Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer

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Pediococci are found in foods and on plants and as beer-spoilage agents. The goal of the present study was to use the DNA sequences of the first three variable regions of the 16S rRNA gene, the 16S–23S rRNA internally transcribed spacer region sequence and approximately a third of the 60 kDa heat-shock protein gene to elucidate phylogenetic groupings within the genus *Pediococcus*. Phylogenetic trees were created with sequence data from 31 *Pediococcus* and three *Lactobacillus* isolates. Complete 16S rRNA gene sequences from selected *Pediococcus* isolates were also examined. The results were interpreted in relation to the currently accepted *Pediococcus* species. We found that, where previously done, speciation of many *Pediococcus* isolates is inaccurate. Also, one grouping of seven isolates did not include any currently recognized *Pediococcus* species type isolate. Our phylogenetic analyses support the conclusion that these seven isolates, all of brewing spoilage origin, belong to a novel species, for which the name *Pediococcus claussenii* sp. nov. is proposed (type strain P06^T = ATCC BAA-344^T = DSM 14800^T). Phylogenetic analysis has therefore helped to resolve problems surrounding species identification of *Pediococcus* isolates.

Keywords: beer-spoilage bacteria, lactic acid bacteria, *Pediococcus claussenii*, *Pediococcus* phylogenetics

INTRODUCTION

Pediococci are lactic acid bacteria found on plants and in many fermented foods (Garvie, 1986). Pediococci have been added as nutritional enhancers of animal feeds and, more recently, have been used in polymer research for production of biodegradable packaging materials. In addition to beneficial aspects, when present in beer, pediococci produce diacetyl, causing a quality problem for brewers. *Pediococcus* speciation

has been based on habitat, DNA–DNA hybridization results and tolerance of temperature, pH and NaCl (Garvie, 1974, 1986). However, speciation of *Pediococcus* isolates has been difficult.

While phenotypic patterns are useful in classifying variable organisms, bacteria often exhibit similar characteristics, making speciation difficult. As protein and nucleic acid sequences allow direct assessment of evolutionary relationships, phylogenetics is increasingly being used to redefine bacterial taxonomy. The most extensive studies regarding the phylogenetics of bacteria are based on the 16S rRNA gene. Using 16S rRNA gene sequences, the genus *Pediococcus* was reported to fall within the *Lactobacillus casei* branch of the lactic acid bacteria, with *Pediococcus dextrinicus* as a distantly related species (Collins *et al.*, 1990, 1991; Collins & Wallbanks, 1992). Within the *Pediococcus* taxon, *Pediococcus damnosus* and *Pediococcus parvulus* formed one cluster, while *Pediococcus acidilactici* and

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Abbreviations: HSP60, 60 kDa heat-shock protein; ITS, internally transcribed spacer; MRS, Man–Rogosa–Sharpe; OTU, operational taxonomic unit; RDP, Ribosomal Database Project.

The GenBank accession numbers for the 16S rRNA gene, ITS and HSP60 sequences of isolate P06^T are AF404716, AF405360 and AF405395. Accession numbers of other sequences obtained in this study are listed in Table 1.

Pediococcus pentosaceus formed a second cluster. Currently, seven *Pediococcus* species are recognized, including *P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *Pediococcus inopinatus*, *P. parvulus*, *P. pentosaceus* and *Pediococcus urinaeequi*. It should be noted that Collins *et al.* (1990) have suggested that *P. urinaeequi* should be reclassified in the genus *Aerococcus*; however, no official opinion on this suggestion has been rendered.

Using 16S rRNA gene sequences to infer organismal phylogeny has been criticized, as it assumes that one molecule reflects organismal evolutionary history. Variability in the 16S–23S rRNA internally transcribed spacer (ITS) region has also been used for phylogenetic analyses (Frothingham & Wilson, 1994; Nour, 1998). To study the ITS region, conserved flanking regions of the 16S and 23S rRNA genes are used as PCR primer-binding sites. Further corroboration of phylogeny can be based on highly conserved proteins that are essential for survival (i.e. have properties of a molecular chronometer; Woese, 1987). One such protein is the 60 kDa heat-shock protein (HSP60), which performs an essential protein-folding function in cells (Gupta *et al.*, 1995; Braig, 1998).

In our examination of the phylogeny of the genus *Pediococcus*, we used sequence information from three genomic regions, including the 16S rRNA gene, 16S–23S rRNA ITS region and HSP60 gene. We also examined a larger number of *Pediococcus* isolates than previous studies of this genus. Each of the three phylogenetic trees created have similar topologies. The results are used to redefine the current species designations of *Pediococcus* isolates and to define a novel *Pediococcus* species.

METHODS

Bacteria. *Lactobacillus* and *Pediococcus* isolates examined are listed in Table 1. Each isolate was first streaked on Man–Rogosa–Sharpe (MRS) agar and grown at 27 °C. Colony morphology, exopolysaccharide production and Gram-staining properties were determined and five colonies with uniform properties were combined and grown in MRS broth. Substrate fermentation was assessed using the API 50 CH kit (bioMérieux). Incubation was at 27 °C, with results recorded daily for 7 days to ensure that a minor contaminant was not growing as well. *Escherichia coli* ATCC 11030 and *Staphylococcus aureus* ATCC 25923 were propagated at 37 °C in Oxoid nutrient broth 2. All bacteria were stored at –70 °C in double-strength skim milk.

G + C content determination. Cells from 10 ml medium were lysed at 60 °C for 30 min in 400 µl 10 mM Tris/HCl, pH 8.0, 10 mM EDTA and 0.5% SDS, to which 6 µl proteinase K (20 mg ml⁻¹ in 10 mM Tris/HCl, pH 7.5, containing 20 mM CaCl₂ and 50% glycerol) was added. After boiling for 5 min, DNA was isolated using an Epicentre Masterpure DNA extraction kit. Extracted DNA was digested according to the standard method of Mesbah *et al.* (1989). Nucleoside determination was done with a Waters HPLC System fitted with a Waters C18 column (5 µm, 300 Å, 3.9 × 150 mm). The column was developed for 3 min with 50 mM potassium phosphate buffer, pH 5.6. Over the next 17 min, methanol content was increased linearly from 0 to 20%. The last 4 min

of each run was 20% methanol in buffer. The column was run at 25 °C for one analysis, while two analyses were done at 30 °C. As the HPLC conditions do not resolve uracil from dC, G + C content (mol%) was calculated as $100 \times [dG]/([dG] + [T])$.

DNA extraction. Bacterial cells from 2 ml overnight culture were collected by centrifugation and resuspended in 200 µl of a solution of 5 mg lysozyme ml⁻¹. After 30 min at room temperature, DNA was extracted using the universal procedure of Golbang *et al.* (1996). The extracted DNA was allowed to air dry for 5 min before being dissolved in 50 µl autoclaved, 0.2 µm-filtered reverse-osmosis deionized water. Samples of each batch of MRS broth were subjected to DNA extraction and PCR amplification to ensure that contamination by unknown microbial contaminants had not occurred.

PCR amplification. Each sample contained 1 × *Taq* buffer (Invitrogen), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer, 1 µl template bacterial DNA and 2.5 U Platinum *Taq* polymerase (Invitrogen) in 100 µl reverse-osmosis deionized water. Primers 8F (5'-AGAGTTTGAT-CCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTG-CTGG-3') were used to amplify the first 526 bp of the 16S rRNA gene (Relman, 1991; Muyzer *et al.*, 1993). To obtain the whole sequence of the 16S rRNA gene for isolates P03 and P06, primers 515F (5'-TGCCAGCAGCCGCGGTAA-3') and 1542R (5'-GGCTACCTTGTTACGACTT-3') were used (Weisburg *et al.*, 1989). Primers S2 (5'-TTGTACAC-ACCGCCCGTCA-3') and S7 (5'-GGTACTTAGATGTT-TCAGTTC-3') were used to amplify the ITS region (Gürtler & Stanisich, 1996). The primers used to amplify a portion of the HSP60 gene were H729 [5'-cgccagggttttccagtcacgacG-AIIIIGCIGGIGA(T/C)GGIACIACIAC-3'] and H730 [5'-agcggataacaatttcacacagga(T/C)(T/G)I(T/C)(T/G)-ITCICC(A/G)AAICCIIGGIGC(T/C)TT-3'] (Goh *et al.*, 2000). The conserved residues in the HSP60 gene are flanked by M13 sequences M13–40 and M13–48 (lower-case).

With primers 8F and 534R, the PCR conditions were one cycle of 5 min at 95 °C, 35 cycles of 1.25 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C and one cycle of 10 min at 72 °C. With primers 515F and 1542R, the annealing temperature was reduced to 42 °C. When the ITS region was amplified, the annealing temperature was 55 °C and last cycle lasted for 8 min (Gürtler & Stanisich, 1996). For amplification of the HSP60 gene, the first cycle was for 3 min, 40 cycles were used and the annealing temperature was 37 °C (Goh *et al.*, 1996, 2000).

PCR product purification. PCR amplification of the 16S rRNA gene and HSP60 gene segments gave single bands of 526 and 652 bp, respectively. The products were purified using a QIAquick Spin PCR purification system (Qiagen). The ITS region PCR resulted in one bright band at 530 bp and two weaker bands, at 650 and 750 bp. The latter two bands are of the appropriate sizes for ITS regions containing one and two tRNAs, respectively (Gürtler & Stanisich, 1996; Nour, 1998). As an ITS region containing no tRNAs would yield a product of approximately 530 bp (Nour, 1998), this band was selected. After the band was excised from the gel, the DNA was purified as described by Zhen & Swank (1993).

DNA sequencing. PCR products were sequenced using the fluorescence-based dideoxy chain-termination method with a thermocycler model GeneAmp PCR system 9700. A Big Dye Terminator kit was used, with an aliquot of 10 µl containing approximately 30 ng PCR product and 3.2 pM of

Table 1. Bacterial isolates used in this study

Culture collections: BSO, Dr B. Kirsop, Institute for Biotechnology, Cambridge, UK; CCC, Coors Brewing Company, Golden, CO, USA; Molson, Molson Breweries of Canada Ltd, Montreal, Quebec, Canada; ATCC, American Type Culture Collection, Manassas, VA, USA; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

Isolate	Received as	Revised nomenclature	GenBank accession numbers		
			16S rRNA	ITS	HSP60
P01	<i>Pediococcus</i> sp. BSO 77	<i>P. acidilactici</i>	AF404711	AF405355	AF405390
P02	<i>Pediococcus</i> sp. BSO 54	<i>P. acidilactici</i>	AF404712	AF405356	AF405391
P03	<i>Pediococcus</i> sp. CCC B1208	<i>P. clausenii</i> sp. nov.	AF404713*	AF405357	AF405392
P04	<i>P. damnosus</i> Molson B48	<i>P. damnosus</i>	AF404714	AF405358	AF405393
P05	<i>P. pentosaceus</i> Molson B49	<i>P. damnosus</i>	AF404715	AF405359	AF405394
P06 [†]	<i>Pediococcus</i> sp. Molson B71	<i>P. clausenii</i> sp. nov.	AF404716*	AF405360	AF405395
P07	<i>Pediococcus</i> sp. Molson B72	<i>P. acidilactici</i>	AF404717	AF405361	AF405396
P08	<i>Pediococcus</i> sp. Molson B76	<i>P. damnosus</i>	AF404718	AF405362	AF405397
P09	<i>Pediococcus</i> sp. Molson B77	<i>P. acidilactici</i>	AF404719	AF405363	AF405398
P10	<i>Pediococcus</i> sp. Molson 77b	<i>P. acidilactici</i>	AF404720	AF405364	AF405399
P11 ^T	<i>P. damnosus</i> ATCC 29358 ^T	<i>P. damnosus</i>	AF404721	AF405365	AF405400
P13	<i>P. damnosus</i> ATCC 25249	<i>P. damnosus</i>	AF404722	AF405366	AF405401
P14	<i>P. damnosus</i> ATCC 11308	<i>P. damnosus</i>	AF404723	AF405367	AF405402
P15	<i>P. acidilactici</i> NCIMB 6990	<i>P. acidilactici</i>	AF404724	AF405368	AF405403
P16 ^T	<i>P. pentosaceus</i> ATCC 33316 ^T	<i>P. pentosaceus</i>	AF404725	AF405369	AF405404
P18	<i>P. damnosus</i> CCC B1056	<i>P. clausenii</i> sp. nov.	AF404727	AF405371	AF405406
P19	<i>Tetragenococcus</i> sp. CCC B1098	<i>P. clausenii</i> sp. nov.	AF404728	AF405372	AF405407
P20	<i>P. dextrinicus</i> CCC B1099	<i>P. clausenii</i> sp. nov.	AF404729	AF405373	AF405408
P21	<i>P. pentosaceus</i> CCC B1100	<i>P. clausenii</i> sp. nov.	AF404730	AF405374	AF405409
P22	<i>Pediococcus</i> sp. CCC B1260	<i>P. clausenii</i> sp. nov.	AF404731	AF405375	AF405410
P23	<i>P. parvulus</i> Spain 2.6 [‡]	<i>P. parvulus</i>	AF404732	AF405376	AF405411
P24	<i>P. pentosaceus</i> ATCC 33314	<i>P. pentosaceus</i>	AF404733	AF405377	AF405412
P25	<i>P. acidilactici</i> ATCC 12697	<i>P. acidilactici</i>	AF404734	AF405378	AF405413
P27	<i>P. acidilactici</i> ATCC 8081	<i>P. pentosaceus</i>	AF404735	AF405379	AF405414
P28	<i>P. acidilactici</i> ATCC 25740	<i>P. acidilactici</i>	AF404736	AF405380	AF405415
P29	<i>P. damnosus</i> ATCC 43013	<i>P. parvulus</i>	AF404737	AF405381	AF405416
P31	<i>P. pentosaceus</i> ATCC 10791	<i>P. pentosaceus</i>	AF404738	AF405382	AF405417
P35	<i>P. damnosus</i> ATCC 11309	<i>P. pentosaceus</i>	AF404739	AF405383	AF405418
P36	<i>P. damnosus</i> ATCC 25249	<i>P. damnosus</i>	AF404740	AF405384	AF405419
P37 ^T	<i>P. inopinatus</i> ATCC 49902 ^T	<i>P. inopinatus</i>	AF404741	AF405385	AF405420
P38	<i>P. acidilactici</i> Pac 1.0 ^b	<i>P. acidilactici</i>	AF404742	AF405386	AF405421
L27	<i>Lactobacillus casei</i> ATCC 334 ^c	<i>L. casei</i>	AF404708	AF405352	AF405387
L44 ^T	<i>Lactobacillus brevis</i> ATCC 14869 ^T	<i>L. brevis</i>	AF404709	AF405353	AF405388
L71 ^T	<i>Lactobacillus plantarum</i> ATCC 14917 ^T	<i>L. plantarum</i>	AF404710	AF405354	AF405389

* Sequence is for the full-length 16S rRNA gene.

[†] Proposed type strain of *P. clausenii* sp. nov. (= ATCC BAA-344^T = DSM 14800^T).

[‡] Strains supplied by: *a*, Dr K. Fernandez, Department of Applied Chemistry, University del Pais Vasco, Gipuzka, Spain; *b*, Dr R. Wheatcroft, Agriculture and Agri-food Canada, Guelph, Ontario, Canada; and *c*, Dr Gregor Reid, Lawson Research Institute, London, Ontario, Canada.

primers in each reaction. After 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, sequencing was done with an Applied Biosystems 373 DNA sequencer STRETCH model. Sequencing and PCR primers were the same except for HSP60 DNA, where primers M13–40F and M13–48R were used for sequencing.

Sequence analysis. For the 16S rRNA gene and ITS region, the PCR products for all isolates were sequenced in both directions. The HSP60 sequence data were of sufficiently high quality that, for most isolates, only one direction of

sequence was completed. The HSP60 DNA sequence data were translated into amino acid sequence and both the DNA and protein sequence data were examined. Each sequence was queried for homologies with BLAST (Altschul *et al.*, 1990). Sequence results for the 16S rRNA gene were also queried against entries in the Ribosomal Database Project (RDP; Maidak *et al.*, 2000) (<http://www.cme.msn.edu/RDP/html/index.html>).

Phylogenetic analysis. Sequence alignments were created using CLUSTAL X (Thompson *et al.*, 1994) applying default

parameters. Each alignment was visualized and edited using GeneDoc (Nicholas *et al.*, 1997). Alignments were edited to conform with structural information and inferred locations of variable and conserved regions (Neefs *et al.*, 1993). Phylogenetic trees were constructed using programs from the PHYLIP package (Felsenstein, 1989) including SEQBOOT (1000 replicates), DNADIST (Jukes–Cantor model), PROTDIST (Kimura model), NEIGHBOR (neighbour-joining model) and CONSENSE (outgroup option). Phylogenetic trees were visualized using TreeView (Page, 1996). Reliable nodes were also found with maximum-likelihood, Jin–Nei and Kimura's two-parameter analysis followed by neighbour-joining (Saitou & Nei, 1987).

RESULTS AND DISCUSSION

The three genomic regions targeted were successfully amplified and sequenced for all isolates listed in Table 1. Distance matrix-based phylogenetic trees for the 16S rRNA gene, 16S–23S rRNA ITS region and HSP60 gene deduced amino acid sequences are presented in Fig. 1(a–c). Similar phylogenetic trees were obtained using maximum-likelihood and parsimony methods (not shown). Also, when the first three variable regions of the 16S rRNA gene were examined individually, no significant differences were found compared with the tree in Fig. 1(a) (not shown). In all analyses, three *Lactobacillus* isolates were included as outgroups and to illustrate the relationship between the genera *Lactobacillus* and *Pediococcus*.

The similarity of the trees in Fig. 1 strongly supports the assignment of *Pediococcus* isolates to one of six phylogenetically based operational taxonomic units (OTUs), which form three groups or clusters. OTU is a generic term (Clewley, 1998) that can mean a group of organisms, a single species, a gene or a region of a gene. For the present paper, an OTU corresponds to a species designation. In our distance-matrix analysis of 16S rRNA gene sequences, distance values < 5.0 were taken to indicate a cluster (i.e. of OTUs), while distances < 1.0 were taken to indicate an OTU or species [comparable OTUs for the ITS region and HSP60 sequences are respectively given in Fig. 1(b) and (c)]. Each sequence from the 16S rRNA gene was queried against the RDP (Table 2). Complete 16S rRNA gene sequences from two novel isolates included in this study were compared with 16S rRNA gene sequences from pediococci already in GenBank and a phylogenetic tree was created (Fig. 2).

Pediococcus cluster 1 consists of a single OTU containing isolates collected by two independent breweries. For each of the phylogenetic trees in Fig. 1, OTU 1 forms a distinct group (with a high bootstrap value at the branch point) containing isolates P03, P06^T, P18, P19, P20, P21 and P22. Except for isolates P03 and P21, all of these isolates produce an exopolysaccharide. However, this property can be lost with repeated culturing in broth medium (unpublished results). 16S rRNA gene sequencing of ropy and non-ropy colonies from isolate P06^T found no sequence differences, indicating that the ropy property does not affect, nor is affected by, the 16S rRNA gene and its function(s).

A comparison was made of isolates P03 and P06^T with other GenBank entries based on the whole 16S rRNA gene. The phylogenetic tree created (Fig. 2) shows that isolates included in OTU 1 are distant from *Pediococcus* entries included from GenBank, while still remaining within the *Pediococcus*–*L. casei* cluster (Collins *et al.*, 1991). Table 2 shows that OTU 1 isolates have low similarity to *Pediococcus* type isolates based on an RDP similarity search (the closest is *P. pentosaceus* ATCC 33316^T, with a similarity value of 0.74). Finally, Table 3 shows that OTU 1 isolates have a distinct substrate fermentation pattern. These results, along with a G + C content in the range 34–42 mol% expected for pediococci (Garvie, 1986) (Table 4), document the uniqueness of the bacteria in OTU 1 and support our contention that OTU 1 represents a novel *Pediococcus* species.

Pediococcus cluster 2 contains two OTUs. OTU 2A includes isolates identified as *P. acidilactici* or not yet speciated. The current type isolate of *P. acidilactici* was not included in this study, but the sequence of *P. acidilactici* DSM 20284^T, available in the RDP, was found to be similar to OTU 2A 16S rRNA sequences (Table 2) and *P. acidilactici* DSM 20284^T (accession no. M58833) groups closely with the OTU 2A isolates in the 16S rRNA phylogenetic tree (not shown). OTU 2B includes isolate P16^T (= *P. pentosaceus* ATCC 33316^T). Isolates P24, P27 and P35 all have 16S rRNA gene and ITS region sequences similar to those of isolate P16^T (Fig. 1a, b), while isolate P35 is slightly more divergent in the HSP60 protein phylogenetic tree (Fig. 1c). According to the RDP query, OTU 2B 16S rRNA gene sequences are similar to *P. pentosaceus* ATCC 33316^T. Overall, these data suggest that isolates comprising OTUs 2A and 2B respectively be assigned to *P. acidilactici* and *P. pentosaceus*.

It is not surprising that isolates comprising OTUs 2A and 2B are related closely enough to form a taxonomic cluster, as these two species are similar based on morphological, biochemical and physiological properties (Garvie, 1986). Previously, these two species have only been distinguished by DNA–DNA hybridization studies (Back & Stackebrandt, 1978) and whole 16S rRNA gene sequence data (Collins *et al.*, 1990). Our results show that *P. acidilactici* and *P. pentosaceus* can be separated using the first 526 bp of the 16S rRNA gene and using ITS region and HSP60 protein sequence data as well.

The remaining *Pediococcus* isolates analysed form three OTUs comprising the third taxonomic cluster in our analyses. OTU 3A contains isolate P11^T (= *P. damnosus* ATCC 29358^T). This isolate was also most similar to *P. damnosus* in an RDP query (Table 2). Further verification comes from isolates P13 and P36 (both received as *P. damnosus* ATCC 25249), which were obtained in different years from different sources. We found only 1 bp change for the 16S rRNA gene and ITS region and none in the HSP60 protein gene for these two isolates. As such, all OTU 3A isolates should be assigned to *P. damnosus*.

Fig. 1. Unrooted trees showing phylogenetic distances based on (a) DNA sequences from the first three variable regions of the 16S rRNA gene, (b) DNA sequences from the 16S rRNA gene (3' end), the 16S-23S rRNA ITS region and the 23S rRNA gene (5' end) and (c) a 184 amino acid sequence from a portion of the HSP60 gene. In (b), the sequences from the ITS region, which varied in length from 193 to 236 bp, were subjected to a BLAST search (<http://www.ncbi.nlm.nih.gov/blast>) to ensure that *Lactobacillus* and *Pediococcus* isolates were involved and that no tRNAs were present in the sequences (Altschul *et al.*, 1990). Bars, 1 nucleotide difference per 100 bp (a, b) or 1 difference per 100 amino acids (c). Bootstrap values based upon 1000 replicates are included at the major branch points.

Table 2. RDP similarity query

OTUs corresponds to OTU designations indicated in Fig. 1. Mean scores were calculated based on sequence similarity (Maidak *et al.*, 2000).

OTU	Isolates in OTU	Most similar <i>Pediococcus</i> sequence in the RDP	Mean score (SD)
1	P03, P06 ^T , P18, P19, P20, P21, P22	<i>P. pentosaceus</i> ATCC 33316 ^T	0.74 (0.01)
2A	P01, P02, P07, P09, P10, P15, P25, P28, P38	<i>P. acidilactici</i> DSM 20284 ^T	0.90 (0.03)
2B	P16 ^T , P24, P27, P31, P35	<i>P. pentosaceus</i> ATCC 33316 ^T	0.95 (0.03)
3A	P04, P05, P08, P11 ^T , P13, P14, P36	<i>P. damnosus</i> JCM 5886 ^T	0.97 (0.04)
3B	P23, P29	<i>P. parvulus</i> JCM 5889 ^T	0.96 (0.01)
3C	P37 ^T	<i>P. damnosus</i> JCM 5886 ^T	0.88 (0.00)

OTU 3B consists of isolates P23 and P29. These two isolates are most similar to *P. parvulus* JCM 5886^T according to sequences in the RDP (Table 2). Consequently, we believe that both isolates are *P. parvulus*.

Based on 16S rRNA gene data, *P. damnosus* (OTU 3A) and *P. parvulus* (OTU 3B) have previously been acknowledged as closely related (Collins *et al.*, 1990). Although each of the three regions we studied indicates

Fig. 2. Unrooted tree showing phylogenetic distances between *Pediococcus* isolates based on the full-length 16S rRNA gene. Data for isolates P03 and P06^T are from this study. Other sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). Accession numbers are listed in parentheses. Bar, 1 nucleotide difference per 100 bp. Bootstrap values based upon 1000 replicates are included at the major branch points.

that OTUs 3A and 3B are distinct, the HSP60 protein sequence had the greatest discriminating power (Fig. 1c).

OTU 3C includes isolate P37^T (= *P. inopinatus* ATCC 499902^T). For the 16S rRNA gene and ITS region (Fig. 1a, b), isolate P37^T exhibited a close genetic relatedness to both *P. damnosus* (OTU 3A) and *P. parvulus* (OTU 3B), although more divergence was found when the

Table 4. DNA G + C contents

Values are given in mol %.

Isolate	Mean $\pm$ SD (n)
<i>P. claussenii</i> P06 ^T (non-exopolysaccharide)	40.2 $\pm$ 0.2 (3)
<i>P. claussenii</i> P06 ^T (exopolysaccharide)	41.1 $\pm$ 0.6 (3)
<i>P. damnosus</i> P11 ^T	42.4 $\pm$ 0.4 (3)
<i>E. coli</i> ATCC 11030	51.7 $\pm$ 1.0 (2)
<i>S. aureus</i> ATCC 25923	35.9 $\pm$ 0.6 (2)
Salmon-sperm DNA	46.5 $\pm$ 0.7 (2)

HSP60 gene sequence was analysed (Fig. 1c). Genome DNA–DNA hybridization studies suggest a close relationship between *P. inopinatus* and *P. damnosus* (Back & Stackebrandt, 1978). Also, although *P. inopinatus* and *P. parvulus* are similar on the basis of their growth habitats, these species have been separated based on electrophoretic patterns of their lactose dehydrogenases (Back & Stackebrandt, 1978). Previous phylogenetic studies have not included any *P. inopinatus* DNA sequences and rRNA gene sequences are unavailable in GenBank. Nonetheless, our data show that isolate P37^T is distant from the other cluster 3 OTUs, indicating that it represents an independent species, i.e. *P. inopinatus*.

We originally expected all of the currently accepted *Pediococcus* species, including *P. dextrinicus*, to be represented by the bacterial isolates analysed here. This expectation was based on isolate P20 coming to us

Table 3. API 50 CH substrate utilization by *P. claussenii* sp. nov.

Maximal changes were recorded on day 2 for non-ropy isolates and on day 3 for ropy isolates. *N*-Acetylglucosamine, amygdalin, cellobiose, aesculin, D-fructose, β -gentiobiose, D-glucose, D-mannose and trehalose gave positive results for all of the *Pediococcus* isolates examined. Negative fermentation results for all isolates were obtained with adonitol, starch, D- and L-arabitol, D-arabinoose, dulcitol, erythritol, D-fucose, gluconate, 2- and 5-ketogluconate, methyl α -D-glucoside, glycerol, glycogen, inositol, inulin, D-lyxose, methyl α -D-mannoside, melibiose, melezitose, rhamnose, sorbitol, L-sorbose, D-turanose, L-xylose, methyl β -xyloside and xylitol. Both ropy and non-ropy strains of *P. claussenii* isolates P06^T, P18, P19, P20 and P22 were examined and the fermentation results were the same. *P. claussenii* isolates P03 and P21 were also examined.

Substrate	<i>P. claussenii</i> (7 isolates)	<i>P. damnosus</i> P11 ^T	<i>P. acidilactici</i> P15	<i>P. pentosaceus</i> P16 ^T
L-Arabinose	—	—	+	+
Arbutin	+	+	—	+
Galactose	—	+	+	+
Lactose	—	—	—	+
Maltose	+*	+	—	+
Mannitol	+*	—	—	—
D-Raffinose	—	—	—	+
Ribose	+	—	+	+
Sucrose	—	—	—	+
Salicin	+	+	—	+
D-Tagatose	—	—	+	+
D-Xylose	—	—	+	+

* Not fermented by *P. claussenii* P03.

identified as *P. dextrinicus*. However, in all three phylogenetic trees, this isolate fell into OTU 1, namely *Pediococcus clausenii* sp. nov. The inclusion of *P. dextrinicus* ATCC 33087^T in the phylogenetic tree created with full-length 16S rRNA gene sequence data (Fig. 2) reveals that *P. dextrinicus* is an outlier compared with other pediococci. This fits with the RDP, which has *P. dextrinicus* within the *Lactobacillus perolens* subgroup, not the *Pediococcus* subgroup, of the *Lactobacillus* division. Results obtained by Collins *et al.* (1990) and Stiles & Holzapfel (1997) also group *P. dextrinicus* within the lactobacilli. These findings appear to support the reclassification of *P. dextrinicus* within *Lactobacillus*. However, to date, no formal request has been made for this change.

The phylogenetic analyses presented here establish that the 16S rRNA gene, ITS region and HSP60 gene sequences are all useful in delineating species of the genus *Pediococcus*. We believe that the total congruence of the three phylogenetic trees allows taxonomic assignments to be made with much more conviction than if based on just one DNA sequence (e.g. the 16S rRNA gene). More specifically, the consistency of OTU placement for the 31 isolates included in this study strongly supports the conclusion that many *Pediococcus* species designations should be revised (as indicated in Table 1) and the conclusion that OTU 1 does indeed represent a novel *Pediococcus* species, for which the name *Pediococcus clausenii* sp. nov. is proposed.

Description of *Pediococcus clausenii* sp. nov.

Pediococcus clausenii (claus'sen.i.i. N.L. n. *clausenii* in honour of N. H. Clausen, who first used the genus name *Pediococcus* in 1903 for isolates of beer-spoilage bacteria).

The proposal of the name *Pediococcus clausenii* avoids any confusion that may have arisen with the revival of obsolete names such as '*Pediococcus cerevisiae*', '*Pediococcus flavus*', '*Pediococcus gadidarum*', '*Pediococcus odoris*' or '*Pediococcus tetrus*'.

Cells are cocci, 0.6–1 µm in diameter. They are Gram-positive, non-motile, do not form spores and occur singly, in pairs or in tetrads. Colonies are white, convex, smooth, circular and opaque if exopolysaccharide is not present. When exopolysaccharide is produced, colonies are grey, convex, circular, smooth, glistening, opaque and sticky. In broth, growth causes turbidity. The broth clears in a few days if the cells are non-ropy. For ropy cells, the turbidity remains much longer, as the cells settle slowly. Acid is produced from *N*-acetylglucosamine, amygdalin, arbutin, cellobiose, aesculin, D-fructose, β-gentiobiose, D-glucose, D-mannose, ribose, salicin and trehalose. Most strains ferment mannitol and maltose. Acid is not produced from adonitol, starch, L-arabinose, D- or L-arabitol, D-arabinose, dulcitol, erythritol, D-fucose, galactose, gluconate, 2- or 5-ketogluconate, methyl α-D-glucoside, glycerol, glycogen, inositol, inulin, lactose, D-xylose, methyl α-D-mannoside, melibiose, melezitose, D-raffinose, rhamnose, sucrose, sorbitol, L-sorbose, D-tagatose, D-turanose, D- or L-xylose, methyl β-xyloside or xylitol. Fermentation patterns for ropy and non-ropy variants are identical.

All isolates reported here were brewery isolates. The G+C content is 40.0–41.7 mol%. The type strain is isolate P06^T (= ATCC BAA-344^T = DSM 14800^T). The description of the type isolate corresponds to that of the species. This isolate can be obtained in an exopolysaccharide-producing form; however, this characteristic can be lost upon repeated broth culture. Colonies showing a ropy phenotype should be used frequently to initiate broth cultures to maintain the ropy phenotype in liquid medium.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Back, W. & Stackebrandt, E. (1978). DNS/DNS homologie studien innerhalb der Gattung *Pediococcus*. *Arch Microbiol* **118**, 79–85.
- Braig, K. (1998). Chaperonins. *Curr Opin Struct Biol* **8**, 159–165.
- Clewley, J. P. (1998). A user's guide to producing and interpreting tree diagrams in taxonomy and phylogenetics. Part I. Introduction and naming of parts. *Commun Dis Public Health* **1**, 64–66.
- Collins, M. D. & Wallbanks, S. (1992). Comparative sequence analyses of the 16S rRNA genes of *Lactobacillus minutus*, *Lactobacillus rimae* and *Streptococcus parvulus*: proposal for the creation of a new genus *Atopobium*. *FEMS Microbiol Lett* **95**, 235–240.
- Collins, M. D., Williams, A. M. & Wallbanks, S. (1990). The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. *FEMS Microbiol Lett* **70**, 255–262.
- Collins, M. D., Rodriguez, U., Ash, C., Aguirre, M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M. & Wallbanks, S. (1991). Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol Lett* **77**, 5–12.
- Felsenstein, J. (1989). PHYLIP – Phylogeny Inference Package (version 3.2). *Cladistics* **5**, 164–166.
- Frothingham, R. & Wilson, K. H. (1994). Molecular phylogeny of the *Mycobacterium avium* complex demonstrates clinically meaningful divisions. *J Infect Dis* **169**, 305–312.
- Garvie, E. I. (1974). Nomenclatural problems of the pediococci. Request for an Opinion. *Int J Syst Bacteriol* **24**, 301–306.
- Garvie, E. I. (1986). Genus *Pediococcus* Clausen 1903, 68^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1075–1079. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Goh, S. H., Potter, S., Wood, J. O., Hemmingsen, S. M., Reynolds, R. P. & Chow, A. W. (1996). HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *J Clin Microbiol* **34**, 818–823.

- Goh, S. H., Facklam, R. R., Chang, M. & 8 other authors (2000). Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. *J Clin Microbiol* **38**, 3953–3959.
- Golbang, N., Burnie, J. P., Klapper, P. E., Bostock, A. & Williamson, P. (1996). Sensitive and universal method for microbial DNA extraction from blood products. *J Clin Pathol* **49**, 861–863.
- Gupta, R. S. (1995). Evolution of the chaperonin families (Hsp60, Hsp10 and Tep-1) of proteins and the origin of eukaryotic cells. *Mol Microbiol* **15**, 1–11.
- Gürtler, V. & Stanisch, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rRNA spacer region. *Microbiology* **142**, 3–16.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 9 other authors (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.
- Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S. & De Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res* **21**, 3025–3049.
- Nicholas, K. B., Nicholas, H. B. J. & Deerfield, D. W. I. (1997). GeneDoc: analysis and visualization of genetic variation. *EMB News* **4**, 14.
- Nour, M. (1998). 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Res Microbiol* **149**, 433–448.
- Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Relman, D. A. (1991). Universal bacterial 16D rDNA amplification and sequencing. In *Diagnostic Molecular Microbiology: Principles and Applications*, pp. 489–495. Edited by D. H. Persing, T. F. Smith, F. C. Tenover & T. J. White. Washington, DC: American Society for Microbiology.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Stiles, M. E. & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36**, 1–29.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.
- Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* **51**, 221–271.
- Zhen, L. & Swank, R. T. (1993). A simple and high yield method for recovering DNA from agarose gels. *Biotechniques* **14**, 894–898.