Vogesella mureinivorans sp. nov., a peptidoglycan-degrading bacterium from lake water

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A novel, non-pigmented, rod-shaped, Gram-negative strain was isolated from mesotrophic lake water in Zealand, Denmark. Phylogenetic analysis of the 16S rRNA gene sequence of the bacterium, designated strain 389^T, indicated that the strain belonged to the genus *Vogesella* and formed a monophyletic group with *Vogesella perlucida* DS-28^T (99.1 % nucleotide similarity); it was less related to *Vogesella indigofera* ATCC 19706^T (96.9 % similarity) and *Vogesella lacus* LMG 24504^T (96.8 % similarity). Hybridization of DNA from strain 389^T and *V. perlucida* demonstrated a reassociation of 50.6 ± 9.6 %. The DNA G + C content of strain 389^T was 61.2 mol%. The fatty acid profile of the strain differed from those of the other strains representing the genus *Vogesella* by a high content of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (71.6 %) and a lower content of C_{16:0}. Strain 389^T was capable of degrading peptidoglycan and had chitinase and lysozyme activities, possibly associated with the degradation of peptidoglycan, and had capacity for degradation of several other polymer compounds. Based on phenotypic and genotypic characteristics, strain 389^T represents a novel species, for which we propose the name *Vogesella mureinivorans* sp. nov. The type strain is 389^T (=DSM 21247^T =LMG 25302^T).

The biomass of prokaryotes on Earth is estimated to equal the entire plant biomass (Whitman *et al.*, 1998). This implies that there is also a considerable global production of peptidoglycan (PG), although some prokaryotes do not contain PG, e.g. *Archaea*. Specific mechanisms involved in degradation of extracellular PG remain to be characterized, but studies of aquatic bacteria indicate that natural microbial populations exist that can degrade free PG, although at lower rates than other macromolecules, such as proteins (Nagata *et al.*, 2003). In this study, a PG-degrading bacterium belonging to the genus *Vogesella* was isolated from lake water. The genus *Vogesella* belongs to the class *Betaproteobacteria* and includes three recognized heterotrophic species that were all isolated from freshwater sites (sediment or water). The earliest described species of the genus, *Vogesella indigofera* ATCC 19706^T, produces distinct blue-pigmented colonies while the other two strains, *Vogesella perlucida* LMG 24214^T and *Vogesella lacus* LMG 24504^T are non-pigmented (Chou *et al.*, 2008,

2009; Grimes *et al.*, 1997). Strain 389^T was isolated from freshwater in Denmark by initial cultivation on agar medium enriched with purified PG. Our studies confirmed the capacity to degrade PG and several other polymeric substances. This contrasts the present understanding that members of the genus *Vogesella* are mainly capable of degrading low-molecular-mass organic compounds.

Lake water was collected from the mesotrophic Lake Sjølsø, northern Zealand, Denmark, and filtered through 0.8 µm pore-size filters. The filtered water was enriched with PG from *Bacillus* sp. strain A6 (Frette *et al.*, 2004) to a final concentration of 2 mg l⁻¹. PG was purified according to a procedure indicated below. After incubation for 7 days on a shaking table at 20 °C, bacteria from the water were cultivated on 1/10 strength Luria–Bertani (LB) agar (Eisenstadt *et al.*, 1994). Isolates from the plates were transferred to 1/10 strength LB agar enriched with PG (or with cell-wall material that had been denatured by heat) of strain A6. Isolates that produced clearing zones on the PG-enriched agar medium were picked for further analysis. Strain 389^T was found to form distinct, turbid clearing zones, and was selected for additional studies. For subsequent analysis, this strain was cultured in LB broth or tryptic soy broth (TSB; Difco, product no. 0370-17-1) in shake flasks incubated overnight at 22 °C.

Abbreviation: PG, peptidoglycan.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 389^T is GQ246809.

An illustration of cultures of strain 389^T grown with and without PG are available with the online version of this paper.

General growth physiology was characterized for strain 389^T and *V. indigofera* DSM 3303^T using individual and multi-substrate kits under identical conditions. The two strains were grown at 22 °C in liquid or on solid 1/10 strength TSB at pH 7.2. The individual tests included: Gram designation (KOH method), staining of cells with Sudan Black to detect poly- β -hydroxybutyrate granules (examined by Nomarski interference contrast microscopy at $\times 1000$ magnification), oxidase activity (BBL Dryslide Oxidase kit), catalase activity (H₂O₂ addition), oxidation or fermentation of glucose (Hugh & Leifson, 1953), casein hydrolysis (detection of clearing zones on LB agar enriched with 3 % skimmed milk), lysozyme activity (EnzChek Lysozyme test kit; Invitrogen), DNase activity (detection of clearing zones made visible after addition of 1 M HCl) in TSB agar (TSB medium with 20 g agar l⁻¹) supplemented with 2 g DNA l⁻¹ (Jeffries *et al.*, 1957), and chitinase, cellulose and starch degradation using the methyl-umbelliferyl (MUF) substrate analogues MUF-*N*-acetyl- β -glucosaminide, MUF- β -glucosidase and MUF- α -glucosidase (Sigma-Aldrich). The MUF substrates were applied as previously described (O'Brien & Colwell, 1987). Briefly, substance from several colonies was rubbed onto 4 cm filter paper discs to which the MUF substrates were added. The discs were incubated at 28 °C for 30 min in closed Petri dishes with a drop of water to eliminate desiccation. Finally, 50 μ l of a saturated solution of NaHCO₃ was added to each disc. The discs were examined under UV light and appearance of blue fluorescence was used to score a positive reaction. Anaerobic growth was examined by incubating the strain on pure TSB agar or TSB agar enriched with 10 mM glucose, nitrate or sulfate, respectively, in an Oxoid AnaeroGen system.

Phenotypic characterization of strain 389^T and *V. indigofera* DSM 3303^T was performed by multi-substrate kits that included API 20 NE, API 50 CH (carbohydrate utilization) and API ZYM (various enzymes) (bioMérieux), and Biolog GN2 MicroPlate and EcoPlate (Biolog). These tests were carried out according to procedures recommended by the manufacturers. For analysis of whole-cell fatty acids, cells of strain 389^T and *V. indigofera* DSM 3303^T were grown in TSB. Cultures for DNA–DNA hybridization were grown in TSB (strain 389^T) or R2A (Becton Dickinson) (*V. perluxida* LMG 24214^T). Genomic DNA G+C content and whole-cell fatty acids were analysed by the DSMZ (Braunschweig, Germany) using HPLC analysis and GC-MS, respectively. DNA–DNA hybridization (duplicate analysis) was performed by the DSMZ according to De Ley *et al.* (1970).

Capacity for degradation of extracellular PG was examined by two different approaches. PG of *Erythrobacter* sp. (class *Alphaproteobacteria*) was purified by the procedure of Pelz *et al.* (1998). Briefly, the extraction included the following steps: treatment with SDS at 100 °C for 1 h for lysis of the cells, sonication for 1 min for additional cell destruction, proteinase treatment (trypsin) to remove proteins, and lipid extraction using organic solvents of decreasing polarity. The purified PG was added to 20 ml samples of

either 1/25 strength liquid TSB or 1/25 strength TSB agar applied to 1/25 strength TSB agar plates as a top agar layer. A final PG concentration of 2 mg l⁻¹ was used in liquid media as well as in the top agar. In the 1/25 strength liquid TSB medium, growth of the inoculum was measured as the increase in cell density (determined as concentration of DNA; see below) compared to controls (1/25 strength TSB without PG). After 3, 5 and 7 days, 50 μ l SYBR Green1 stain for quantification of dsDNA (product no. S7563, diluted 1:10 000; Invitrogen) was added to triplicate subsamples of 2 ml. The fluorescence intensity (excitation 498 nm; emission 520 nm) was read after 10 min of incubation in the dark. For examination of PG degradation on solid media, cells were spread on 1/25 TSB plates with and without PG-containing top agar. Degradation (hydrolysis) of PG was determined from clearing (opaque) zones surrounding the colonies.

Growth capacity of the strains was determined at different temperatures (3, 5, 10, 20, 25, 30 and 37 °C), salinities (0, 1, 3, 6 and 10 %, w/v, NaCl) and pH (5, 6, 7, 8, 9 and 10) in 1/10 or full-strength TSB medium incubated on a shaking table at 20 °C (or at 3–37 °C for the temperature preference experiment). The growth rate of strain 389^T was measured for cultures growing at 20 °C in 1/10 and full-strength TSB.

Cell morphology and cell size of strain 389^T were determined by scanning electron microscopy (SEM) analysis of cells from an overnight culture grown in 1/10 TSB and collected on 0.2 μ m (pore size) polycarbonate filters. Fixation and dehydration was according to the procedure described by Watson *et al.* (1980). Finally the filters were mounted on aluminium stubs and sputter-coated with gold/palladium. The preparations were analysed with a Quanta 200 SEM microscope (FEI). Cell sizes were measured by using LCS Lite software (Leica Microsystems). For transmission electron microscopy (TEM) to detect presence of flagella, a drop of the overnight culture was placed on a Formvar-coated copper grid (mesh 200; Electron Microscopy Sciences). The cells were treated with 1 % uranyl acetate before visualization of the negatively stained cells by using a Phillips TEM, CM 100, equipped with a Morada 11 Mbit pixel camera.

Genomic DNA was isolated from strain 389^T grown overnight in TSB medium as described previously (Brandt *et al.*, 2006). The 16S rRNA gene was amplified by PCR using the primer pair Fd1 and Rd1 (Brandt *et al.*, 1999). The PCR product was purified by using a Qiagen PCR purification kit (cat. no. 28106, Qiagen). The almost complete 16S rRNA gene sequence (1444 bp) was obtained by sequencing the gene in both directions using several forward and reverse sequencing primers (GATC Biotech). Preliminary phylogenetic analysis was carried out by using BLAST (Johnson *et al.*, 2008) and Seqmatch (RDP 10 website; Cole *et al.*, 2009). Based on taxonomic and sequence quality criteria, a subset of sequences was selected for subsequent alignment and final phylogenetic analysis as

Fig. 1. Cells of strain 389^T observed by scanning electron microscopy (SEM; left) and transmission electron microscopy (TEM; right). The polar flagellum could only be visualized by TEM.

described previously (Jørgensen *et al.*, 2009) using BioEdit version 7.0.9.0 (Hall, 1999) and CLUSTAL X 2.0 software (Larkin *et al.*, 2007).

Strain 389^T consists of rod-shaped, motile cells (one polar flagellum was observed; Fig. 1) showing accumulation of poly- β -hydroxybutyrate granules, as previously observed for the other three species of the genus *Vogesella* (Chou *et al.*, 2008, 2009; Grimes *et al.*, 1997). The strain forms transparent colonies and lacks the characteristic blue pigmentation found in *V. indigofera* ATCC 19706^T (Grimes *et al.*, 1997). The fatty acid profile of strain 389^T is dominated by C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH (together also named ‘summed feature 3’) that account for a total of 71.6 % of the fatty acids, followed by C_{16:0} and C_{10:0} 3-OH (Table 1). The proportion of ‘summed feature 3’ is higher than in the other *Vogesella* strains and that of C_{16:0} is lower, but the abundance of the remaining fatty acids equals approximately 20 % as also observed in the other three *Vogesella* strains.

A characteristic feature of strain 389^T is its capacity to degrade PG isolated from bacterial cell walls. When 1/25 strength TSB medium was enriched with PG, a 1.4- to 1.7-fold higher cell density was found (liquid media) and

opaque clearing zones occurred around the colonies (solid agar medium) (Fig. S1, available in IJSEM Online, and Table 2). By contrast, no utilization of PG was observed by *V. indigofera* DSM 3303^T or *V. perlucida* LMG 24214^T. When 1/25 strength TSB was replaced by mineral media, no growth on PG was observed for strain 389^T (data not shown). Strain 389^T was capable of degrading several other polymer compounds, including casein, gelatin, chitin, starch and Tween 80, and had DNase and lysozyme activities (Table 2). Cellulose was not degraded. Phenotypic characteristics with respect to utilization of various polymer compounds and carbon sources by strain 389^T and other *Vogesella* strains are shown in Table 2.

Lysozyme and chitinase activities indicated that strain 389^T was capable of breaking β (1,4) glycosidic bonds in chitin and PG, and this might explain its success in utilizing cell-wall material from Gram-positive and Gram-negative bacteria as observed during the initial screening. Capacity to degrade chitin and PG has not previously been reported for members of the genus *Vogesella*. The opaque rather than transparent clearing zones around the colonies on PG-enriched agar may indicate only partial degradation of PG, possibly supporting the observed, positive reaction with *N*-acetylglucosamine. *N*-acetylglucosamine is part of the polymeric structure of both chitin and PG and is one of the most abundant monomers (amino sugars) in the biosphere (Cauchie, 2002). In contrast to the other species of the genus *Vogesella*, strain 389^T showed only weak growth on D-glucose (Table 2). This suggests that the glucose moiety of PG is not a major source of nutrients for strain 389^T.

Phylogenetic analysis of the 16S rRNA gene sequence clearly showed that strain 389^T belonged to the genus *Vogesella* (Fig. 2). Based on evolutionary distance and bootstrap analysis, strain 389^T formed a monophyletic group with *V. perlucida* DS-28^T (99.1 % nucleotide similarity), whereas it was less related to *V. indigofera* ATCC 19706^T (96.9 % similarity) and *V. lacus* LMG 24504^T (96.8 % similarity). The DNA G + C content of strain 389^T was 61.2 mol%. Due to the close relationship with *V.*

Table 1. Dominant whole-cell fatty acid composition (%) of strain 389^T and related species of the genus *Vogesella*

Strains: 1, strain 389^T; 2, *V. indigofera* DSM 3303^T; 3, *V. perlucida* DS-28^T (data from Chou *et al.*, 2008); 4, *V. lacus* LMG 24504^T (Chou *et al.*, 2009). Fatty acids representing <0.5 % of the total are not shown.

Fatty acid	1	2	3	4
C _{10:0}	2.3	2.6	2.4	<0.5
C _{10:0} 3-OH	5.5	5.1	4.8	4.2
C _{12:0}	3.4	2.7	2.9	1.8
C _{14:0}	2.0	0.6	1.2	0.9
C _{16:1} ω 7c and/or iso-C _{15:0} 2-OH*	71.6	56.9	61.7	60.0
C _{16:0}	8.2	26.4	18.1	13.6
C _{18:1} ω 7c	2.0	4.8	3.8	12.5

*Also known as ‘summed feature 3’.

Table 2. Selected differential characteristics of strain 389^T and the three recognized species of the genus *Vogesella*

Strains: 1, strain 389^T; 2, *V. indigofera* ATCC 19706^T; 3, *V. perlucida* DS-28^T (data from Chou *et al.*, 2008, except for DNA–DNA similarity); 4, *V. lacus* LMG 24504^T (Chou *et al.*, 2009). +, Positive reaction; (+), weakly positive reaction; –, no reaction; NA, no data available.

Characteristic	1	2	3	4
Isolation source	Lake water	Pond sediment	Spring water	Pond water
Colony colour	Colourless to greyish	Blue	Colourless to white	Colourless
DNA G + C content (mol%)	62.1	65.4	61.8	63.0
DNA–DNA similarity relative to strain 389 ^T (%) [*]	NA	NA	50.6 ± 9.6	NA
Hydrolysis of polymers				
Casein	+	–	–	+
Cellulose	–	NA	–	–
Gelatin (liquefaction)	+	–	+	–
Chitin	+	–	–	NA
Starch	+	–	–	–
Tween 80	+	–	+	+
DNase	(+)	+†	–	(+)
Lysozyme	+	NA	NA	NA
Assimilation of carbon sources				
D-Glucose	(+)	+	+	+
D-Fructose	+	–	+	+
Glycogen	+	NA	+	+
Gluconate	+	–	–	–
Propionate	+	+	–	–
L-Ornithine	–	+	–	+
Degradation of PG				
Liquid culture‡	+	–	NA	NA
Agar§	+	–	–	NA

^{*}Mean ± SD (*n*=2).

†Data on DNase from Grimes *et al.* (1997).

‡After 3 days, cell densities of strain 389^T were 1.4- to 1.7-fold higher than densities of *V. indigofera* DSM 3303^T in the PG-supplemented cultures. No differences between the two strains were found in the cultures without PG. No changes in cell densities were observed at days 5 and 7, relative to day 3. Densities of cells were determined with the SYBR Green1 assay.

§Opaque zones of 4–8 mm were found around colonies of strain 389^T on PG-enriched agar, but no similar zones were observed for *V. indigofera* DSM 3303^T or *V. perlucida* LMG 24214^T on agar supplemented with PG. Colonies on agar without PG did not produce opaque zones for any of the strains.

perlucida DS-28^T, based on 16S rRNA gene sequence and genomic DNA G + C content (Table 2), a DNA–DNA hybridization experiment was performed to compare strain

389^T and *V. perlucida* LMG 24214^T. The DNA reassociation showed 50.6 ± 9.6 % similarity between the two strains and indicated that strain 389^T belongs to a novel species

Fig. 2. Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences from strain 389^T and related type strains. Numbers at nodes indicate bootstrap values based on 1000 resampled datasets; values below 500 are not shown. Bar, 0.01 nt substitutions per site. GenBank accession numbers are shown in parentheses.

(Wayne *et al.*, 1987) (Table 2). Based on the phenotypic and genotypic characters, we propose to classify strain 389^T, isolated from lake water, as the type strain of *Vogesella mureinivorans* sp. nov.

Description of *Vogesella mureinivorans* sp. nov.

Vogesella mureinivorans (mu.re.i.ni.vo'rans. N.L. n. *mureinum* murein, cell wall; L. part. adj. *vorans* eating, devouring; N.L. part. adj. *mureinivorans* murein-devouring).

Cells are Gram-negative, short rods, non-sporulating and $2.7 \pm 0.9 \times 0.8 \pm 0.1 \mu\text{m}$ (mean $\pm$ SD, $n=16$) in size. Cells are motile by means of a single polar flagellum and accumulate poly- β -hydroxybutyrate granules. Growth occurs on TSB from 5–37 °C and pH 6–9, with optima at 20–30 °C and pH 6–7, respectively. Doubling times in 1/10 and full strength TSB at optimum conditions are 4.3 and 1.6 h, respectively. Concentrations of up to 1 % NaCl are tolerated, but optimum growth occurs at 0.1 % NaCl. After 24 h of growth on LB or TSB, colonies are circular with a lobate margin, low convex, smooth, nonfluorescent and transparent to slightly greyish. Hydrolyses aesculin, gelatin, glycogen, casein, PG and Tweens 40 and 80. Positive enzyme activities include lysozyme, DNase, casein protease, β -1,4-glucanase, chitinase, chitobiase, leucine and valine arylamidases, and acid and alkaline phosphatases. The following compounds support growth: D-glucose (weak reaction), D-fructose, L-fucose, glycerol, *myo*-inositol, D-mannitol, methyl-pyruvate, mono-methyl succinate, acetic acid, cis-aconitic acid, formic acid, D-gluconic acid, α -, β - and γ -hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, L-pyrroglutamic acid, L-threonine, γ -aminobutyric acid, N-acetylglucosamine and urocanic acid. Compounds that do not support growth are L- or D-arabinose, L or D-xylose, D-galactose, L-rhamnose, L-arginine, L-ornithine, L-tryptophan and urea. Nitrate is reduced to nitrite, but the reduction is not coupled to anaerobic growth. Anaerobic growth is not observed, and oxidation and fermentation of D-glucose are negative (Hugh–Leifson test). The DNA G + C content of the type strain is 61.2 mol%. The predominant fatty acids are C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

The type strain is 389^T (=DSM 21247^T =LMG 25302^T) isolated from the clear-water Lake Sjølsø, Zealand, Denmark.

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