Environmental Microbiology (2004) **6**(6), 611–621 doi:10.1111/j.1462-2920.2004.00614.x

Lentisphaera araneosa gen. nov., sp. nov, a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, *Lentisphaerae*

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Summary

Two phylogenetically distinct marine strains producing transparent exopolymers (TEP), designated HTCC2155^T and HTCC2160, were cultivated from Oregon coast seawater by dilution to extinction in a high throughput culturing format. When cultured in low-nutrient seawater media, these strains copiously produced Alcian Blue-stainable viscous TEP. Growing cells were attached to each other by the TEP in a three dimensional network. Polymerase chain reaction employing 16S rDNA primers specific for the novel isolates indicated that they are indigenous to the water column of the Atlantic and Pacific oceans. The abundance of the isolates as determined by 16S rRNA dot blots, however, indicated that they are less than 1% of the total bacterial community. In phylogenetic analyses, the strains consistently formed a new phylum-level lineage within the domain *Bacteria*, together with members of the candidate phylum VadinBE97, which consists of *Victivallis,* the first cultured genus in the candidate phylum, and 16S rRNA gene clones from DNA extracted from marine or anaerobic terrestrial habitats. Five putative subgroups were delineated within this phylum-level lineage, including a marine group and an anaerobic group. The isolates are Gram negative, strictly aerobic, chemoheterotrophic, and facultatively oligotrophic sphere-shaped bacteria. The DNA G+C content of strain HTCC2155^T was 48.3 mol% and the genome size was 2.9 mb. It is proposed from these observations that the strains be placed into a new genus and a new species named *Lentisphaera araneosa* (type strain HTCC2155^T = ATCC BAA-859^T = KCTC 12141^T) gen. nov., sp. nov., the cultured marine representative of the *Lentisphaerae* phyl. nov., and the phylum be divided into two novel orders named the *Lentisphaerales* ord. nov. and the *Victivallales* ord. nov.

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Introduction

Molecular ecological and phylogenetic analyses of 16S rRNAs, both with and without cultivation, have significantly expanded our view of microbial diversity. The number of major groups (lineages or phyla) within the domain *Bacteria* (Woese, 1987) increased from the 11 groups Carl Woese delineated in 1987 to 36 identifiable bacterial phyla in 1998 (Hugenholtz *et al*., 1998a). Today, the domain *Bacteria* is composed of approximately 52 recognized phyla, including 26 candidate phyla which have exclusively 16S rRNA gene clone sequences obtained from environments without cultivation (Rappé and Giovannoni, 2003). Whereas the number of candidate phyla increased from 14 in 1998 to 26 in 2003, only two phylum-level lineages, the phyla *Gemmatimonadetes* (Zhang *et al*., 2003) and *Caldithrix* (Miroshnichenko *et al*., 2003), have had representatives cultured during this period.

We recently isolated several novel bacterial strains (Rappé *et al*., 2002; Cho and Giovannoni, 2003a, b, c; 2004) from the coast of Oregon and the Sargasso Sea using high throughput culturing (HTC) approaches (Connon and Giovannoni, 2002) that are based on the concept of dilution-to-extinction in very low nutrient media. This study is the first description of the isolation, phylogeny, and ecophysiology of strains HTCC2155^T and HTCC2160 ('HTCC′ stands for the high throughput culture collection that is being maintained at Oregon State University, USA), which are shown by phylogenetic analysis to belong to the candidate phylum VadinBE97. The nomenclature candidate phylum VadinBE97 (the VadinBE97 clade) did not appear in the literature until Rappé and Giovannoni (2003) included it in their list of 26 candidate phyla. Although they did not discuss the VadinBE97 clade, they delineated the clade as a candidate phylum by comprehensive phylogenetic analyses. In their analyses, the phylum was phylogenetically located most closely to the phylum *Verrucomicrobia*. Here we show that the candidate phylum VadinBE97 now includes at least 11 full-length (>1300 nt) sequences obtained from environments

without cultivation, two undescribed isolates from marine worms, *Victivallis vadensis*, a recently described isolate from human faeces (Zoetendal *et al*., 2003), and two HTCC strains isolated from the Pacific Ocean.

The two HTCC strains, interestingly, produced transparent exopolymers (TEP) (Alldredge *et al*., 1993; reviewed by Passow, 2002), which are polymeric gel particles that form spontaneously from dissolved polysaccharides or colloidal organic matter (Chin *et al*., 1998; Engel and Passow, 2001). Marine TEP is an essential component of marine snow, a term used to describe the flocculent organic matter that commonly forms in the marine water column. Marine snow plays an important role in the recycling of organic carbon particles and the movement of organic matter from the euphotic zone to the deep ocean (Alldredge *et al*., 1993; Passow *et al*., 1994; Logan *et al*., 1995). It has been thought that marine TEP forms from DOM excreted by phytoplankton (Passow and Alldredge, 1994; Passow *et al*., 2001), mainly diatoms. This is the first report, to our knowledge, of a TEP originating from a marine bacterium cultivated with oligotrophic conditions. We describe two HTCC strains as TEP-producing novel marine bacteria and propose their inclusion in a new genus and a species named *Lentisphaera araneosa* gen. nov., sp. nov. within the *Lentisphaerae* phyl. nov.

Results

Isolation and morphology

Strains HTCC2155^T and HTCC2160 were isolated by HTC using a oligotrophic seawater medium amended with seven different carbon compounds (0.001%–0.002% final concentration of each), and selected from among 33 HTCC isolates obtained after screening 480 extinction wells. Sequences of the nearly full-length 16S rRNA gene for the two strains were identical. Therefore, phenotypic and genotypic characterizations were performed only using the type strain HTCC2155^T. Strain HTCC2155^T was a Gram-negative, non-motile sphere-form bacterium, 0.6–1.1 (average 0.8) μm in diameter, dividing by binary fission (Fig. 1A and C). Neither flagellation, endospore formation, nor poly β-hydroxybutyrate granules were observed. A faint layer of extracellular slime was seen around the cells (Fig. 1B), and buds or appendages were sometimes detected around the cells (Fig. 1C and D). A mucoid phenotype was not observed in the colonies.

Phylogenetic analysis

Nearly complete 16S rRNA gene sequences (1478 nt) were determined for strains HTCC2155^T and HTCC2160 and only unambiguously aligned positions were used for phylogenetic analyses. The most closely related cultured microorganisms to the strains were *Victivallis vadensis* (84.4% similarity), an anaerobic bacterium that was recently isolated from a human faecal sample (Zoetendal *et al*., 2003), and two marine worm isolates Dex80–43 (85.7%, AJ431234, GenBank description) and Dex80–64 (85.8%, AJ431235, GenBank description) which are as yet undescribed. Several 16S rRNA gene clones retrieved from anaerobic digesters, rumen, termite guts, seawater, and marine sediments without cultivation – all from the recently proposed candidate phylum VadinBE97 – were also related to the HTCC strains (82–86% sequence similarity; Fig. 2). In phylogenetic trees based on maximum likelihood, maximum parsimony, and evolutionary distance, the HTCC strains formed a clade together with *Victivallis vadensis* and sequences of the candidate phylum VadinBE97 obtained from environments without cultivation. This clade was strongly supported by high bootstrap values (100% in a neighbour-joining tree and 99% in a maximum parsimony tree) and clearly separated from the nearest phylum, *Verrucomicrobia*. Members of this clade shared only 72.9–82.1% 16S rRNA gene sequence similarity to members of adjacent phyla (*Verrucomicrobia*, *Chlamydiae*, *Planctomycetes* and candidate division OP3), and less than 80% sequence similarity to members of the other major bacterial phyla. Hugenholtz *et al*. (1998b) suggested that 85% similarity be used as a cutoff for distinguishing new phyla; thus, by this criterion together with phylogenetic position, this clade was considered to be a novel phylum-level lineage in the domain *Bacteria* (Fig. 2).

Cellular fatty acids, DNA base composition and genome size

A total of 14 different kinds of fatty acids with two fatty acid mixtures, containing 10–28 carbon atoms, were observed in strain HTCC2155^T (Table 1). The most abundant fatty acids were *cis*-9-hexadecenoic acid (16 : 1ω9c, 50.8%) and *n*-tetradecanoic acid (14 : 0, 25.9%). The DNA G+C content of the strain HTCC2155^T was 48.3 ± 0.6 mol%. Based on pulsed-field gel electrophoresis (PFGE) of digested or linearized chromosomes genome size was estimated to be 2.9 ± 0.6 mb.

Phenotypic characteristics

Strain HTCC2155^T is an obligately aerobic, NaCl-requiring, marine chemoheterotroph. The strain grew under neither strictly anaerobic conditions nor microaerobic conditions, even with prolonged incubations for 50 days at 16°C. The temperature range for growth was 4–25°C, with optimum growth at 16–20°C. The pH range for growth was pH 7.0–9.0, with optimum growth at pH 8.0. The strain was moderately halophilic, showing good

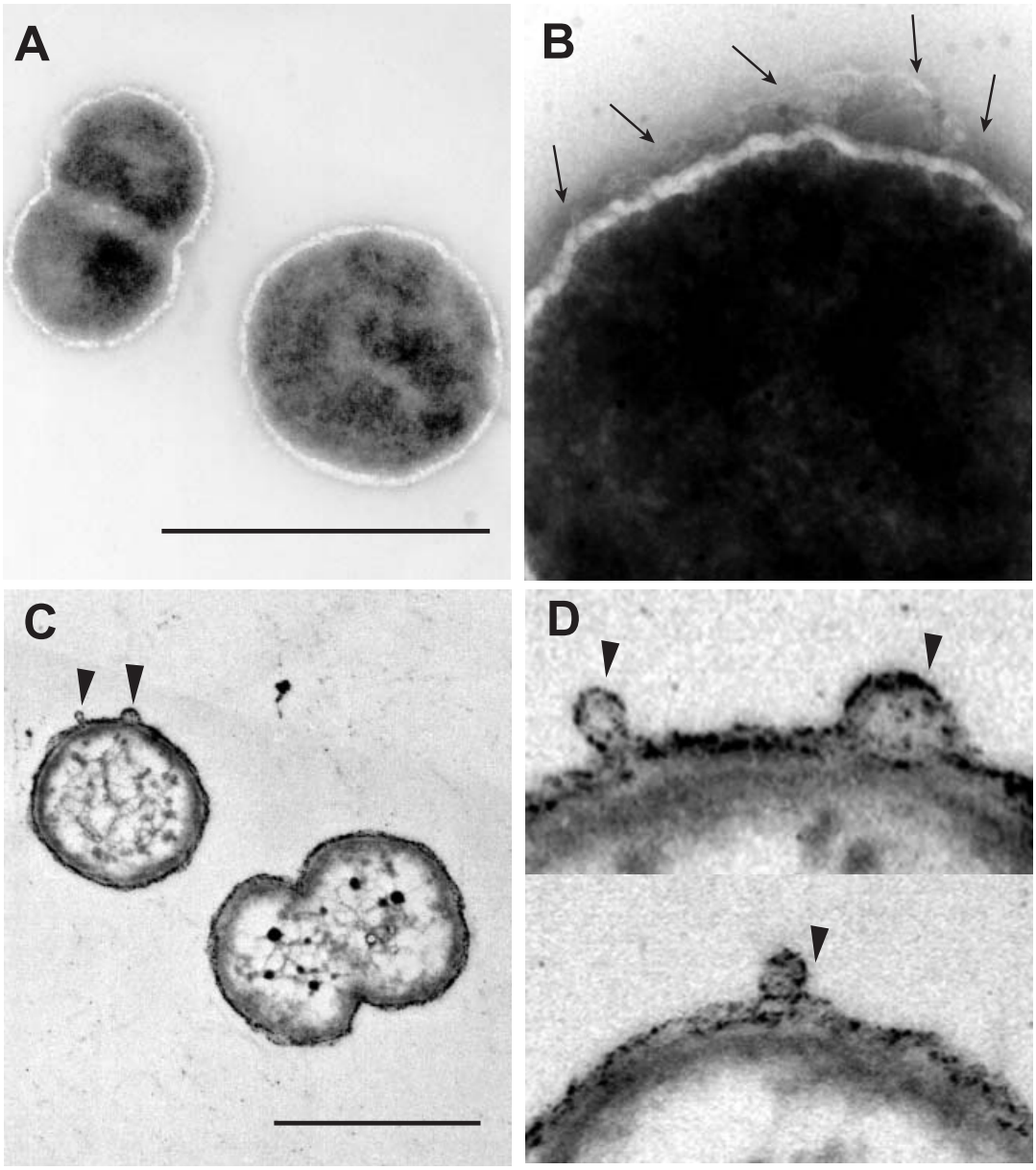

Fig. 1. Negatively stained (A and B) and ultra thin-sectioned (C and D) transmission electron micrographs of cells of stain HTCC2155^T. Arrows in panel B indicate 'capsular' EPS around the cells. Triangles in panels C and D indicate buds or appendages around the cells. Scale bars; 1 μm.

growth at NaCl concentrations of 0.75–15% (w/v), and optimal growth at 3.0% (w/v) NaCl. Neither carotenoid pigments nor bacteriochlorophyll peaks were detected in acetone-extracted cell lysates. The major biochemical properties, sole carbon utilization patterns and susceptibility to antibiotics are described in Table 1. The strain utilized a limited range of substrates (concentration of each compound; 0.02%) as sole carbon sources, including hexoses, disaccharides, sugar alcohols, and sugar acids (custom 48-well plate tests). No amino acids were utilized by the strain as sole carbon sources.

Growth characteristics and production of transparent exopolymers (TEP)

During batch growth of strain HTCC2155^T, viscosity of the growth media was first observed at the end of exponential growth phase and reached a maximum in stationary phase (Fig. 3). The maximum cell densities and viscosity in ASW plus 10× MC medium were much higher than in LNHM plus 1× MC medium (Fig. 3) (*see Experimental procedures* for media abbreviations, DOC concentration and composition in detail). The specific growth rates, vis-

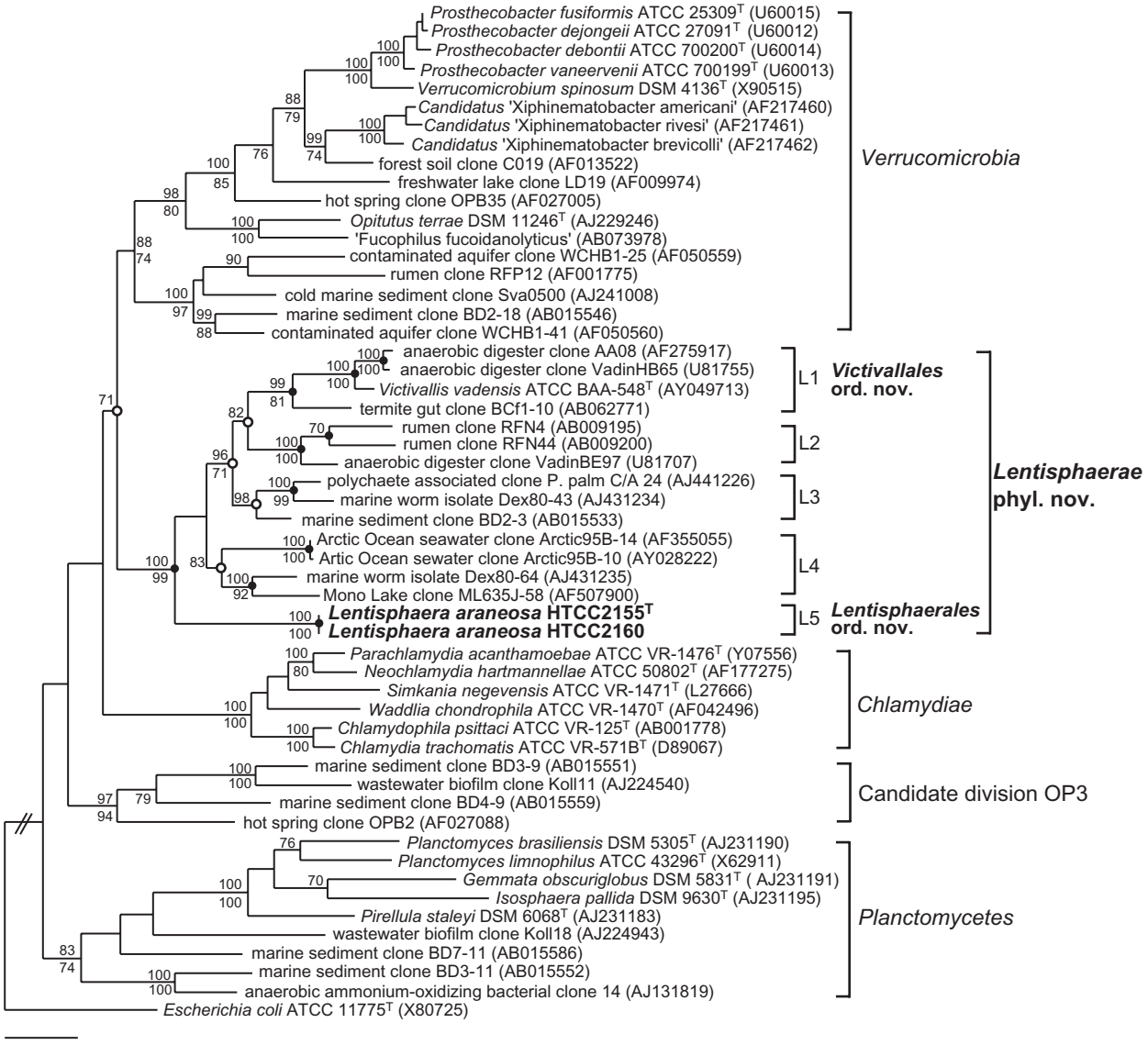

Fig. 2. Maximum likelihood 16S rRNA phylogenetic tree showing the phylogenetic positions of the genus *Lentisphaera*, the orders *Lentisphaerales* and *Victivallales*, and the phylum *Lentisphaerae* within the other adjacent phyla. Bootstrap proportions over 70% from both neighbour-joining (above nodes) and maximum parsimony (below nodes) are shown. The closed circles and open circles at each node in the *Lentisphaerae* indicate recovered nodes in all three treeing methods and recovered nodes in two treeing methods respectively. The letter 'L' of subgroups L1 to L5 represents the initial letter of the phylum *Lentisphaerae*. *Escherichia coli* ATCC 11775^T was used as an outgroup to define the root of the tree. Scale bar, 0.1 substitutions per nucleotide position.

cosities, and cell densities in the series of ASW basal media generally increased proportionally with increasing carbon concentrations up to their maxima, whereas relatively high concentrations of DOC blocked TEP production and inhibited the cellular growth (Fig. 4). In the series of LNHM basal media, the highest viscosity (1.14 mm^2 s^{-1}) was observed in LNHM plus 0.75× MC medium (*ca.* 27.3 mg l^{-1} of DOC), and the maximum cell density in this medium was 8.4×10^6 cells ml^{-1}. Although maximum cell density (3.9×10^7 cells ml^{-1}) was highest in LNHM plus 5 × MC medium, interestingly the strain did not produce TEP in this medium. Similarly, the strain did not produce TEP in 1/10 R2A medium in spite of a maximum cell density of 7.6×10^6 cells ml^{-1}. These results suggest that TEP production by strain HTCC2155^T is inhibited by high DOC concentrations and depends on the phase of growth in batch culture.

The TEP produced by strain HTCC2155^T stained bright to deep blue with Alcian Blue, which stains negatively charged polysaccharides. Staining revealed that the cells were interconnected by the TEP, which had three dimensional web-like morphology (Fig. 5). The effect of desic-

Table 1. Major phenotypic characteristics of *Lentisphaera araneosa* HTCC2155^T.

Characteristics	Results
Biochemical properties	
PNPG (β-galactosidase)	+
Catalase, oxidase, denitrification, indole production, glucose acidification, arginine dihydrolase, urease, hydrolysis of esculin and gelatin	–
Sole carbon source utilization (0.02% of each carbon compound)	
D-glucose, D-galactose, D-fructose, β-lactose, D-trehalose D-cellobiose, D-maltose, D-mannose, D-glucosamine, D-mannitol, D-sorbitol, pyruvic acid, succinic acid, D-malic acid	+
D,L-glyceraldehyde, D-ribose, D-arabinose, D-xylose, L-rhamnose, L-sorbose, sucrose, D-melibiose, D-raffinose, D-melezitose, *m*-inositol, adonitol, methanol, ethanol, glycerol, *N*-acetyl-D-glucosamine, itaconic acid, gluconic acid, malonic acid, formic acid, propionic acid, lactic acid, L-glutamate, L-ornithine, L-proline, L-lysine, L-alanine, L-serine, L-leucine, L-isoleucine, glycine, L-arginine	–
Susceptibility to antibiotics (μg ml^{-1})	
Chloramphenicol (25), carbenicillin (25), ampicillin (10), erythromycin (25), benzylpenicillin (100 U ml^{-1})	+
Nalidixic acid (25), kanamycin (25), tetracycline (25), streptomycin (10), puromycin (25), vancomycin (25), rifampicin (25), gentamicin (25), cycloheximide (25)	–
Fatty acid composition (FAME analyses)	% of total
10 : 0	0.09
12 : 0	3.29
12 : 0 3-OH	3.07
14 : 0	25.93
15 : 1ω8c	0.44
15 : 0	0.53
16 : 1ω9c	50.83
16 : 1ω5c	0.57
16 : 0	6.00
16 : 0 3-OH	0.35
18 : 1ω9c	0.38
18 : 0	0.26
14 : 0 3-OH + 16 : 1 Iso I	6.46
15 : 0 Iso 2-OH + 16 : 1ω7c	1.18

cation during slide preparation on TEP morphology is unknown; however, it is clear that EPS of strain HTCC2155^T is different from typical cell capsules. The electron micrographs (Fig. 1B) and Alcian-Blue staining (Fig. 5) indicate that strain HTCC2155^T produces TEP as both integral ('capsular' or 'envelope', closely attached to the cell surface) and peripheral ('free' or 'slime', diffusible polymeric substances in solution) forms of EPS (Wrangstadh *et al*., 1990). The sticky cell-capsular EPS complex recovered by centrifuging at 20 000 r.p.m. had a hard, gel-like, consistency, and the viscous slime EPS in the supernatants formed white granules when precipitated by the addition of ethanol. After 20 days of incubation, the amount of ethanol-precipitated EPS was 0.6 g l^{-1} and 1.6 g l^{-1}, in LNHM plus 1 × MC and ASW plus 10 × MC respectively. The sugars rhamnose (62.2%), galactose (14.2%), mannose (12.2%), and glucose (11.4%) were identified by GC/MS analysis as the major components of this EPS.

Abundance of Lentisphaera araneosa *in seawater*

Bulk nucleic acid hybridization values from Oregon coast and oligotrophic Atlantic seawater samples indicate that *Lentisphaera araneosa* is a common marine bacterium which occurs in relatively low numbers, accounting for less than 1% of total bacterial 16S rRNA. The proportion of *Lentisphaera araneosa* rRNA to total bacterial rRNA reached a maximum of 0.32% (NH127, 500 m) in the Pacific Ocean (Fig. 6A), with significantly lower values in the Atlantic Ocean (Fig. 6B). *Lentisphaera araneosa* was also detected in both Pacific and Atlantic samples by PCR employing HTCC2155^T-specific primer sets (data not shown).

Discussion

The most remarkable ecophysiological feature of strain HTCC2155^T is TEP production in oligotrophic conditions. Many bacterial species have been shown to produce copious amounts of exopolymers (reviewed by Decho, 1990; Costerton, 1999), especially when they are attached to particles or solid surfaces. Generally, exopolymer productions have been observed in cultures adapted to laboratory conditions, such as high-nutrient growth medium and artificially designed support material for biofilm formation. In contrast, the highest rate of TEP production of strain HTCC2155^T was found in relatively low DOC-amended LNHM whereas high levels of DOC inhibited TEP production as well as cellular growth (Fig. 4). The LNHM media used in this study (no MC to 1× MC) contained dissolved

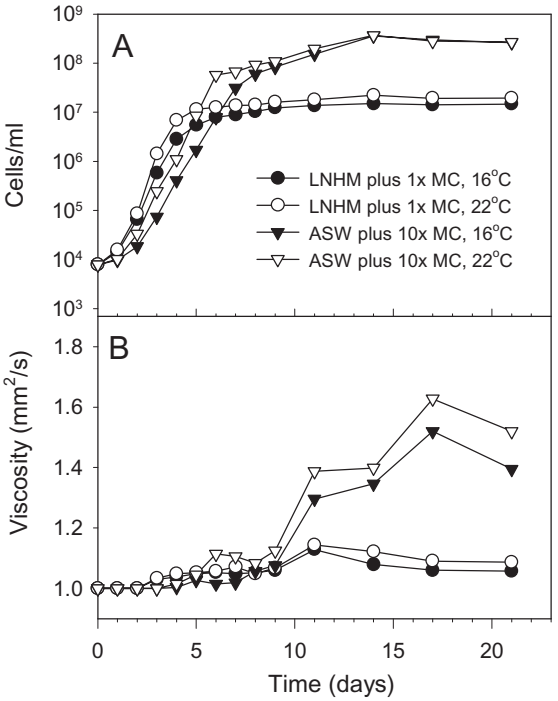

Fig. 3. Cell densities (A) and viscosity of medium (B) during growth of strain HTCC2155^T at two different temperatures and growth media. For abbreviations of media in detail, see *Experimental procedures*.

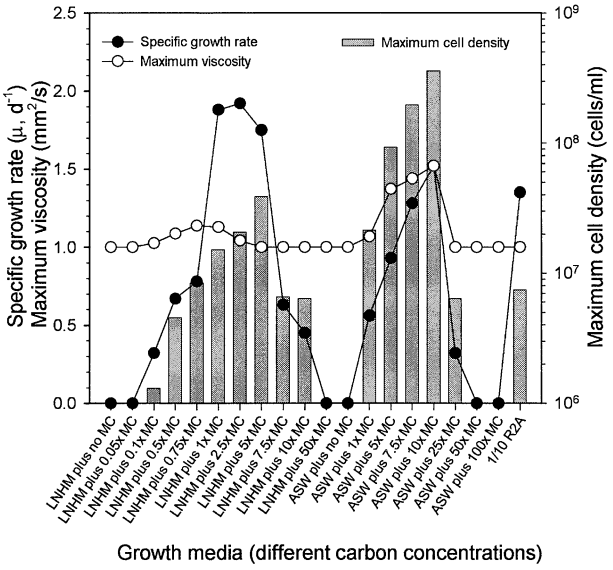

Fig. 4. Specific growth rates, maximum cell densities, and maximum viscosity of strain HTCC2155^T during growth at different level of DOC-containing growth media. For abbreviations of media in detail, see *Experimental procedures*.

organic carbon at concentrations that ranged from *in situ* levels to slightly higher concentrations, but were two to three orders of magnitude less than in commonly used laboratory media (Connon and Giovannoni, 2002).

Most EPS excreted by microorganisms have been known to exist as cell surface-bound capsular EPS rather than freely floating slime EPS (Cowen, 1992; Heissenberger *et al*., 1996). Although the production of EPS, thought to be the main precursors of TEP formation in aquatic environments, is mainly associated with phytoplankton (Passow *et al*., 2001), capsular EPS of bacterial origin also has been observed by transmission electron microscopy in marine snow and seawater samples (Cowen, 1992; Heissenberger *et al*., 1996). In addition, capsular EPS were found to comprise a significant portion of the marine DOM pool (Stoderegger and Herndl, 1998). The cultivation of strain HTCC2155^T from Pacific seawater and its slime EPS production in oligotrophic growth conditions provide tantalizing evidence about TEP. The new species we describe is potentially a source of marine TEP and may play a role in DOC production and recycling in oligotrophic marine environments. The unusual morphology of the EPS produced by strain HTCC2155^T suggests that it may produce TEP as an adaptive strategy for particle trapping. However, further study will be required to define its ecological role. Although the exact number of cells of *Lentisphaera araneosa* present in seawater is unknown, and the abundance of the species appears to be low, their occurrence in different oceans, and in coastal and pelagic seawater samples, suggests that this bacterium is widely distributed. The specific detection of intact cells of this species, combined with Alcian Blue staining in a variety of diverse marine environments, including marine snow sediments and the seawater column, might help elucidate their ecological role.

Here we designate the candidate phylum VadinBE97 the phylum *Lentisphaerae* phyl. nov., named after a novel genus and a species *Lentisphaera araneosa* gen. nov.,

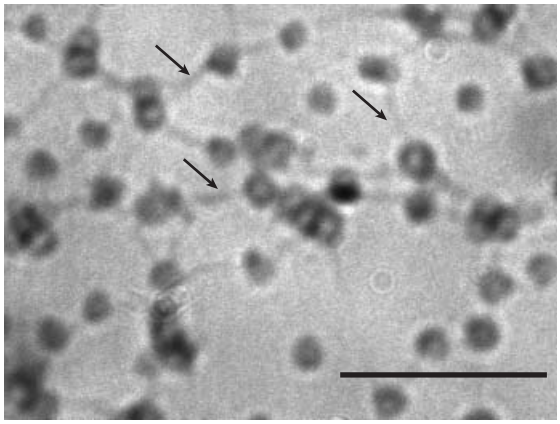

Fig. 5. Alcian Blue-stained transparent exopolymer particles (TEP) produced by strain HTCC2155^T. Arrows indicate 'slime' extracellular polysaccharides (EPS). Microscopic images of TEP were taken using a CCD camera and imaging software at 1000× magnification under light microscopy. Scale bar, 5 μm.

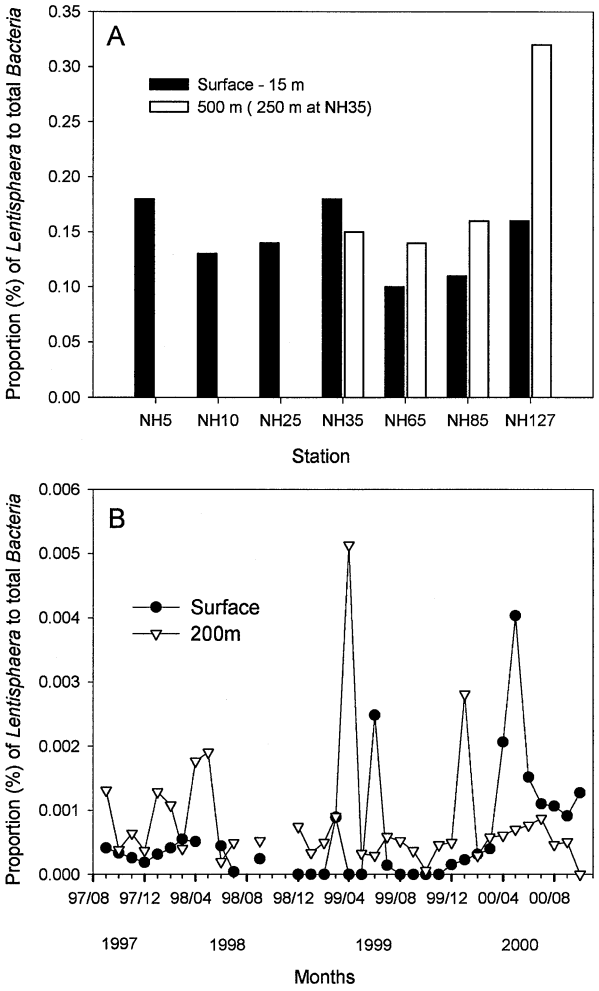

Fig. 6. The distribution of *Lentisphaera araneosa* in the Pacific Ocean (A) and the Atlantic Ocean (B) evaluated by 16S rRNA dot blot hybridization.
A. Pacific Ocean samples collected from surface (15 m) and deep (250 or 500 m) seawater along the Newport Hydroline stations in May 2002.
B. Monthly time-series samples from the BATS site for surface and 200 m depths over a 3- year period.

sp. nov. (see description; type strain = HTCC2155^T), a representative marine isolate in this phylum. In comparative sequence analyses, the phylum *Lentisphaerae* appeared to comprise uncultured environmental clone sequences or isolates retrieved from mainly marine habitats and animal (or human)-related anaerobic terrestrial environments. Marine habitats include seawaters of the Arctic Ocean (Bano and Hollibaugh, 2002) and the Pacific Ocean (this study), deep-sea sediments (Li *et al*., 1999), mucous of the hydrothermal vent polychaete *Paralvinella palmiformis* (Alain *et al*., 2002), and marine white tubes *Alvinella pompejana* (GenBank description; AJ431234 and AJ431235). Terrestrial (mainly anaerobic) environments include human faeces (Zoetendal *et al*., 2003), rumen fluid (GenBank description; AB009195, AB009200), termite gut (GenBank description; AB062771), and anaerobic digesters (Godon *et al*., 1997; Delbes *et al*., 2000). Five putative subgroups from L1 to L5 were delineated within the *Lentisphaerae* and supported by different methods of phylogenetic analysis (Fig. 2). Subgroups L1–L2, including *V. vadensis* consist of bacterial sequences originated from anaerobic habitats, whereas subgroups L3–L5 appear to be predominantly of marine origin (exception: Mono Lake clone ML635J-58). The two marine HTCC isolates (subgroup L5) were closely related to subgroup L3–L4 (84.2–86.1% of similarity) rather than subgroups L1–L2 (82.5–83.4%). Thus HTCC2155^T represents the marine bacterial group in L3–L5, and *V. vadensis* represents anaerobic bacteria in subgroup L1–L2. However, clear supragroup differentiation between subgroups L1–L2 and L3–L5 was not supported by the phylogenetic analysis. The two cultured representatives, *V. vadensis* and *L. araneosa* differed in rRNA sequence similarity at about the order-level. In addition to the phylogenetic difference, strain HTCC2155^T was clearly differentiated from *V. vadensis* based on habitat (seawater versus human), oxygen requirement (obligately aerobic versus obligately anaerobic), mode of glucose utilization (oxidation versus fermentation), DNA G+C composition (48.3 mol% versus 59.2 mol%), and optimum growth temperature (20°C versus 37°C). Therefore, we propose that these cultures be designated as members of the two novel orders, the *Lentisphaerales* ord. nov. (L5) and the *Victivallales* ord. nov. (L1), within the phylum *Lentisphaerae*.

In conclusion, polyphasic approaches detailed above demonstrate that strain HTCC2155^T cannot be classified as a member of any of the known genera or phyla in the bacterial domain and, thus, represents a new genus within a novel phylum, for which we propose the name *Lentisphaera araneosa* gen. nov., sp. nov.

Description of Lentisphaerae *phyl. nov.*

Lentisphaerae (Len.ti.sphae'rae. N.L. fem. pl. n. *Lentisphaera* type genus of the type order of the phylum; N.L. fem. pl. n. *Lentisphaerae* phylum of the genus *Lentisphaera*). The phylum *Lentisphaerae* is defined by phylogenetic analysis based on 16S rRNA gene sequences of cultured strains and environmental clone sequences retrieved mainly from marine and several anaerobic environments. The phylum includes two Gram-negative bacterial genera, *Lentisphaera* and *Victivallis*, both of which produce extracellular slime material. The type order is *Lentisphaerales* ord. nov.

Description of Lentisphaerales *ord. nov.*

Lentisphaerales (Len.ti.sphae'ral.es. N.L. fem. pl. n. *Lentisphaera* type genus of the order; *-ale*s ending to denote

an order; N.L. fem. pl. n. *Lentisphaerales* the order of the genus *Lentisphaera*).

The order *Lentisphaerales* encompasses Gram-negative bacteria retrieved from marine habitats, within the phylum *Lentisphaerae*. The order comprises two strains of the genus *Lentisphaera*. The delineation of the order is primarily determined based on phylogenetic information of 16S rRNA sequences. The type genus is *Lentisphaera* gen. nov.

Description of Lentisphaera *gen. nov.*

Lentisphaera (Len.ti.sphae'ra. L. adj. *lentus* sticky; L. fem. n. *sphaera* sphere; N.L. fem. n. *Lentisphaera* a sticky sphere). Gram-negative, non-motile spherical cells. Strictly aerobic, chemoheterotrophic, and facultatively oligotrophic bacteria. Requires NaCl for growth. The major fatty acids are 16 : 1ω9c (50.8%) and 14 : 0 (25.9%). Produces transparent exopolymers. The type and only species of the genus is *Lentisphaera araneosa*.

Description of Lentisphaera araneosa *sp. nov.*

Lentisphaera araneosa (a.ra.neo'sa. L. fem. adj. *araneosa* similar to cobwebs, pertaining to the morphology of transparent exopolymer particles produced by the strain). Description is the same as the genus. Cells are 0.6–1.1 μm in diameter. Growth occurs at 4–25°C (optimum; 16–20°C). The pH and salinity ranges for growth are 7.0–9.0 and 0.75–15% (w/v). Colonies are 0.6–1.1 mm in diameter, milkish, uniformly circular, convex, and opaque, with smooth surfaces and entire margins (marine agar 2216, 3 weeks incubation). Utilize hexoses, disaccharides, sugar alcohols and sugar acids. No amino acids were utilized by the strain. Biochemical properties, sole carbon sources utilization patterns, carbon source oxidation and antibiotics susceptibility are described in Table 1. The DNA G+C content of the species is 48.3 ± 0.6 mol% (HPLC method). Genome size is 2.9 ± 0.6 mb. Two strains (HTCC2155^T and HTCC2160) were isolated from the coast of Oregon, Pacific Ocean. The type strain is strain HTCC2155^T (= ATCC BAA-859^T = KCTC 12141^T).

Description of Victivallales ord. nov.

Victivallales (Vic.ti.val'lal.es. N.L. fem. pl. n. *Victivallis* type genus of the order; -*ale*s ending to denote an order; N.L. fem. pl. n. *Victivallales* the order of the genus *Victivallis*). The order *Victivallales* encompasses Gram-negative bacteria retrieved from mainly anaerobic environments, including human faeces, termite gut, rumen, and anaerobic digester, within the phylum Lentisphaerae. The order comprises the genus *Victivallis* and several uncultured clones retrieved from anaerobic digester and termite gut. The delineation of the order is primarily determined by phylogenetic information of 16S rRNA gene sequences. The type genus is *Victivallis* (Zoetendal *et al*., 2003).

Experimental procedures

Isolation and bacterial cultures

A seawater sample was collected from a depth of 10 m at a station (NH5) 27.6 km off of the coast of Oregon (44°39.1′-N, 124°24.7′-W) in September 2001 and used for the HTC. The seawater sample was diluted to 10 cells per ml in a low nutrient heterotrophic medium [LNHM] (0.2 μm-filtered and autoclaved seawater supplemented with 1.0 μM NH_4Cl and 0.1 μM KH_2PO_4) amended with 1× mixed carbons [MC] (LNHM plus 1× MC); 1× MC was composed of 0.001% (w/v) each carbon compound of D-glucose, D-ribose, succinic acid, pyruvic acid, glycerol, N-acetyl D-glucosamine, and 0.002% (v/v) of ethanol. The liquid cultures of two strains designated HTCC2155^T and HTCC2160 were obtained after incubating dilution-to-extinction cultures for 21 days at 16°C. The liquid cultures were spread on LNHM plus 1× MC agar medium and colonies were isolated after incubation for 20 days at 16°C.

16S rRNA gene sequencing and phylogenetic analyses

Genomic DNA extraction, PCR amplification of 16S rRNA gene, and sequencing were performed as previously described (Cho and Giovannoni, 2003a). Sequences were aligned against representative sequences of most of the major recognized phyla using the ARB software package (Ludwig *et al*., 1998) and only 1066 unambiguously aligned nucleotide positions were used for phylogenetic analyses with PAUP* 4.0 beta 10 (Swofford, 2002). Sequence similarities between 16S rRNA gene sequences of the major bacterial phyla were calculated from a *Lentisphaerae*-specific mask (1291 bp) generated in ARB. Phylogenetic trees were inferred by maximum likelihood with a heuristic search, a transition/transversion ratio of 1.5009, and nucleotide frequencies that were estimated from the data. The tree topology was also compared with the phylogenetic trees inferred by both neighbour-joining with Kimura-2 parameter model and maximum parsimony with a heuristic search. The neighbour-joining and parsimony trees were evaluated by bootstrap analyses based on 1000 and 100 resamplings respectively. The 16S rDNA sequences of strains HTCC2155^T and HTCC2160 have been deposited in GenBank under accession no. AY390428 and AY390429 respectively.

DNA base composition, genome size and cellular fatty acid composition

The DNA G + C content was determined by a HPLC-based method (Mesbah *et al*., 1989) using a Platinum EPS reverse-phase C18 column (150 mm, 4.6 mm, 5 μm pore size, Alltech). Genome size was measured by pulsed-field gel

electrophoresis (PFGE), specifically, the contour-clamped homogenous electric field (CHEF) method (Lanoil *et al*., 1996). Cellular fatty acid methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI).

Phenotypic characterizations

Tests used for phenotypic characterizations have been detailed in the previous studies (Cho and Giovannoni, 2003a,b) except for differences in growth media and incubation temperature. Biochemical tests were carried out using API 20NE strips (BioMérieux) following the manufacturer's instructions. Custom-made 48-well microplates containing 47 different carbon compounds at a final concentration of 0.02% (w/v, or v/v) were used for sole carbon source utilization tests. In this study, an artificial seawater medium (ASW; 30 g NaCl, 1.0 g $MgCl_2{\cdot}6H_2O$, 4.0 g Na_2SO_4, 0.7 g KCl, 0.15 g $CaCl_2{\cdot}2H_20$, 0.5 g NH_4Cl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2{\cdot}6H_2O$, 0.025 g H_3BO_3, 0.001 g NaF, 10 ml Tris-Cl (pH 8.0), and 1 ml of SN trace metal solution (Waterbury *et al*., 1986) per 1 litre deionized water) amended with 10 × MC and marine agar 2216 were used for liquid cultures and plate cultures, respectively, and incubated at 20°C.

Characterization of growth properties and viscometric analysis

Growth characteristics of strain HTCC2155^T were examined under different culture conditions, including different temperatures and DOC concentrations. For determination of optimum growth temperature, cell densities were adjusted to approximately 1000 cells ml^{-1} in LNHM plus 1× MC, ASW plus 10 × MC, and 1/10 R2A in aged seawater (Reasoner and Geldreich, 1985; Suzuki *et al*., 1997). The cultures were incubated at 4, 10, 16, 20, 25 and 30°C. In the same way, cultures in the exponential phase of the strains were inoculated to LNHM and ASW media amended with different concentrations of carbon mixtures ranging from no MC to 100× MC and incubated at 20°C. During incubation, medium viscosity and cell density were measured at 1–2 day intervals. Cell densities, sizes, and morphology were examined by DAPI staining using an epifluorescence microscope (DMRB, Leica) equipped with a cooled charge-coupled device (CCD) camera (ORCA-ER, Hamamatsu) and IPLab v3.5 scientific imaging software (Scanalytics). The kinematic viscosity of liquid culture was measured at room temperature using a Cannon-Ubbelohde viscometer, which had a viscometric constant of 0.003779 mm^2/s^2 (Cannon Instrument).

Transparent exopolymer particles (TEP) and extracellular polysaccharide (EPS)

In order to examine the morphology of TEP produced by strain HTCC2155^T, a modified method of Alldredge *et al*., 1993) was used. Triplicate 1 ml cultures grown in LNHM plus 1× MC medium for 10 days were transferred to microcentrifuge tubes containing a glass disc (diameter 6 mm) and centrifuged for 15 min at 10 000 r.p.m. in an ultracentrifuge (Beckman). This process let all particles in the cultures attach to the surface of the glass disc. After removal of glass discs from microcentrifuge tubes, glass discs were stained for 5 s with a 0.2 μm-prefiltered solution of 0.02% Alcian Blue in 0.06% acetic acid, a stain specific for negatively charged polysaccharides. The air-dried glass discs were mounted on microscope slides with a drop immersion oil and examined at 1000× magnification under light microscopy (Leica, DMRB).

Extracellular polysaccharide was collected from the culture medium after 20 days of incubation. Cells were removed from the medium by centrifugation at 20 000 r.p.m. for 30 min, and the EPS was precipitated from the supernatant by addition of equal volume of 100% ethanol. The EPS was then washed five times with increasing ratios of ethanol to water. The precipitated EPS was lyophilized and the dry-weight was measured. Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the lyophilized samples by acidic methanolysis (Merkle and Poppe, 1994).

PCR and RNA hybridization in seawater samples

Polymerase chain reaction amplification for specific detection of HTCC2155^T. was carried out using the primer sets designed in this study, HTCC2155–195F (5′-AAGGTTAC GCTTAGGGATGA-3′) and HTCC2155–1345R (5′-GTAGCT GATGCCCATTTACT-3′). For rRNA hybridization, Pacific Ocean samples from surface (15 m) and deep (250 or 500 m) seawater were collected along the Newport Hydroline (NH: 44°39.1′-N), at stations 10 (124°17.7′-W), 25 (124°39′-W), 35 (124°53′-W), 65 (125°36′-W), 85 (126°-3′-W), and 127 (127°W), in May 2002. Monthly samples from the BATS were collected over a three year period from the upper (1 m) and lower (200 m) surface layers. Specific hybridization values for probes HTCC2155–1110R (5′-TTAGCAAGTAAGGATATG GGT-3′), and EUB-338 pL (5′-GCWGCCWCCCGTAGGW GT-3′) were determined as previously described (Giovannoni *et al*., 1990). Strains HTCC2155^T, *Cytophaga* sp., *Synechococcos* sp., and *Roseobacter* sp. controls were used to calculate percentage contribution of *Lentisphaera araneosa* to total bacterial 16S rRNA abundance.

Acknowledgements

We are grateful to Dr Jean Euzéby for his helpful recommendations about etymology. We thank to crews of the R/V Elakha for helping us collect seawater samples and Dr Skip Rochefort for his help with viscometric analyses. This study was supported by grants from Diversa Corporation and the National Science Foundation grant DEB-0207085. This study was also supported in part by the Department of Energy-funded (DE-FG09–93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

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