.biorxiv.org/content/10.1101/2023.08.12.553040v2>).

Moreover, several genes involved in complex polysaccharide degradation have been found, such as three genes encoding for endoglucanases (T8CH_0107; T8CH_0381; T8CH_2387). Numerous carbohydrate-active enzymes and transporters, and genes involved in transport and metabolism of organic acids, for example of lactate (T8CH_1820–1822, located on the plasmid sequence), have been found as well. These findings highlight the capacity of the strain to metabolize a large variety of substrates.

Interestingly, several heavy metal translocating ATPases and heavy metal efflux pumps were predicted in the genome of strain 8C15b^T, which suggests heavy metal resistance properties of the strain. For example, the genome contains a *nikMNQ* operon encoding for a Co/Ni transporter (T8CH_1107–1109), and an operon encoding for a Cu transporter (T8CH_0472–0473). A gene encoding for a Cu/Mn oxidase (T8CH_3311), similar to the Cu resistance protein A [31], is also specifically present in the genome. In addition, it contains an arsenic resistance operon (T8CH_0475–0477), present in the genome of *Alkaliphilus metal-liredigens* QYMF [32]. Two copies of the *aroA* gene (T8CH_0935 and _2011), encoding the enzyme 5-enopyruvylshikimate-3-p phosphate synthase, were also found in the genome. This enzyme has the potential in developing glyphosate-resistant crops [33].

Overall, the genome analysis highlights the high potential of strain 8C15b^T for environmental applications, which may thus contribute to organic matter mineralization and heavy-metal bioremediation processes.

Metal tolerance properties of strain 8C15b^T

Gram-positive bacteria, that have high adsorptive capacity due to high peptidoglycan and teichoic acid content in their cell walls, have the potential to bind and sequester metals [34, 35]. We were particularly interested in the tolerance properties of strain 8C15b^T to nickel, because genes involved in resistance to this metal are present in the genome (see above) and it has been found to be present at high concentration in the Red River Delta. Indeed, nickel has been found at a concentration of around 60 mg kg⁻¹ of dry sediments in the Bach Dang Estuary, from which the strain was isolated (personal data, S. Chifflet). This represents a concentration tenfold higher than that found in pristine areas [36]. Under our growth conditions, using NiCl₂ as a source of nickel, the minimal inhibitory concentration of strain 8C15b^T was 1 mM (Fig. 7) and the LD50 was up to 20 mM after 40 h of growth at 30°C (Fig. S2), indicating a significant tolerance of the strain to high nickel concentration. While further molecular studies will help in understanding the mechanisms involved in its metal tolerance, it is conceivable that strain 8C15b^T could be further exploited in the biotechnology arena for the bioremediation of heavy-metal contaminated environments.

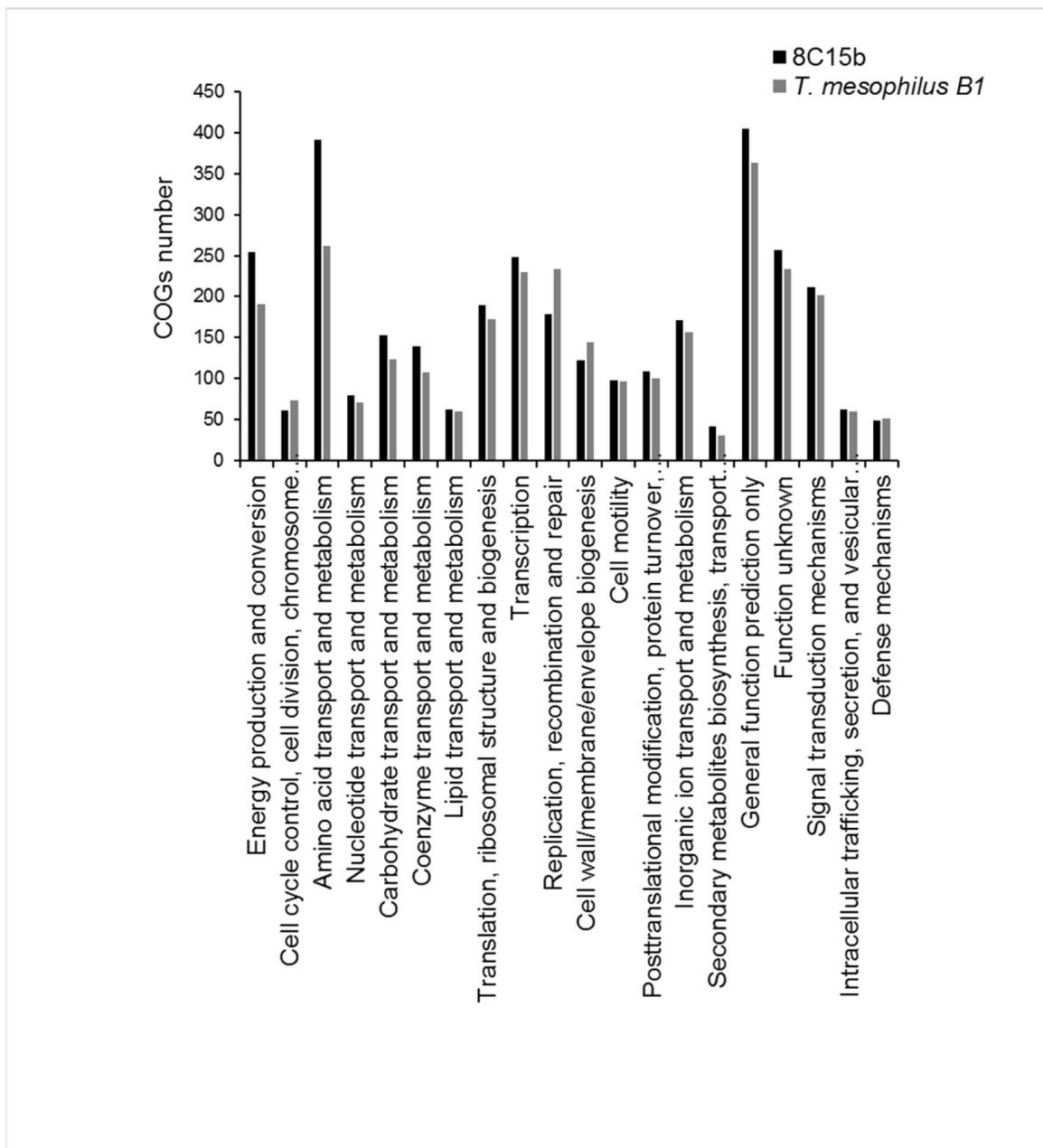


Fig. 6. Distribution of the COGs number in the genomes of strain 8C15b^T and *T. mesophilus* B1^T.

CONCLUSION

At present, a few strains of the genus *Tepidibacter* have been isolated, but none from Asian coastal marine areas (which are typically submitted to elevated anthropic influence). Here, we report strain 8C15b^T, isolated from the heavy metal polluted marine environment of the Bach Dang Estuary, Haiphong, Vietnam. Though this strain exhibited maximum similarity to *T. mesophilus* B1^T in terms of 16S rRNA gene sequences (99.2% identity), it differed at the whole genome sequence level (89.5% identity). Plus, an extra plasmid was observed in 8C15b^T [10]. Moreover, strain 8C15b^T possesses several genes involved in metal resistance and was shown to be tolerant to high nickel concentrations using NiCl₂. These characteristics suggest that the organism has developed adaptation strategies according to the physico-chemical conditions of the environment from which it has been isolated. Thus, spatially separated environments for which the physico-chemical parameters greatly differ may play an important role in the functioning of bacteria, as previously suggested for *Clostridium* species [37]. Properties such as the capacity to use a wide range of complex substrates and to resist toxic concentrations of heavy metals make strain 8C15b^T a suitable candidate for use in industrial technologies.

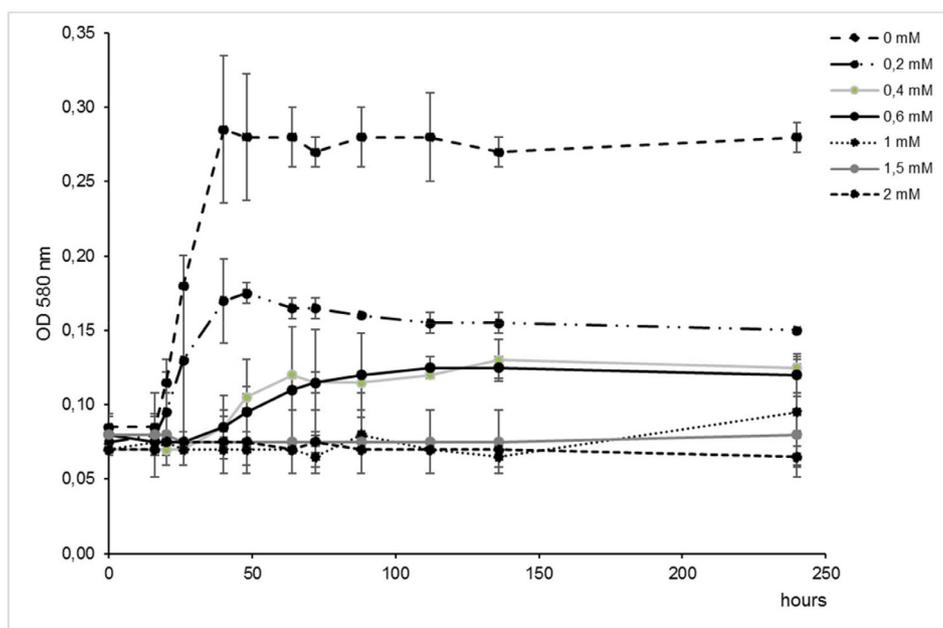


Fig. 7. Effect of NiCl_2 on the growth of strain 8C15b^T ($\text{OD}_{580\text{nm}}$ /hours).

Tepidibacter species belong to the family *Peptostreptococcaceae*, in the class *Clostridia*. The physiological features of the genus *Tepidibacter* stem from their capacity to reduce S^0 and from their thermophilic characteristics, except for *T. mesophilus* B1^T, assigned as a *Tepidibacter* but which is mesophilic. Based on the data obtained from the present study, we propose that strain 8C15b^T represents a novel species. We propose the name *Tepidibacter aestuarii* sp. nov. for the type strain 8C15b^T.

DESCRIPTION OF *TEPIDIBACTER AESTUARI* SP. NOV.

Tepidibacter aestuarii (aes.tu.a'ri.i. L. gen. n. *aestuarii*, of an estuary).

Cells are Gram-positive, anaerobic, rod-shaped and motile by means of peritrichous flagella. Terminal spores are observed. Growth occurs between 20 and 40°C (optimum, 30°C), between pH 6.0 and 9.5 (optimum, pH 7.5) and with NaCl % (w/v) between 1 and 4.5 (optimum, 2.5 %, w/v). Heterotrophic, able to ferment mainly sugars, complex substrates and some organic acids. Utilizes fructose, glucose, maltose, rhamnose, ribose, sucrose, trehalose, beef extract, peptone, tryptone, yeast extract, casamino acids, alanine+proline, arginine, serine, valine, lactate, malate and pyruvate. The main products of fermentation are acetate, formate and CO_2 . The main cellular fatty acids are iso- $\text{C}_{15:0}$ (51%), iso- $\text{C}_{15:1}$ ω7c (32%) and iso- $\text{C}_{13:0}$ (5%). Genomic G+C content is 29.79mol%. The results of 16S rRNA gene analysis locate the strain within the class of *Clostridia* that encompasses the genus *Clostridium* and related genera such as the genus *Tepidibacter*. The 16S rRNA gene sequence (accession number OQ970588) is 99.2% similar to that of *Tepidibacter mesophilus* B1^T.

The type strain is 8C15b^T, deposited in JCM and KCTC culture collections as JCM 35983^T and KCTC 25692^T, respectively. Strain 8C15b^T was isolated from a costal sediment sample collected on the Bach Dang Estuary, Haiphong, Vietnam (20° 45' 28.39" N 106° 46' 22.91" E).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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