Emticicia oligotrophica gen. nov., sp. nov., a new member of the family 'Flexibacteraceae', phylum Bacteroidetes

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An aquatic bacterium, strain GPTSA100-15^T, was isolated on nutritionally poor medium TSBA100 (tryptic soy broth diluted 100 times and solidified with 1.5 % agarose) and characterized using a polyphasic approach. The isolate was unable to grow on commonly used nutritionally rich media such as tryptic soy agar, nutrient agar and Luria–Bertani agar. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolate was affiliated with the family 'Flexibacteraceae' in the phylum Bacteroidetes. Phylogenetically, it showed closest similarity (94.0 %) with an uncultured bacterial clone, HP1A92, detected in a sludge microbial community. Among the culturable bacteria, the isolate had highest 16S rRNA gene sequence similarity with *Leadbetterella byssophila* 4M15^T (87.8 %). Sequence similarities with other members of the phylum Bacteroidetes were less than 85 %. The fatty acid profile of the isolate grown on TSBA100 indicated that the major fatty acid was iso-C_{15:0}, which is also present in many members of the family 'Flexibacteraceae'. Cells of strain GPTSA100-15^T are Gram-negative, strictly aerobic rods. The DNA G + C content of the isolate is 36.9 mol%. Results of phenotypic, chemotaxonomic and phylogenetic analyses clearly indicate that strain GPTSA100-15^T represents a new genus within the family 'Flexibacteraceae'; the name *Emticicia* gen. nov. is proposed for the genus, with *Emticicia oligotrophica* sp. nov. as the type species. The type strain of *Emticicia oligotrophica* is GPTSA100-15^T (=MTCC 6937^T=DSM 17448^T).

Members of the phylum Bacteroidetes (Ludwig & Klenk, 2001; Garrity & Holt, 2001) are widely distributed in nature, particularly in aquatic systems (Glöckner *et al.*, 1999; Kirchman, 2002; Yoon *et al.*, 2005), and are known to play important roles in biogeochemical cycling and degradation of complex biopolymers like cellulose, chitin (Cottrell & Kirchman, 2000) and agar (Nedashkovskaya *et al.*, 2003). Recently, a member of this phylum associated with decay of a planktonic bloom, *Aquiflexum balticum*, was described (Brettar *et al.*, 2004). Over the past few years taxonomic reclassification of many taxa has been reported (Bernardet *et al.*, 1996; Bowman, 2000). In the second edition of *Bergey's Manual of Systematic Bacteriology*, the phylum Bacteroidetes includes three classes, 'Bacteroides', *Flavobacteria* and 'Sphingobacteria'. The class 'Sphingobacteria' includes five families, of which 'Flexibacteraceae' is the largest, represented by ten genera (Garrity & Holt, 2001). Since then, a few more

genera have been described. In this paper, taxonomic characterization is described of a novel bacterial strain belonging to the family 'Flexibacteraceae', GPTSA100-15^T, isolated from Assam, India.

Strain GPTSA100-15^T was isolated on TSBA100 medium (normal strength tryptic soy broth diluted 100 times and solidified with 1.5 %, w/v, agarose) by dilution plating and incubating the plates at 37 °C. Since the strain could not grow on commonly used nutritionally rich media, its ability to grow on media of different compositions was checked. Media used in this study are as follows (compositions expressed in %, w/v): TSBA (tryptic soy broth, 3; agar, 1.5), YEA (yeast extract, 0.5; CaCl₂, 0.1; agar, 1.5; pH 7.2), modified R2A (MR2A) agar (Casamino acids, 0.025; yeast extract, 0.05; peptone, 0.075; glucose, 0.05; soluble starch, 0.05; K₂HPO₄, 0.03; MgSO₄·7H₂O, 0.0024; sodium pyruvate, 0.03; agar, 1.5; pH 7.2), R3A agar, MHA (Mueller–Hinton agar), TSIA (triple-sugar iron agar), MAC (MacConkey agar), NA (nutrient agar), NB (nutrient broth), NA-NaCl (NA devoid of NaCl), CA (cytophaga agar), LY agar [1 mM phosphate buffer, pH 7.0; yeast extract, 0.01; (NH₄)₂SO₄, 0.1; MgSO₄·7H₂O, 0.05; CaCl₂·2H₂O, 0.005; standard trace elements; agar, 1.5], LBA (Luria–Bertani agar), ZMA (ZoBell marine agar) and

Abbreviation: CMC, carboxymethylcellulose.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GPTSA100-15^T is AY904352.

An extended phylogenetic tree based on 16S rRNA gene sequences of members of the phylum Bacteroidetes is available as supplementary material in IJSEM Online.

PCA (plate count agar). Media were made nutritionally poor by diluting these media with distilled water and solidifying them with 1.5 % agarose. Dilutions are indicated by the abbreviated name of the medium followed by 100. For acid production, modified MR2A broth (MR2A without starch, glucose and sodium pyruvate) supplemented with 0.5 % of the desired carbon source and phenol red (as pH indicator) was used; for methyl red/Voges-Proskauer tests, the same basal medium as above was used, supplemented with 0.5 % glucose. Sole carbon source utilization medium (pH 7.2) contained the following, supplemented with 1 % carbon source (%): $(\text{NH}_4)_2\text{SO}_4$, 0.2; K_2HPO_4 , 0.024; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.024; KCl, 0.01; yeast extract, 0.01; agarose, 1.5.

Tests for phenotypic properties were carried out by standard methods (Smibert & Krieg, 1994; Murray *et al.*, 1994; Powers, 1995; Cowan & Steel, 1965). Growth at different temperatures, pH and NaCl concentrations was checked on basal TSBA100 medium. Growth on various media (TSBA, CA, LY agar, YEA, R2A agar, R3A agar, MR2A agar, MHA, TSA, NA, NA-NaCl, ZMA, PCA, NA100, LBA100, ZMA100, MAC100, MHA100 and TSI100) was also checked at 37 °C. Determination of gliding motility was made essentially according to Bowman (2000) but on MR2A agar. Flexirubin pigment production was determined by exposing bacterial cell mass to 20 % (w/v) KOH solution according to Reichenbach (1989). All biochemical tests were done at 37 °C. Phenotypic properties of the strain are given in the genus and species descriptions. Hydrolysis of cellulose (filter paper) and chitin was tested by overlaying the respective substrates on MR2A agar solidified with 1.2 % agar. Hydrolysis of carboxymethylcellulose (CMC) was checked on modified MR2A agar supplemented with 0.5 % (w/v) CMC (Sigma) for up to 7 days. Plates were flooded with 0.2 % aqueous Congo red dye solution followed by removal of extra dye and flooding the plate with 1 M NaCl solution. A clear zone around bacterial growth indicated positive hydrolysis. For determining hydrolysis of various other substrates, MR2A agar supplemented with substrates at concentrations normally recommended in standard methods (Smibert & Krieg, 1994; Cowan & Steel, 1965) was used. Sensitivity to antibiotics was tested on MR2A agar using antibiotic susceptibility discs (Hi-Media) of ampicillin (10 µg), bacitracin (8 U), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (2 µg), neomycin (30 µg), novobiocin (30 µg), norfloxacin (10 µg), penicillin G (10 U), polymyxin B (300 U), rifampicin (2 µg), streptomycin (10 µg), sulfasomidine (300 µg) and tetracycline (3 µg).

For cellular fatty acid analysis, the isolate was grown on TSBA100 medium at 30 °C for 48 h. Extraction and analysis of cellular fatty acids were done according to procedures of the SHERLOCK Microbial Identification system (MIDI), as described previously (Pandey *et al.*, 2002). Major fatty acids were iso- $\text{C}_{15:0}$ (42.9 %), iso- $\text{C}_{17:0}$ 3-OH

(12.5 %), anteiso- $\text{C}_{15:0}$ (12.2 %), summed feature 3 (iso- $\text{C}_{15:0}$ 2-OH and/or $\text{C}_{16:1}\omega 7c$, 11.3 %), iso- $\text{C}_{15:0}$ 3-OH (5.6 %), $\text{C}_{15:1}\omega 6c$ (5.5 %) and $\text{C}_{16:1}\omega 5c$ (3.8 %). The presence of iso- $\text{C}_{15:0}$ fatty acid has been reported for many members of the family 'Flexibacteraceae'. Genomic DNA of GPTSA100-15^T was isolated according to Marmur (Johnson, 1994). The G + C content of genomic DNA was determined spectrophotometrically as described previously (Saha *et al.*, 2005). Genomic G + C content of strain GPTSA100-15^T was 36.9 mol%. Amplification of the 16S rRNA gene from the isolate was done using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Amplification, purification of the amplicon and sequencing of the 16S rRNA gene were done as described by Pandey *et al.* (2002). A nearly complete (1385 nt continuous stretch) 16S rRNA gene sequence of the isolate was used to search for similar sequences from GenBank and the RDP. Various online sequence analysis tools available from RDP release 9.1 (<http://rdp.cme.msu.edu/index.jsp>, RDP analysis tools) were also used to get an idea of the systematic position of the strain. Sequence analysis revealed that GPTSA100-15^T is a member of the phylum *Bacteroidetes* showing closest similarity (94.0 %) with an uncultured bacterial clone, HP1A92, which was a representative sequence of a sludge microbial community (McMahon *et al.*, 2002). Among the culturable bacteria, it showed closest similarity to *Leadbetterella byssophila* 4M15^T (87.8 %). Sequence similarities with other members of the phylum *Bacteroidetes* were less than 85 %. Since 16S rRNA gene sequence similarity of 95 % or less is generally regarded as a borderline for describing a new genus (Ludwig *et al.*, 1998), low sequence similarity (87.8 % and less) with cultured bacterial relatives clearly indicated that GPTSA100-15^T should be treated as a representative of a new genus. For determination of the phylogenetic position of GPTSA100-15^T, the sequence of its closest uncultured bacterial relative as well as 73 strains from different representative families within the phylum *Bacteroidetes* were retrieved (RDP analysis tools Hierarchy Browser; NCBI taxonomy browser), aligned by the program CLUSTAL_X (Thompson *et al.*, 1997) and edited manually. Aligned sequences were analysed by the TREECON software package (Van de Peer & De Wachter, 1997). Neighbour-joining (NJ) analysis (Saitou & Nei, 1987) with the correction of Jukes & Cantor (1969) was carried out with *Bacillus subtilis* as an outgroup. The stability of the tree was estimated by bootstrap analysis of 1000 replications. The analyses indicated phylogenetic positioning of the strain in the family 'Flexibacteraceae' (see Supplementary Fig. S1 in IJSEM Online). Using the parameters and software mentioned above and 16S rRNA gene sequences from 22 representative culturable genera of the family 'Flexibacteraceae' and the sequence of uncultured bacterial clone HP1A92, an NJ tree was constructed to reveal the position of strain GPTSA100-15^T within this family. A phylogenetic tree was also constructed by the maximum-parsimony method using DNAPARS from the PHYLIP package (Felsenstein, 1993). Irrespective of the tree-generating

method used, strain GPTSA100-15^T always formed a clade with the uncultured bacterial clone HP1A92 that was separate from the *Leadbetterella* clade within the family 'Flexibacteraceae' with a very high bootstrap value (Fig. 1). Strain GPTSA100-15^T could also be differentiated from its closest relatives on the basis of phenotypic properties and G + C content (Table 1). Moreover, the similarity of strain GPTSA100-15^T with its closest cultured bacterial relatives at the 16S rRNA gene sequence level was low enough (87.8% and less) to justify it as a member of a new genus. Thus, on the basis of polyphasic characterization, it is concluded that GPTSA100-15^T represents a novel species in a new genus for which the name *Emticicia oligotrophica* gen. nov., sp. nov. is proposed.

Description of *Emticicia* gen. nov.

Emticicia (Em.ti.ci'cia. N.L. fem. n. *Emticicia* arbitrarily formed from the acronym MTCC for Microbial Type Culture Collection and Gene Bank, where this investigation was carried out).

Gram-negative, strictly aerobic, non-motile rods occurring mostly singly, sometimes in pairs. Positive for oxidase and nitrate reduction; weakly positive for catalase. Major whole-cell fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH, anteiso-C_{15:0}, summed feature 3 (iso-C₁₅: 2-OH and/or C_{16:1}ω7c), iso-C_{15:0} 3-OH and C_{15:1}ω6c. On the basis of 16S rRNA gene

sequence analysis, the genus is affiliated to the family 'Flexibacteraceae' in the phylum *Bacteroidetes*. The type species is *Emticicia oligotrophica*.

Description of *Emticicia oligotrophica* sp. nov.

Emticicia oligotrophica (o.li.go.tro'phi.ca. Gr. adj. *oligos* few; Gr. adj. *trophikos* nursing, tending or feeding; N.L. fem. adj. *oligotrophica* eating little, referring to a bacterium living on low-nutrient media).

In addition to properties described for the genus, the species has the following characteristics. Colonies on TSBA100 medium after 2 days growth at 37 °C appear round, mucoid, light pink and convex with almost entire margins; with prolonged incubation, the colony colour deepens, it produces slime and appears glistening. Cells are usually 2–5 µm long and 0.3–0.4 µm wide; in an aged culture, some cells may be longer. No gliding motility observed. No flexirubin pigment detected. Grows at 15–42 °C and pH 5.0–11.0; cannot grow in 1% NaCl. Positive for methyl red, but negative for Voges–Proskauer test. Cannot grow on most of the common nutritionally rich culture media (e.g. TSBA, NA, LBA, PCA, MHA, TSIA and ZMA), but grows on R2A, R3A and 100-fold dilutions of all these media. It can hydrolyse gelatin, starch, Tween 60 and urea, but not casein, ONPG, hypoxanthine, chitin, cellulose (CMC or filter paper), xylan, tyrosine or Tween 20, 40

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the relative position of GPTSA100-15^T among various representative members of the family 'Flexibacteraceae'. Bootstrap values greater than 50% (percentages of 1000 replications) are shown at nodes. Bar, 0.1 substitutions per site. The sequence from *Bacillus subtilis* subsp. *subtilis* DSM 10^T was used as an outgroup.

Table 1. Differential characteristics of GPTSA100-15^T and some of its close phylogenetic relatives

Taxa: 1, strain GPTSA100-15^T; 2, *Leadbetterella byssophila* (data from Weon *et al.*, 2005); 3, *Runella slithyformis* (Larkin & Borral, 1984; Chelius & Triplett, 2000); 4, *Runella zeae* (Chelius & Triplett, 2000; Chelius *et al.*, 2002); 5, *Dyadobacter fermentans* (Chelius & Triplett, 2000; Chaturvedi *et al.*, 2005); 6, *Dyadobacter crusticola* (Reddy & Garcia-Pichel, 2005; Chaturvedi *et al.*, 2005); 7, *Dyadobacter hamtensis* (Chaturvedi *et al.*, 2005). +, Positive; w+, weakly positive; –, negative; v, variable; SL, slight; ND, data not available; NM, not mentioned.

Characteristic	1	2	3	4	5	6	7
Colony colour*	LP	O	S	S	Y	Y	LY
Flexirubin	–	+	–	–	+	+	+
Growth on TSA	–	+	–	ND	ND	+	ND
pH range for growth	5.0–11.0	6.0–8.0	ND	ND	ND	6.0–8.0	6.0–8.0
Nitrate reduction to nitrite	+	–	–	–	–	–	–
Methyl red test	w+	ND	–	ND	ND	–	–
NaCl tolerance (% w/v)	<1	1 but not 3	<1.5	<1.5	1.5	1	11.6
Amylase	+	+	SL	–	–	–	–
Gelatinase	+	+	–	–	–	–	–
Urease	+	–	–	ND	ND	–	–
Degradation of tyrosine	–	+	ND	ND	ND	ND	ND
Acid production from:							
Sucrose	w+	ND	+	+	+	–	–
Ribose	–	ND	–	–	+	–	–
Galactose	w+	+ / w+	v	ND	ND	–	–
Fructose	–	ND	–	ND	–	+	–
Maltose	w+	+ / w+	+	ND	+	–	–
Isolation source†	WS	CWC	FW	SSS	SSS	BSC	GW
DNA G+C content (mol%)	36.9	33	49	49	48	48	49
Presence of anteiso-C _{15:0} (%)	+ (12.2)	+ (0.4)	+ (4.3)	+ (4.0)	NM	NM	NM

*LP, Light pink; LY, light yellow; O, orange; S, salmon; Y, yellow.

†BSC, Biological soil crust sample; CWC, cotton waste compost; FW, freshwater; GW, glacial water; SSS, surface-sterilized stem of *Zea mays*; WS, warm spring water.

or 80. Can utilize D-cellobiose, D-lactose, D-melezitose, D-raffinose and arbutin; weakly utilizes L-arabinose, L-arabitol, D-amygdalin, D-fructose, D-galactose, D-glucose, glycerol, *myo*-inositol, D-melibiose and sucrose as sole carbon sources. Cannot utilize adonitol, dulcitol, D-mannitol, D-sorbitol, L-sorbose or xylitol as sole carbon sources. Produces acids from arbutin, D-cellobiose, D-glucose, D-lactose, methyl α -D-glucoside, salicin, D-trehalose and D-xylose and weakly from D-galactose, D-maltose, D-mannose, D-melibiose, D-melezitose and sucrose, but not from adonitol, D-amygdalin, L-arabinose, L-arabitol, dulcitol, aesculin, erythritol, D- or L-fucose, D-fructose, glycerol, glycogen, *myo*-inositol, inulin, 2-ketogluconate, D-mannitol, D-raffinose, D-ribose, D-sorbitol, L-sorbose or xylitol. Sensitive to ampicillin, bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, lincomycin, neomycin, novobiocin, norfloxacin, penicillin G, polymyxin B, rifampicin, streptomycin, sulfasomidine and tetracycline. The genomic DNA G+C content of the type strain is 36.9 mol%.

The type strain is GPTSA100-15^T (= MTCC 6937^T = DSM 17448^T), isolated from a warm spring water sample from Assam, India.

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