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TAXONOMIC DESCRIPTION

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Emcibacter congregatus sp. nov., isolated from sediment cultured *in situ*

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Abstract

A Gram-negative, aerobic, motile, rod-shaped, pale-yellow bacterial strain, designated as ZYL^T, isolated from a cultured *in situ* sediment sample collected from the East China Sea coast, was studied using a polyphasic taxonomic approach. Strain ZYL^T grew at 4–30 °C (optimum, 25 °C), at pH 6.0–8.5 (pH 7.0) and with 0–7.0 % (w/v) NaCl (2.0 %). Results of phylogenetic analysis based on 16S rRNA gene sequences clearly showed that strain ZYL^T and *Emcibacter nanhaiensis* HTCJW17^T, which was most closely related to strain ZYL^T with 93.6 % sequence similarity, clustered together. The genomic DNA G+C content was 51.5 % (genome sequence). The quinone system was composed only of ubiquinone-10. Strain ZYL^T possessed $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8), iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ (summed feature 3), $C_{14:0}$ 2-OH and $C_{14:0}$ as the major fatty acids. The content of summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) in strain ZYL^T was far greater than that in *E. nanhaiensis*. The polar lipid profile consisted of phosphatidylethanolamine, phosphatidylglycerol, three unidentified aminophospholipids, three unidentified phospholipids, one unidentified aminolipid and four unidentified lipids. One unidentified aminophospholipid and two unidentified lipids present in strain ZYL^T were not found in *E. nanhaiensis* in this research. On the basis of phenotypic, phylogenetic and chemotaxonomic data, strain ZYL^T (=KCTC 62328^T=JCM 32378^T=MCCC 1K03526^T) represents a novel species of the genus *Emcibacter* for which the name *Emcibacter congregatus* sp. nov. is proposed.

In traditional marine bacterial isolation methods, samples were always collected directly from natural conditions. After long-term storage, the microbial diversity in these samples might decrease a great deal. To obtain more marine microorganisms that can be cultured in the laboratory, we designed long-term culture *in situ* experiments. In these experiments, bacteria that can grow in artificial medium are enriched *in situ*, so that the diversity of cultivable microorganisms might increase in these samples. The new order *Emcibacterales* in the phylum *Proteobacteria* was established by Iino *et al.* in 2016, and the order contains only one family *Emcibacteraceae*, and this family consists of only one genus *Emcibacter* [1]. This genus, characterized by a large proportion of summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) as the major cellular fatty acid, formed a special branch on the phylogenetic tree [1]. The unique member of the genus *Emcibacter*, *Emcibacter nanhaiensis*, was isolated from sediment of the South China Sea by Liu *et al.* [2]. In this paper, we present a polyphasic taxonomic study describing a novel strain, ZYL^T, which is a new species within the genus *Emcibacter*.

We used YTP medium, which contained (per litre distilled water): 20 g NaCl, 0.5 g yeast extract, 1.0 g tryptone, 10 g peel of pemelo (*Citrus maxima*) and 20 g agar 20 g, pH 7.0. YTP agar medium was made and divided into 50 ml PP tubes sealed by gauze. After autoclaving, the gauze was replaced by single layer aseptic linen, and the tubes were placed into autoclaved barrels aseptically. We then buried this device on a coast in the East China Sea (29° 56′ N, 122° 05′ E), and dug it out after 1 year of culturing. For subsequent experiments, 1cm-thick medium on the upper layer was cut off. To isolate the strain, the standard dilution plating technique on modified marine agar 2216 (MA; BD) medium was used. The modified MA, replaced Na_2SO_4 with 6.64 g $MgSO_4 \bullet 7H_2O$ and decreased the amount of $MgCl_2 \bullet 6H_2O$ to 12.6 g. Whitish yellow colonies formed after 15 days' incubation at 20 °C, and were purified by repeated restreaking. Unless otherwise stated, strain ZYL^T was

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Abbreviations: AL, unidentified aminolipid; APL, unidentified aminophospholipid; L, unidentified lipid; MA, marine agar 2216; MB, marine broth 2216; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PL, unidentified phospholipid; Q10, ubiquinone-10.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ZYL^T is MG051279.

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

routinely cultured in marine broth 2216 (MB; BD) or on MA at 20 °C and preserved at −80 °C with 30.0 % (v/v) glycerol.

The genome of strain ZYL^T was sequenced using the Illumina HiSeq2500 platfrom. Assembly was performed using ABySS version 2.0.2 [3]. The quality of the assembled genome sequence was checked by CheckM version 1.0.7 [4].

The complete 16S rRNA sequence of strain ZYL^T was predicted by the RNAmmer 1.2 server in full genome sequences [5]. To get a proofreading sequence, a quick bacteria genomic DNA extraction kit (DongSheng Biotech) was used to collect genomic DNA as a template. The 16S rRNA gene was amplified by PCR using bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCT-3′) and 1492R (5′-GGTT ACCTTGTTACGACTT-3′). The almost-complete 16S rRNA sequence (1490 bp) was compared with sequences of closely related reference organisms obtained from the EzBiocloud database [6] via the EzTaxon-e tool. Multiple sequence alignment was performed with CLUSTAL_W 2.1 [7]. Phylogenetic trees were reconstructed using the neighbour-joining (NJ) [8], maximum-likelihood (ML) [9] and the maximum-parsimony (MP) [10] methods with the MEGA 7 program package. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model [11] for the NJ method. On the basis of 16S rRNA gene sequence alignment, strain ZYL^T was most closely related to *E. nanhaiensis* with 93.6 % sequence similarity, and were below 90.0 % to other type strains. Phylogenetic analysis, based on full-length 16S rRNA gene sequences, showed that strain ZYL^T clustered with *E. nanhaiensis* in the NJ, ML and MP trees (Fig. 1). According to the result of 16S rRNA gene sequence alignment, *E. nanhaiensis* MCCC 1A06723^T (obtained from the Marine Culture Collection of China) was chosen as a reference strain. It was cultured and preserved under the same conditions as strain ZYL^T, except that it was incubated at 37 °C.

Cell morphology was observed by optical microscopy and transmission electron microscopy using cells incubated on MA for 3 days (Fig. S1, available in the online version of this article). Motility was determined by microscopic observation and inoculation in 0.5 % semi-solid agar medium. The Gram reaction was performed as described by Reddy [12]. The optimal conditions for growth were tested in MB. The growth temperature was tested by incubation at 4, 15, 20, 25, 28, 30, 35, 40, 45 and 50 °C. The pH range was tested in MB adding four kinds of buffering agents (MES for pH 5.5–6.5, PIPES for pH 6.5–7.5, Tricine for pH 7.5–8.5, Capso for pH 9.0–10.0) at a concentration of 30 mM. The salt tolerance was determined in NaCl-free MB (prepared according to the MB formula, but without NaCl) with various NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 15.0 and 18.0 %). Anaerobic growth was

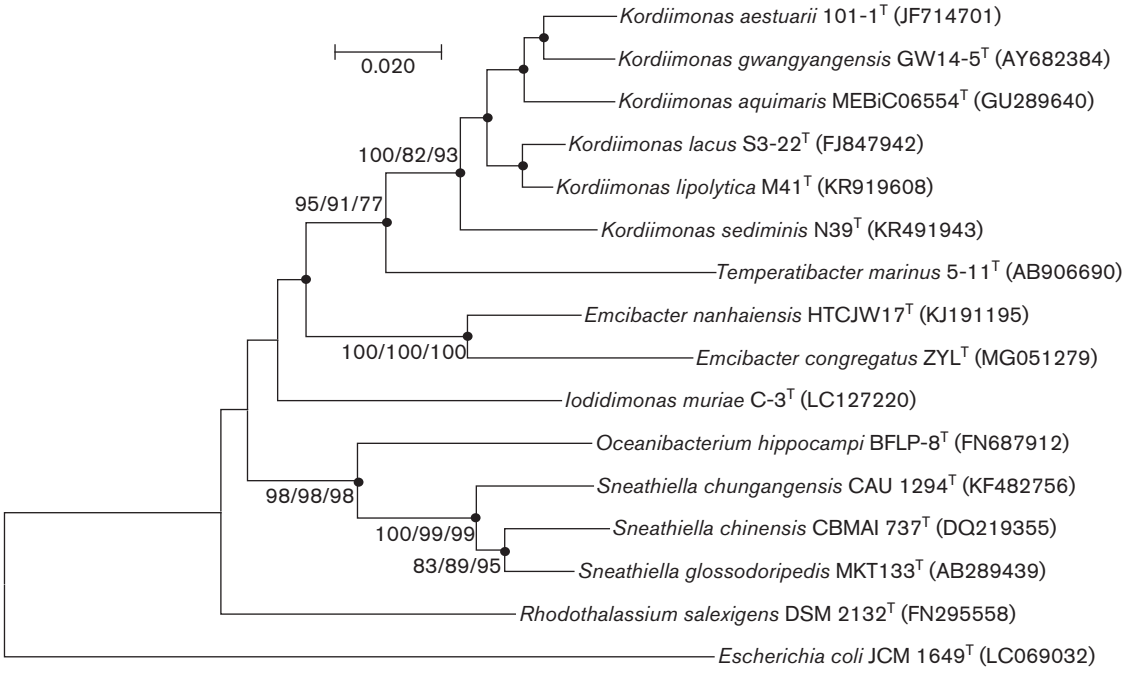

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain ZYL^T and some closely related taxa. Filled circles indicate nodes also obtained in both maximum-likelihood and maximum-parsimony trees. Bootstrap values (expressed as percentage of 1000 replications) >70 % in all three trees are shown at the branching points in the order: NJ/ML/MP. *Escherichia coli* JCM 1649^T (GenBank accession no. LC069032) was used as an outgroup. Bar, 0.020 substitutions per nucleotide position.

determined on MA with an anaerobic system (AnaeroPack-Anaero, 2.5 L, MGC) and in MB, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate as electron acceptors, and 5 g l^{-1} L-arginine, and 1 g l^{-1} cysteine as reductant were added [13], by omitting air by N_2 and CO_2 gases.

Oxidase activity was detected using 1.0 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by observing bubble production in 3.0 % (v/v) H_2O_2 solution. The hydrolysis of Tweens 20, 40, 60, and 80, H_2S production, methyl red and Voges–Proskauer reactions were tested based on methods of Sun *et al.* [14]. All experiments were performed in triplicate. For the acid production test, Leifson modified MOF medium [15] was used to suspend the cells for the inoculation of API 50CH (bioMérieux) strips. Other biochemical characteristics and enzyme activities were tested using API 20NE and API ZYM strips (bioMérieux) following the manufacturer's instructions. The phenotypic characteristics of strain ZYL^T are given in Table 1, and in the species description. Antibiotic sensitivity was detected on MA plates with discs containing one of the following antibiotics (μg per disc unless stated otherwise; 0.5 cm in diameter): ciprofloxacin (5), clindamycin (2), lincomycin (2), erythromycin (15), streptomycin (10), tetracycline (30), gentamicin (10), chloramphenicol (30), penicillin (10 IU), ofloxacin (5), co-trimoxazole (25), kanamycin (30), vancomycin (30), norfloxacin (10), polymyxin B (300 IU), ampicillin (10), neomycin (30), novobiocinum (30), piperacillin (100), enoxacin (10), rifampicin (5), ceftriaxone (30), cefradine (30), cefalexin (30), amoxicillin (10), minocycline (30) and oxacillin (1). The size of inhibition zones was measured and the diameter of zones which >1.5 cm, 1–1.5 cm or <1 cm was regarded as positive, weakly positive or negative inhibition, respectively.

Cells used for polar lipid, fatty acid and isoprenoid quinone analyses were grown in MB at 20 °C for 2 days to reach the exponential stage of growth. Isoprenoid quinones were determined by reversed-phase high-performance liquid chromatography [16]. A respiratory quinone detected in strain ZYL^T was Q10, which was same as *E. nanhaiensis* [1]. The fatty acids were analysed according to the standard protocol of the Sherlock Microbial Identification System (MIDI, Microbial ID) with the standard MIS Library Generation Software version 4.5, and identified by using the TSBA40 database. The major cellular fatty acids (>10.0 %) of strain ZYL^T were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 25.7 %), summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ $\omega7c$; 22.7 %), $C_{14:0}$ 2-OH (17.1 %) and $C_{14:0}$ (11.0 %). Polar lipids were extracted according to Cui *et al.* [17], and separated by two-dimensional thin-layer chromatography. Polar lipids were identified as described by Minnikin *et al.* [18]. Phosphomolybdic acid (5.0 %, heating at 160 °C), ninhydrin (0.5 %, heating at 55 °C) and molybdenum blue (Sigma) to

Table 1. Differential characteristics between strain ZYL^T and related species

Strains: 1, ZYL^T; 2, *Emcibacter nanhaiensis* MCCC 1A06723^T. +, Positive reaction; −, negative reaction; W, weakly positive; ND, no data. Data were obtained from this study unless stated otherwise. Both strains were positive for oxidase and H_2S production, and negative for hydrolysis of Tweens 20, 40, 60 and 80, and starch.

Characteristic	1	2
NaCl range (%) for growth (optimal)	0–7.0 (2.0)	1.0–7.0 (3.0)
Temp range (°C) for growth (optimal)	4–30 (25)	15–45 (30-37)
pH range for growth (optimal)	6.0–8.5 (7.0)	6.0–9.0 (7.0)
Catalase	weak	+
API ZYM:		
Cysteine arylamidase, trypsin, chymotrypsin	+	−
API 20NE:		
Reduction of nitrate to nitrite, β-galactosidase	+	−
Hydrolysis of aesculin and gelatin	−	W
API 50CH:		
Glycerol, D-arabinose, D-ribose	W	−
D-Glucose, D-fructose, L-rhamnose, Inositol, *N*-acetylglucosamine, cellobiose, maltose, lactose (bovine origin)	+	−
Starch	W	−
Potassium 5-ketogluconate	−	+
Antibiotic susceptibility:		
Ofloxacin, piperacillin, streptomycin, gentamicin, neomycin, penicillin, ampicillin, kanamycin	+	−
Chloramphenicol	−	+
Voges–Proskauer reaction	+	−
DNA G+C content (%)	51.5	56.3*

*Data from Liu *et al.* [2].

detect total lipids, aminolipids and phospholipids, were used, respectively. The polar lipid profile of strain ZYL^T comprised one phosphatidylethanolamine, one phosphatidylglycerol, three unidentified aminophospholipids (APL1–3), three unidentified phospholipids (PL1–3), one unidentified aminolipid (AL1) and four unidentified lipids (L1–4) (Fig. S2).

The DNA base composition (G+C content) was determined by counting G+C in full genome sequences. The genomic DNA G+C content of strain ZYL^T was 51.5 %, which could be used to discriminate the new isolate from *E. nanhaiensis* HTCJW17^T (56.5 % [2]).

In the fatty acid data, summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) was one of the most abundant fatty acids, which was in accordance with the *E. nanhaiensis* MCCC 1A06723^T (Table 2). Summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$) was the major fatty acid in strain ZYL^T, but in *E. nanhaiensis*, its ratio was less than 10.0 %. The polar lipid profile of strain ZYL^T was almost same as that of *E. nanhaiensis* MCCC 1A06723^T. One unidentified aminophospholipid (APL3) and two unidentified lipids (L1–L2) present in strain ZYL^T were not found in *E. nanhaiensis* in this experiment. But one unidentified phospholipid (PL4), one unidentified aminolipid (AL2) and two unidentified lipids (L5–L6) were present in *E. nanhaiensis*, but not found in strain ZYL^T in this study (Fig. S2). Strain ZYL^T showed a lower optimum growth temperature (25 °C) and growth range (4–30 °C) than *E. nanhaiensis*, which grows optimally at 30–37 °C and cannot grow at 4 °C. When cultured in MB for 2 days, strain ZYL^T formed flocculent mass, and the medium maintained transparent. *E. nanhaiensis* MCCC 1A06723^T also clustered in liquid medium, but the medium became turbid. More detailed differences, including physiological and biochemical characteristics, are shown in Table 1.

On the basis of the phenotypic, phylogenetic and chemotaxonomic characteristics described above, strain ZYL^T is considered to represent a novel species within the genus *Emcibacter*, for which the name *Emcibacter congregatus* sp. nov. is proposed.

Table 2. Cellular fatty acid composition (%) of strain ZYL^T and related species

Strains: 1, ZYL^T; 2, *Emcibacter nanhaiensis* MCCC 1A06723^T. All data are from this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not included. TR, trace (<1 %). Major components (≥10 %) are highlighted in bold.

Fatty acid	1	2
Straight chain:		
$C_{12:0}$	TR	2.0
$C_{14:0}$	**11.0**	7.5
$C_{16:0}$	9.0	6.5
Unsaturated:		
$C_{16:1}\omega5c$	1.2	TR
$C_{17:1}\omega6c$	5.8	4.6
$C_{19:0}$ cyclo $\omega8c$	1.1	4.6
Hydroxy:		
$C_{14:0}$ 2-OH	**17.1**	**21.4**
$C_{16:0}$ 3-OH	TR	1.6
Summed features*		
2	1.3	TR
3	**22.7**	8.2
8	**25.7**	**38.8**

*As indicated previously [19], summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately. Summed feature 2 was listed as iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH; summed feature 3 was listed as iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$; summed feature 8 was listed as $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

DESCRIPTION OF *EMCIBACTER CONGREGATUS* SP. NOV.

Emcibacter congregatus sp. nov. (con.gre.ga′tus. L. part. adj. *congregatus* clustered, always clustered when cultured in lipid medium).

Cells are Gram-negative, aerobic, rod-shaped, 0.4–0.6 µm in diameter and 1.4–2.6 µm in length, motile, and non-spore-forming. Colonies are pale yellow, circular, slightly convex with a shiny, smooth surface and entire edges. After prolonged incubation, the colonies become mucoid and irregular. Sticky filaments with a length of 1.0–4.0 µm between cells can be seen under transmission electron microscopy. The temperature range for growth is 4–30 °C (optimum, 25 °C). The pH range for growth is 6.0–8.5, and the optimum is pH 7.0. Growth occurs at NaCl concentrations below 7.0 % (w/v) (optimum, 2.0 %). Cells are positive for oxidase activity and weakly positive for catalase activity. Nitrate is reduced to nitrite, and H_2S is produced. Positive in Voges–Proskauer tests. Negative for Tween 20, Tween 40, Tween 60, Tween 80 and starch hydrolysis. With API 50CH strips, acid is produced from glycerol (weakly), D-arabinose (weakly), D-ribose (weakly), D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, *N*-acetylglucosamine, cellobiose, maltose, lactose (bovine origin), starch (weakly) and potassium 2-ketogluconate (weakly). In the API 20NE system, β-galactosidase activity is positive. Indole production, fermentation of glucose, hydrolysis of urea, hydrolysis of aesculin, hydrolysis of gelatin and arginine dihydrolase activity are negative. In API ZYM tests, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive. Esterase lipase (C8) is weakly positive. Lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase are negative. The predominant respiratory quinone is Q10. The major cellular fatty acids include $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8), iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$ (summed feature 3), $C_{14:0}$

2-OH, and $C_{14:0}$. The polar lipids include phosphatidylethanolamine, phosphatidylglycerol, three unidentified aminophospholipids (APL1–3), three unidentified phospholipids (PL1–3), one unidentified aminolipid (AL1) and four unidentified lipids (L1–4).

The type strain ZYL^T (=KCTC 62328^T=JCM 32378^T =MCCC 1K03526^T) was isolated from a cultured *in situ* sediment sample collected on the coast of the East China Sea. The genomic DNA G+C content is 51.5 %.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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