Megasphaera paucivorans sp. nov., *Megasphaera sueciensis* sp. nov. and *Pectinatus haikarae* sp. nov., isolated from brewery samples, and emended description of the genus *Pectinatus*

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Seven unidentified strictly anaerobic, Gram-negative, non-spore-forming bacteria from spoiled beer or the brewery environment were characterized. Based on 16S rRNA gene sequence analyses, all strains were affiliated to the *Sporomusa* sub-branch of the class 'Clostridia'. Three of the strains were non-motile cocci, on average $1.5 \times 1.2 \mu\text{m}$ or $1.2 \times 1.0 \mu\text{m}$, occurring mainly singly or in pairs. They shared nearly identical ($>99\%$) 16S rRNA gene sequences, being most closely related to the species of the *Megasphaera*–*Anaeroglobus* group ($\leq 93.9\%$ similarity). According to DNA–DNA hybridization results, the coccoid strains represented two genospecies, neither of which was related to any of the recognized *Megasphaera* species. Several phenotypic characteristics and/or DNA G + C content also differentiated the strains from each other and from their closest relatives. The other four novel strains were motile, slightly curved to helical rods, $0.6\text{--}0.8 \times 3\text{--}50 \mu\text{m}$ or more in size. They shared identical 16S rRNA gene sequences and ribofragment patterns. The highest 16S rRNA gene similarity was found between these isolates and *Pectinatus cerevisiiphilus* ATCC 29359^T (95.6%) and *Pectinatus frisingensis* ATCC 33332^T (93.6%). The novel strains also differed from recognized *Pectinatus* species in their sugar utilization, proteolytic activity, catalase activity, antibiotic resistance and temperature tolerance. The results suggest that the bacteria belong to three novel species, for which the names *Megasphaera paucivorans* sp. nov. (type strain VTT E-032341^T = DSM 16981^T), *Megasphaera sueciensis* sp. nov. (type strain VTT E-97791^T = DSM 17042^T) and *Pectinatus haikarae* sp. nov. (type strain VTT E-88329^T = DSM 16980^T) are proposed.

The genera *Megasphaera* and *Pectinatus* include several beer-spoilage bacteria (for a review see Haikara & Helander, 2002). These Gram-negative, strictly anaerobic bacteria are affiliated to the *Sporomusa* sub-branch of the class 'Clostridia' in the phylum *Firmicutes* (Willems & Collins, 1995; Strömpl *et al.*, 1999; Marchandin *et al.*, 2003). At present, the genus *Megasphaera* Rogosa 1971 emend. Engelmann and Weiss 1985, emend. Marchandin *et al.* 2003 comprises three species: the type species *Megasphaera elsdenii* Rogosa 1971,

first described as '*Peptostreptococcus elsdenii*' (Gutierrez *et al.*, 1959), *Megasphaera cerevisiae* Engelmann and Weiss 1986 and *Megasphaera micronuciformis* Marchandin *et al.* 2003. *M. cerevisiae* is the only brewery-associated species (Engelmann & Weiss, 1985; Suihko & Haikara, 2001). It spoils mainly low-alcohol beers by producing turbidity, H₂S and short-chain fatty acids. At the time of writing, the genus *Pectinatus* Lee *et al.* 1978 comprises two brewery-contaminating species, *Pectinatus cerevisiiphilus* Lee *et al.* 1978 emend. Schleifer *et al.* 1990 and *Pectinatus frisingensis* Schleifer *et al.* 1990. They are common spoilage bacteria of unpasteurized packaged beers. Signs of spoilage include turbidity and off-flavours from the synthesis of organic acids and sulphuric compounds (Haikara & Helander, 2002). In an earlier study, we characterized 32 *Megasphaera* and *Pectinatus* strains of brewery origin, earlier identified by conventional methods, using ribotyping, specific PCR primers and partial 16S rRNA gene sequencing. The results indicated that four of the strains could represent a novel *Pectinatus* species and one strain could be a new member of the genus *Megasphaera* (Suihko & Haikara, 2001).

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Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains VTT E-97791^T, VTT E-032341^T, VTT E-88329^T and VTT E-90406^T are DQ223729, DQ223730, DQ223731 and DQ217599, respectively.

Results of ribotyping analysis and volatile fatty acid profiles of the novel strains are available as supplementary material in IJSEM Online.

Supporting our results, one of the *Pectinatus* strains, DSM 20764, has also been reported to exhibit low DNA relatedness (<20 % DNA–DNA reassociation value) with the previously described *Pectinatus* species and to contain cadaverine in its cell-wall peptidoglycan (<http://www.dsmz.de/strains/no020764.htm>), a typical feature for many members of the *Sporomusa* sub-branch (Schleifer *et al.*, 1990; Strömpl *et al.*, 1999). The aim of the present study was to investigate in further detail the taxonomic affiliation of the atypical brewery-contaminating strains, tentatively identified as *Megasphaera* and *Pectinatus* spp., and two novel coccoid-shaped isolates from spoiled beer.

Strains

Strains VTT E-032341^T and VTT E-042576 originated from a spoiled Italian lager beer (pH 4.3) with an alcohol content of 5 % (v/v). Strain VTT E-97791^T was isolated from a spoiled Swedish lager beer (pH 4.9) containing 2.8 % (v/v) alcohol. Strains VTT E-88329^T and VTT E-88330 originated from air of a brewery bottling hall in Finland and VTT E-89371 from a spoiled Finnish lager beer containing 2.7 % (v/v) alcohol. The isolates were obtained from the samples by plating on peptone/yeast extract/fructose (PYF) medium (Suihko, 1999) followed by anaerobic incubation at 30 °C. Strain VTT E-97914 (= DSM 20764) was originally isolated from spoiled German beer and it was obtained from the DSMZ. The following reference strains were used: *M. cerevisiae* VTT E-79111^T, *M. elsdenii* VTT E-84221^T, *M. micronuciformis* VTT E-042113^T (= AIP 412.00^T, provided by H. Marchandin, CHU-Montpellier, France), *P. frisingensis* VTT E-79100^T and *P. cerevisiiphilus* VTT E-79103^T. The strains were subcultured on PYF or peptone/yeast extract/glucose (PYG) (for VTT E-97791^T) medium (Suihko, 1999) at 30 °C, with the exception of VTT E-042113^T, for which MRS agar (Oxoid) with 1 % fructose (BDH) and 25 % fermented wort without hops and an incubation temperature of 37 °C were used. Anaerobic conditions were created using a Whitley Anaerobic Cabinet MK III (Don Whitley Scientific) with 80 % N₂, 10 % CO₂ and 10 % H₂ or Anoxomat WS8000 (Mart Microbiology) with 85 % N₂, 5 % CO₂ and 10 % H₂.

Morphological characterization

Cell morphology (including motility) and Gram staining of 1-, 2- and 3-day-old broth cultures were examined by phase-contrast and normal light microscopy, respectively, at 800–1250 × magnification (Polyvar; Reichert-Jung). Cell size ranges were expressed as mean width and length of 20 cells ± SD. Colony morphologies were determined after 3–4 and 7 days incubation on PYF or PYG plates. The results of the morphological characterization are presented in Table 1 and in the species descriptions below. The cellular characteristics of VTT E-97791^T, VTT E-032341^T and VTT E-042576 together with their isolation source and beer-spoilage ability suggested that they belong to the genus *Megasphaera*, which contains Gram-negative, non-motile, strictly anaerobic cocci (Rogosa, 1971; Engelmann & Weiss, 1985; Marchandin *et al.*,

2003). Cells of the novel isolates were, however, smaller than those of *M. elsdenii* or *M. cerevisiae* and bigger than those of *M. micronuciformis* (Table 1). Strains VTT E-88329^T, VTT E-88330, VTT E-89371 and VTT E-97914 resembled members of the genus *Pectinatus* morphologically (Schleifer *et al.*, 1990). They formed an 'X' shape during movement, which is characteristic for *P. frisingensis* and *P. cerevisiiphilus* (Lee *et al.*, 1978; Haikara *et al.*, 1981) and discriminates them from morphologically similar *Selenomonas* species (Hespell *et al.*, 1999).

Genetic characterization

Automated ribotyping (RiboPrinter Microbial Characterization System; DuPont Qualicon) with *EcoRI* was performed as described by Bruce (1996). The strains were ribotyped until the same pattern was generated three times. For 16S rRNA gene sequencing, DNA was isolated from 0.5–1 ml liquid cultures by bead-beating with 200 µm glass beads (0.1 g ml⁻¹; Sigma) in a Ribolyser instrument (2 min at 6.5 m s⁻¹; Hybaid) after washing of cells in ultra-pure water. Extracts (2–5 µl) were used directly as templates in 50 µl PCRs. The 16S rRNA gene was amplified according to Carlier *et al.* (2002) except that DyNAzyme II DNA polymerase (Finnzymes) was used. Resulting PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and the manufacturer's procedure. Both strands of the gene were sequenced using the PCR primers and the primers described by Wilmotte *et al.* (1993) and ABI Prism BigDye Terminator version 3.0 or 3.1 Ready Reaction cycle sequencing kit (Applied Biosystems). Sequencing products were electrophoresed in the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences were compiled with the software DNAMAN version 4.1 (Lynnon BioSoft) and compared to GenBank/EMBL/DBJ databases using BLAST (Altschul *et al.*, 1997). The 16S rRNA gene sequences were aligned using Fast aligner. Phylogenetic relationships were inferred for representative strains (VTT E-97791^T, VTT E-032341^T and VTT E-88329^T) using neighbour-joining (NJ; Saitou & Nei, 1987), maximum-parsimony (MP; Kluge & Farris, 1969) and maximum-likelihood (ML; Olsen *et al.*, 1994) algorithms in the software package ARB (Strunk *et al.*, 2000). Vertical gaps and ambiguous nucleotides were excluded from the analyses. Evolutionary distances for the NJ analysis were based on the correction of Jukes & Cantor (1969). Robustness of the NJ and MP tree topologies was estimated by bootstrapping with 1000 replications. DNA–DNA hybridization and G+C content analysis were done at DSMZ. Cells were disrupted with a French pressure cell (Thermo Spectronic) and purified on hydroxyapatite (Cashion *et al.*, 1977). Hybridization was done according to De Ley *et al.* (1970) with the modifications of Huß *et al.* (1983) using a Cary 100 Bio UV/VIS spectrophotometer with *in situ* temperature probe (Varian). The DNA G+C content was determined by HPLC (adapted from Tamaoka & Komagata, 1984) of P1-nuclease-digested, dephosphorylated DNA (Mesbah *et al.*, 1989) and calculated according to Mesbah *et al.* (1989).

Table 1. Differential characteristics of the novel *Megasphaera* species and closely related Gram-negative, strictly anaerobic cocci

Strains: 1, VTT E-032341^T and VTT E-042576; 2, VTT E-97791^T; 3, *M. micronuciformis* VTT E-042113^T; 4, *M. cerevisiae* VTT E-79111^T; 5, *M. elsdenii* VTT E-84221^T; 6, *A. geminatus* AIP 313.00^T. Data for *M. micronuciformis* are from Marchandin *et al.* (2003) except for cell size and utilization of adonitol, dulcitol, DL-erythritol, α -D-melibiose, xylitol, succinate and pyruvate. Data for AIP 313.00^T are from Carlier *et al.* (2002). DNA G+C contents of VTT E-79111^T and VTT E-84221^T are taken from Engelmann & Weiss (1985) and Rogosa (1971), respectively. +, Positive; –, negative; W, weakly positive; $\pm$, variable; ND, not determined; R, resistant; S, susceptible. Additional test results: all strains were negative for reduction of nitrate^{ab}, formation of indole^{ab}, oxidase, catalase^{ab} and urease activities and hydrolysis of aesculin, milk^b, gelatin^b and arginine. All strains were also negative (except those in parentheses) for fermentation of adonitol, D-cellobiose^{ab}, dulcitol, DL-erythritol, aesculin^{ab}, glycerol^a, glycogen (AIP 313.00^T, VTT E-042113^T), i-inositol, inulin (AIP 313.00^T, VTT E-042113^T), lactose^{ab}, α -D-melibiose, melezitose^b (VTT E-042113^T), N-acetylglucosamine (AIP 313.00^T), raffinose^b, rhamnose, D-ribose^a, D-salicin^b, D-xylose^b and xylitol and utilization of succinate^{ab}. All strains were positive for utilization of pyruvate^{ab} and susceptible to 1 mg kanamycin and bile discs.

Characteristic	1	2	3	4	5	6
Cell size (μ m)	1.2–1.9 $\times$ 1.0–1.4	1.0–1.4 $\times$ 0.8–1.2	0.5–0.9 $\times$ 0.5–0.7	1.5–2.1	1.6–2.6	0.5–1.1
Growth at 45 °C	–	–	ND	– ^{a*}	+ ^b	ND
Acid production from:						
L-Arabinose	–	–	–	$\pm$ ^a	– ^b	–
D-Fructose	–	–	–	+ ^a	+ ^b	–
D-Galactose	–	–	–	–	– ^b	+
D-Glucose	–	–	–	– ^a	+ ^b	–
D-Maltose	–	–	–	–	+	–
Mannitol	–	–	–	– ^a	+ ^b	–
D-Mannose	–	–	–	– ^a	– ^b	+
Utilization of DL-lactate	–	–	–	+ ^a	+ ^b	–
Utilization of gluconate	+	+	–	+ ^a	+ ^b	ND
Gas production	+	+	–	+ ^a	+ ^b	–
Production of H ₂ S	+	+	–	+ ^a	+ ^b	–
Production of acetoin	–	W	ND	W	W	ND
Desulfoviridin	–	–	$\pm$	–	–	ND
Susceptibility to:						
Vancomycin (5 μ g)	R	R	S	R	R	R
Colistin (10 μ g)	R	R	S	S	S	S
Short-chain volatile fatty acids†	A, (P), iB, B, <u>iV</u> , V, (iC), <u>C</u>	(A), P, iB, <u>B</u> , <u>iV</u> , <u>V</u> , C	<u>A</u> , <u>P</u> , (iB), B, <u>iV</u> , (V), PhA	A, P, (iB), <u>B</u> , <u>iV</u> , V, <u>C</u> ^a	A, (P), <u>B</u> , iV, V, <u>C</u> ^b	<u>A</u> , P, iB, <u>B</u> , <u>iV</u>
DNA G+C content (mol%)‡	40.5 (<i>T_m</i>)	43.1 (<i>T_m</i>)	46.4 (<i>T_m</i>)	42.4–44.8 (<i>T_m</i>)	53.1–54.1 (Bd)	51.8 (<i>T_m</i>)

*Test was also performed in the following study with the following strain(s) and gave congruent result: *a*, Engelmann & Weiss (1985) with VTT E-79111^T and 11 other strains; *b*, Gutierrez *et al.* (1959), Rogosa (1971) and/or Engelmann & Weiss (1985) with VTT E-84221^T.

†A, Acetic acid; B, butyric acid; C, caproic acid; iB, isobutyric acid; iC, isocaproic acid; iV, isovaleric acid; P, propionic acid; PhA, 2-phenylacetic acid; V, valeric acid. Major products are underlined; parentheses indicate inconsistent production.

‡Bd, Buoyant density; *T_m*, thermal denaturation.

Genetic diversity of the novel isolates was studied by ribotyping in comparison with previously described *Megasphaera* and *Pectinatus* species. The results are available in Supplementary Figs S1 and S2 in IJSEM Online. The fingerprints distinguished VTT E-97791^T from VTT E-032341^T and VTT E-042576 (Supplementary Fig. S1). Strain VTT E-97791^T grouped closely with *M. cerevisiae*, while strains VTT E-032341^T and VTT E-042576 formed a distinct group with the highest similarity to the former strains and *M. micronuciformis* VTT E-042113^T. The fingerprints of the rod-shaped strains (VTT E-88329^T, VTT E-88330, VTT

E-89371, VTT E-97914) grouped together and were clearly distinct from the fingerprints of recognized *Pectinatus* species (Supplementary Fig. S2). Hence, ribotyping results suggested that the coccoid and rod-shaped strains could respectively represent two and one novel species within the genera *Megasphaera* and *Pectinatus*.

Subsequently, nearly complete 16S rRNA gene sequences (1467–1556 nt) of the novel strains were determined except that the sequence of VTT E-97914 was taken from Sakamoto (1997). The 16S rRNA gene of *Zymophilus raffinovorans*

VTT E-90406^T was also sequenced as it was not available from public databases. The sequences (1540 nt) of VTT E-032341^T and VTT E-042576 shared 100 % similarity with each other and 99.3 % similarity with VTT E-97791^T. Similarly, the sequences (1467 nt) of VTT E-88329^T, VTT E-88330, VTT E-89371 and VTT E-97914 were identical. Based on BLAST searches, VTT E-032341^T and VTT E-97791^T shared the highest sequence similarity with *M. micronuciformis* (95.1 and 94.9 %, respectively), *M. cerevisiae* (94.6 and 94.5 %), *M. elsdenii* (93.2 and 93.3 %) and *Anaeroglobus geminatus* (93.2 and 93.3 %) over 1359 unambiguous bases. The sequences of the rod-shaped strains were most similar to those of *P. cerevisiophilus* (96.3 %) and *P. frisingensis* (94.6 %). Thus, based on 16S rRNA gene sequence comparisons, all novel strains are members of the *Sporomusa* sub-branch of the class 'Clostridia', which contains low-G + C-content Gram-positive bacteria with Gram-negative cell walls (Willems & Collins, 1995; Strömpl *et al.*, 1999).

In phylogenetic analyses with the most closely related species, VTT E-032341^T and VTT E-97791^T formed a well-supported group (100 % in the NJ and MP trees) that was linked to *M. cerevisiae* with good and moderate support, respectively, in the NJ (94 %; Fig. 1) and MP (67 %; not shown) trees. Based on the NJ analysis, the most closely related species to VTT E-032341^T and VTT E-97791^T were *M. cerevisiae* (93.9 and 93.9 %, respectively), *M. micronuciformis* (93.2 and 93.1 %), *A. geminatus* (90.6 and 90.5 %) and *M. elsdenii* (89.8 and 89.8 %). In accordance with Marchandin *et al.* (2003), the applied treeing methods could not resolve the exact branching order within the *Megasphaera*–*Anaeroglobus* group. The representative rod-shaped strain VTT E-88329^T grouped with *P. cerevisiophilus* in the NJ (97 %; Fig. 2), MP (57 %; not shown) and ML (not shown) analyses. These strains formed a robust cluster (100 % in the NJ and MP analyses) together with *P. frisingensis* and *Pectinatus* sp. B6. After the NJ analysis (Fig. 2), the sequence similarity of VTT E-88329^T to *P. cerevisiophilus* ATCC 29359^T and *P. frisingensis* ATCC 33332^T was 95.6 and 93.6 %, respectively. In conclusion, the phylogenetic analyses indicate that the new isolates represent novel species because sequence similarities to the closest relatives were below 97 %, the level below which strains are generally assigned to separate species (Stackebrandt & Goebel, 1994).

Fig. 1. Consensus neighbour-joining tree of 16S rRNA gene sequences (1309 nt) showing the phylogenetic positions of the novel coccoid strains VTT E-032341^T and VTT E-97791^T within the *Megasphaera*–*Anaeroglobus* group. Bootstrap values (expressed as percentages of 1000 replications) greater than 60 % are shown at the branch points. Species names are followed by strain numbers and GenBank accession numbers. Bar, 10 % nucleotide substitutions.

Fig. 2. Consensus neighbour-joining tree of 16S rRNA gene sequences (1309 nt) showing the phylogenetic position of the novel rod-shaped strain VTT E-88329^T within the *Pectinatus*–*Selenomonas*–*Sporomusa* group. Other details are as in Fig. 1.

The phylogenetic relationships of the coccoid strains to each other and to recognized *Megasphaera* species were further studied by DNA–DNA hybridization, as it has significantly higher resolving power than 16S rRNA gene sequence analysis (Amann *et al.*, 1992). The results confirmed that VTT E-97791^T and VTT E-032341^T are not related to each other (41.0 %) or to *M. micronuciformis* VTT E-042113^T (28.9 and 17.1 %, respectively), *M. elsdenii* VTT E-84221^T (7.2 and 23.6 %) or *M. cerevisiae* VTT E-79111^T (22.0 and 3.1 %) at the species level when the recommended criterion of 70 % DNA–DNA relatedness is applied for species delineation (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). The G + C content of the DNA of the proposed type strains of the novel species are shown in Table 1 or in the species descriptions below. The DNA G + C contents of VTT E-97791^T and VTT E-88329^T fall within the radiations observed for the genera *Megasphaera* and *Pectinatus*, respectively (Engelmann & Weiss, 1985; Schleifer *et al.*, 1990; Marchandin *et al.*, 2003). The DNA G + C content of VTT E-032341^T is somewhat lower than reported for previously known *Megasphaera* species. The lower DNA G + C content clearly distinguishes both VTT E-97791^T and VTT E-032341^T from the closely related *A. geminatus* (Carrier *et al.*, 2002).

Biochemical and physiological characterization

Biochemical and physiological characterization was performed using actively growing cultures, PYF medium and incubation at 30 °C, unless otherwise stated. All media were pre-reduced and all tests included positive and negative control strains. Nitrate reduction, arginine hydrolysis, urease activity, acetoin production and bile resistance were tested using diagnostic tablets and procedures from Rosco A/S. For antibiotic susceptibility tests, An-ident discs (Oxoid) were used. Gas production was determined in autoclaved PYF and PYG broth with inverted Durham tubes. Growth in MRS (Oxoid) and PY (Holdeman *et al.*, 1977) media, in selective SMMP broth for *Megasphaera* and *Pectinatus* species (Lee, 1994) and in supplemented brucella blood agar (Jousimies-Somer *et al.*, 2002) was also examined. In addition, the ability of selected strains (5×10^8 cells) to grow in different beers (330 ml, 0.3–4.6% v/v alcohol, pH 4.1–4.5) was studied. Aerotolerance was examined by incubating plate cultures for 7 days in atmospheric air, in air with 10% CO₂ (Anoxomat WS8000) and under anaerobic conditions (control). For temperature limits of growth, plate cultures were incubated at 10, 15, 20, 30, 37, 40 or 45 °C and inspected after 7 and 14 days. Other growth tests were performed after Holdeman *et al.* (1977) or Jousimies-Somer *et al.* (2002). Short-chain fatty acids produced by the strains were extracted from 5-day-old broth cultures according to Jousimies-Somer *et al.* (2002). Volatile fatty acids were determined by GC as described by Suihko & Haikara (2001). Non-volatile acids were analysed by HPLC using the method of Rajakylä (1981) with minor modifications (oven temperature 35 °C, 5 mM sulphuric acid as a carrier liquid, flow rate 0.6 ml min⁻¹).

Results of the physiological and biochemical tests are presented in Tables 1 and 2 and in the species descriptions below. None of the strains produced succinic, lactic or pyruvic acids. The volatile fatty acid profiles of the coccoid strains were dominated by C4–C6 acids (Supplementary Table S1 in IJSEM Online), which is characteristic for members of the genus *Megasphaera* (Engelmann & Weiss, 1985; Haikara & Lounatmaa, 1987; Jousimies-Somer *et al.*, 2002; Marchandin *et al.*, 2003), supporting their affiliation to this genus. However, strains VTT E-032341^T and VTT E-97791 differed from the type strains of recognized *Megasphaera* species in that they produced isovaleric acid as the major end product. The fatty acid profiles of the type strains of *M. cerevisiae* and *M. elsdenii* determined in this study are in agreement with the results of Haikara & Lounatmaa (1987) and Suihko & Haikara (2001). The metabolic end products of *M. micronuciformis* VTT E-042113^T were not analysed as it did not grow in PYG or PYF broth. No major differences were found in the volatile fatty acid profiles between the novel rod-shaped strains (VTT E-88329^T, VTT E-88330, VTT E-89371, VTT E-97914) and the type strains of recognized *Pectinatus* species (Supplementary Table S2 in IJSEM Online). All strains also produced H₂S and acetoin, but succinic, lactic and pyruvic acids were not detected. The production of acetate, propionate, H₂S and acetoin as

Table 2. Differential characteristics of *Pectinatus haikarae* sp. nov. and other species of the genus *Pectinatus*

Strains: 1, VTT E-88329^T, VTT E-88330, VTT E-97914 and VTT E-89371; 2a, *P. cerevisiophilus* VTT E-79103^T; 2b, combined data for 11 *P. cerevisiophilus* strains from Haikara *et al.* (1981) and Schleifer *et al.* (1990); 3a, *P. frisingensis* VTT E-79100^T; 3b, combined data for 14 *P. frisingensis* strains from Haikara *et al.* (1981) and Schleifer *et al.* (1990). Symbols: +, 75% or more of the strains are positive; w, 75% or more of the strains are weakly positive; –, 75% or more of the strains are negative; ±, 26–74% of the strains are negative; ND; not determined; R, resistant; S, susceptible. Additional test results: all strains were positive for acid production from adonitol^{ab*}†, L-arabinose^{abc}, DL-erythritol^{abcde}, D-fructose^{abcde}, D-galactose^{abc}, D-glucose^{abcde}, glycerol^{abcde}, mannose^b, rhamnose^{abcde}, D-ribose^{abcde} and utilization of DL-lactate^{bce}. All strains were negative (except those in parentheses) for acid production from glycogen^{abcde}, inulin^{abce}, maltose^{ac} (VTT E-97914, VTT E-79100^T), melezitose^{abce} and raffinose^{abcde}, utilization of succinate†, production of oxidase^{ce}, desulfovibrin and indole^{acde}, hydrolysis of arginine and gelatin^{acde} and reduction of nitrate^{ce}. All strains were susceptible to 1 mg kanamycin and 10 µg colistin and bile discs (VTT E-88330).

Characteristic	1	2a	2b	3a	3b
Catalase activity	+	–	–	–	–
Susceptibility to 5 µg vancomycin	R	S	ND	R	ND
Urease activity	–	+ ^{b*}	±	– ^a	–
Growth at 37 °C	–	+	+	+	+
Acid production from:					
Aesculin	–	+ ^e	–	+ ^c	±
D-Cellobiose	–	+ ^{be}	–	+ ^c	+
Dulcitol	+	+ ^{de}	±	+ ^{ac}	±
i-Inositol	+	– ^{bde}	–	– ^{ac}	+
Lactose	+	w	–	–	–
Mannitol	+	– ^{de}	±	+ ^{ac}	+
α-D-Melibiose	+	w ^{de}	+	– ^{ac}	–
N-Acetylglucosamine	–	– ^{de}	–	+ ^{ac}	+
D-Salicin	–	– ^{de}	+	– ^{ac}	+
Xylitol	+	+ ^d	–	+ ^c	±
D-Xylose	+	w ^b	+	w	–
Utilization of:					
Gluconate	+	+	±	–	±
Pyruvate	–	–	ND	+	ND
Hydrolysis of milk	+	+	ND	–	ND

*Test was also performed in the following study with the following strain and gave congruent result: a, Haikara *et al.* (1981) with VTT E-79100^T; b, Lee *et al.* (1978) with VTT E-79103^T; c, Schleifer *et al.* (1990) with VTT E-79100^T; d, Haikara *et al.* (1981) with VTT E-79103^T; e, Schleifer *et al.* (1990) with VTT E-79103^T.

†Test result only available for the novel strains and *P. frisingensis* VTT E-79100^T and *P. cerevisiophilus* VTT E-79103^T.

the major metabolites supports the affiliation of the novel strains to the genus *Pectinatus* and differentiates them from the closely related *Zymophilus raffinosivorans* and

Zymophilus paucivorans, which do not produce acetoin, and *Selenomonas lacticifex*, which produces large quantities of lactate (Schleifer *et al.*, 1990). The metabolic end products determined for the type strains of *P. frisingensis* and *P. cerevisiiphilus* in this study are in agreement with earlier published data (Lee *et al.*, 1978; Haikara *et al.*, 1981; Haikara, 1989; Schleifer *et al.*, 1990).

Taxonomic considerations

The results of morphological characterization, 16S rRNA gene sequence comparisons, DNA G+C content determinations and metabolic end product analyses show that the novel coccoid and rod-shaped strains are respectively members of the genera *Megasphaera* and *Pectinatus*. Based on the low degree of DNA relatedness (<41%) as measured by DNA–DNA hybridization, the coccoid strains VTT E-97791^T and VTT E-032341^T are not related to each other or to the previously known species of the genus *Megasphaera* at the species level (Wayne *et al.*, 1987). The strains can also be differentiated from each other and from recognized *Megasphaera* species based on cell size, fermentation of carbohydrates, utilization of organic acids, gas production, antibiotic susceptibility, DNA G+C content (Table 1) and/or the sequence of the 16S rRNA gene. Furthermore, they can be discriminated from each other by ribotyping with *EcoRI*. The novel rod-shaped strains (VTT E-88329^T, VTT E-88330, VTT E-89371, VTT E-97914) can be distinguished from the previously described *Pectinatus* species by ribotyping with *EcoRI* and by 16S rRNA gene sequencing. In addition, the following phenotypic characteristics differentiate them from *P. frisingensis* and *P. cerevisiiphilus* strains: inability to grow at 37 °C and ability to hydrolyse milk, positive catalase and negative urease reactions, vancomycin resistance and utilization of different organic acids and carbohydrates (Table 2). Based on these results, we propose two novel *Megasphaera* species, *Megasphaera paucivorans* sp. nov. and *Megasphaera sueciensis* sp. nov., and a novel *Pectinatus* species, *Pectinatus haikarae* sp. nov. As the original description of the genus *Pectinatus* by Lee *et al.* (1978) does not accommodate all of the phenotypic properties of *P. haikarae*, an emended genus description is proposed.

Description of *Megasphaera paucivorans* sp. nov.

Megasphaera paucivorans (pau.ci.vo'rans. L. adj. *paucus* few, little, L. part. adj. *vorans* devouring; N.L. part. adj. *paucivorans* devouring few substrates).

Gram-negative, non-spore-forming and non-motile cocci with a mean size of 1.5×1.2 µm, mainly arranged in pairs. Stationary phase cells may form chains of 20–25 diplococci and cell clumps. Strictly anaerobic. Moderate to good growth (2+ or 3+ on a scale of 0 to 4+) is obtained in autoclaved PYF, PYG, MRS and SMMP media. Growth in SMMP is accompanied by a colour change from violet to yellow. Poor growth in PY medium (1+), but the addition

of 1% (w/v) pyruvate or gluconate results in heavily turbid suspension (4+). Colonies on PYF plates appear after 3 days at 30 °C and after 7 days are yellowish, circular, convex, glossy and opaque with entire margins and a diameter of 1–1.5 mm. Grows at 15–37 °C, with an optimum at around 30 °C, but not at 10 or 45 °C. Major volatile fatty acids produced in beer are butyric and isovaleric acids; H₂S and minor amounts of propionic, isobutyric, valeric and caproic acids are also produced. Other phenotypic properties and characteristics that differentiate the species from other *Megasphaera* species are listed in Table 1.

The type strain, VTT E-032341^T (=DSM 16981^T), was isolated from spoiled Italian beer.

Description of *Megasphaera sueciensis* sp. nov.

Megasphaera sueciensis (sue.ci.en'sis. N.L. fem. adj. *sueciensis* pertaining to Sweden).

Strictly anaerobic, Gram-negative, non-spore-forming and non-motile cocci, mainly arranged in pairs and occasionally in short chains. Mean cell size is 1.2×1.0 µm. Moderate growth (2+ on a scale of 0 to 4+) is obtained in autoclaved PYF, PYG, MRS and SMMP media at 30 °C. The addition of 1% (w/v) pyruvate or gluconate to the PY medium markedly stimulates growth. Colonies on PYF and PYG plates appear after 4 days at 30 °C and after 7 days are slightly yellowish, glossy, convex, opaque, smooth and circular with entire edges and a diameter of 0.5–0.8 mm. Grows at 15–37 °C, with an optimum at around 30 °C, but not at 10 or 45 °C. Other physiological properties and characteristics that differentiate this species from other *Megasphaera* species are shown in Table 1.

The type strain, VTT E-97791^T (=DSM 17042^T), was isolated from spoiled Swedish beer.

Emended description of *Pectinatus* Lee *et al.* 1978

Pectinatus [Pec.ti.na'tus. L. part. adj. *pectinatus* combed (bacteria)].

Cells are non-spore-forming, slightly curved to helical rods, $0.4\text{--}0.9 \times 2\text{--}50$ µm or more, with rounded ends and a Gram-negative cell wall. They occur singly, in pairs or rarely in short chains. Cells are usually motile by means of comb-like flagellation which emanates from only one side of a cell. Cadaverine or putrescine is found in the cell-wall peptidoglycan. Organisms are strictly anaerobic mesophiles with fermentative metabolism. Glucose and fructose are mainly metabolized to acetic and propionic acids. H₂S and acetoin and occasionally minor amounts of succinic acid are produced. Cells do not synthesize cytochrome oxidase, desulfovibrin or indole, hydrolyse arginine or gelatin or reduce nitrate. The G+C content of the DNA is 38–41 mol%. The species of the genus can be separated from each other by using various genetic and phenotypic criteria. Isolated from beer and brewing processes. The type species

is *Pectinatus cerevisiiphilus* Lee *et al.* 1978 emend. Schleifer *et al.* 1990.

Description of *Pectinatus haikarae* sp. nov.

Pectinatus haikarae (hai.ka'rae. N.L. gen. fem. n. *haikarae* of Haikara, named after Dr Auli Haikara for her many contributions to the characterization and detection of *Pectinatus* species).

Cells are Gram-negative, non-spore-forming, straight or slightly curved, flexible rods with rounded ends, $0.6\text{--}0.8 \times 3\text{--}50\text{ }\mu\text{m}$ or more in size. They usually occur singly and occasionally in pairs. Stationary-phase cells may form long, helical filaments and round and loop-shaped formations or have club-shaped, distended ends. Young cells are motile, following an X-like pattern. Old cells exhibit slow, snake-like movements or are non-motile. Strictly anaerobic. Grows at $15\text{--}30\text{ }^{\circ}\text{C}$, but not at 10 or $37\text{ }^{\circ}\text{C}$. The optimum lies between 20 and $30\text{ }^{\circ}\text{C}$. Good growth ($3+$ or $4+$ on a scale of 0 to $4+$) is obtained in PYF, PYG, MRS and SMMP media after $1\text{--}2$ days at $30\text{ }^{\circ}\text{C}$. Weak growth on supplemented brucella blood agar and PY medium. Colonies on PYF and PYG plates after 3 days at $30\text{ }^{\circ}\text{C}$ are convex to pyramidal, glossy, opaque, cream to grey in colour and circular with entire margins and a diameter of $0.5\text{--}2.5\text{ mm}$. Major products of fructose fermentation are propionic and acetic acids. Acetoin and H_2S are also produced. Differential characteristics compared with other *Pectinatus* species are shown in Table 2.

The type strain is VTT E-88329^T (=DSM 16980^T), with the DNA G+C content of $38.8\text{ mol}\%$ (T_m), isolated from a brewery bottling hall in Finland. Strains have also been isolated from spoiled German and Finnish beers.

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