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Characterization of lactobacilli from Scotch malt whisky distilleries and description of *Lactobacillus ferintoshensis* sp. nov., a new species isolated from malt whisky fermentations

Kirsten L. Simpson,[1] Bertil Pettersson[2] and Fergus G. Priest[1]

Author for correspondence: Fergus G. Priest. Tel: +44 131 451 3464. Fax: +44 131 451 3009. e-mail: f.g.priest@hw.ac.uk

[1] International Centre for Brewing & Distilling, Department of Biological Sciences, Heriot-Watt University, Edinburgh EH14 4AS, Scotland, UK

[2] Department of Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden

Sixty-four strains of *Lactobacillus* were isolated from fermentation samples from 23 malt whisky distilleries located in the major whisky producing regions of Scotland. The strains were assigned to 26 ribotype patterns. Strains of some ribotype patterns were widely distributed and recovered from distilleries throughout Scotland, while strains representing other ribotypes were particular to a specific region or even a certain distillery. Repeated sampling of a single distillery over a 12 month period showed that the range of bacteria present, as indicated by ribotyping, was stable, but was influenced by changes in malt supply and the period of closure for annual maintenance. Partial 16S rDNA sequence analysis of ribotype representatives revealed *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus paracasei* and *Lactobacillus pentosus* as the major species present in the distilleries; however, four isolates could not be identified by this procedure. Determination of the full 16S rDNA gene sequence from one of these isolates (strain R7-84) revealed >98·5% similarity to *Lactobacillus buchneri* and its phylogenetic neighbours. DNA from two other strains showed greater than 70% hybridization to DNA from R7-84 under non-stringent renaturation conditions and DNA from strain R7-84 shared less than 65% hybridization with members of the *L. buchneri* group. It is proposed that these three strains should be placed in a new species for which the name *Lactobacillus ferintoshensis* represented by the type strain R7-84^T is suggested.

Keywords: 16S rRNA, lactobacilli, taxonomy, whisky, ribotyping, random-amplified polymorphic DNA

INTRODUCTION

Scotch malt whisky is distilled from the fermented extract of malted barley. In this process, the malt is milled and mixed with water to produce a 'grist' which is mashed at ~63 °C for about 90 min to enable enzymes from the malt to hydrolyse polymeric material, largely starch and protein. The hot liquid or 'wort' is collected, cooled and transferred directly to the fermentation vessel. After fermentation, the 'wash' is distilled and the spirit is matured in oak casks for at least 3 years and generally a lot longer. Since the wort is not boiled, as it is in a brewery, it contains active malt enzymes that continue to hydrolyse starch during yeast fermentation and the alcohol yield is maximized (Palmer, 1997). However, bacteria from the malt that can survive mashing will also be transferred to the fermentation. In practice these are largely lactic acid bacteria and the Scotch whisky fermentation is therefore a mixed yeast/bacterial fermentation (Bryan-Jones, 1975).

The origins of these bacteria within the distillery are unclear. The lactic flora could become resident within

Abbreviation: RAPD, random-amplified polymorphic DNA.

The GenBank accession number for the 16S rRNA sequence of strain R7-84 is AF071856.

Fig. 1. Map of Scotland showing the principal whisky distilling regions.

the pipework and vessels of the plant and comprise a stable population of strains selected from the raw materials by the particular practices adopted in that distillery. In such circumstances, the flora would vary between different distilleries. Alternatively, the lactic acid bacteria could be flushed out by the cleaning and sterilization procedures and regularly reintroduced into the distillery with the raw materials during mashing. Since the malt is sourced from relatively few suppliers, in this case the flora would be expected to be similar between geographically distinct distilleries.

Several attempts have been made to identify the lactic acid bacteria in Scotch whisky fermentations using traditional schemes (MacKenzie & Kenny, 1965; Bryan-Jones, 1975; Makanjuola & Springham, 1984) and numerical taxonomy of phenotypic characters (Priest & Barbour, 1985). These studies have been partially successful and, while many isolates could not be identified, some were equated with established species such as *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum* and *Lactobacillus plantarum* (Bryan-Jones, 1975; Priest & Barbour, 1985). With the introduction of molecular systematics, in particular 16S rDNA sequencing and phylogenetic analysis of the data, the classification and identification of lactic acid bacteria has been revolutionized (Schleifer & Ludwig, 1995). Moreover, molecular methods provide high discriminatory power for epidemiological typing of lactobacilli (Rodtong & Tannock, 1993; Tynkkynen *et al*., 1999). In this paper, we describe the use of these approaches to identify lactic acid bacteria isolated from fermentation samples from 23 malt whisky distilleries from various geographical locations in Scotland (Fig. 1) and show that a distillery does develop its own stable population of lactobacilli.

METHODS

Strains and culture conditions. Lactobacilli were isolated from late fermentation samples (48–72 h after addition of yeast) by plating various dilutions on a modified de Man, Rogosa & Sharpe (MRS) medium comprising MRS broth (Oxoid) solidified with agar, autoclaved and, when cool, supplemented with maltose (final concn 0·5 %), vitamins (10 μg biotin ml^{-1}, 500 μg calcium pantothenate ml^{-1}, 500 μg pyridoxin ml^{-1} and 10 μg thiamin ml^{-1}), and cycloheximide (25 μg ml^{-1}) to suppress yeast growth. Plates were incubated at 30 °C for 48 h. Since similar numbers of colonies were recovered on plates incubated aerobically and anaerobically, we routinely used aerobic incubation. Colonies of different morphological appearance were purified on the same medium and examined microscopically. Gram-positive rods with a negative catalase reaction were retained as presumptive *Lactobacillus* species. Thereafter, strains were routinely cultured on MRS agar and broth at 30 °C unless otherwise stated. Strains were stored at −70 °C in MRS broth containing 20 % glycerol. Reference strains of *Lactobacillus buchneri* CIP 103023^T, *Lactobacillus hilgardii* CIP 103006^T and *Lactobacillus kefiri* CIP 103007^T were obtained from the Collection de l'Institut Pasteur. Strains used in this study are shown in Table 1.

Morphological and physiological characterization. Cellular morphology was determined from Gram-stained cultures grown for 48 h on modified MRS agar. Growth at 15 °C and 45 °C was tested in MRS broth held in water baths set at the corresponding temperatures for up to 1 week. Production of gas from glucose was detected using Durham's tubes in MRS broth supplemented with 0·5 % glucose. Tubes were incubated for up to 14 d. Utilization of starch and gelatin was determined in MRS agar supplemented with 1 % soluble starch (Difco) or 0·4 % gelatin (Sigma). Cultures were incubated for 2 d and starch hydrolysis was detected by exposing the starch plates to iodine vapour while gelatin plates were flooded with 30 % trichloroacetic acid. Production of ammonia following growth in heterofermentation-arginine broth (Pilone *et al*., 1991) for 14 d was detected with Nessler's reagent (BDH). A kit based on L-lactate and D-lactate dehydrogenases (Roche) was used to determine L- and D-lactic acids after growth in MRS broth for 24 h. Resistance to antibiotics was determined on MRS agar as zones of inhibition around impregnated discs (Oxoid) containing colistin (10 μg), erythromycin (60 μg), kanamycin (1000 μg), penicillin (2 units), rifampicin (15 μg) or vancomycin (5 μg). Carbohydrate fermentation tests were carried out using API 50 CHL strips according to the manufacturer's instructions (bioMérieux). Incubations were at 30 °C for 48 h.

Molecular methods. Chromosomal DNA was prepared using a modification of the method described by Johansson *et al*. (1995). Bacteria from 100 ml MRS broth grown for 16 h were harvested by centrifugation at 8000 ***g*** and washed in 10 ml TES buffer (50 mM NaCl, 100 mM Tris/HCl, 70 mM EDTA, pH 8·0) and recentrifuged. The pellet was resuspended in 3 ml TES supplemented with 25 % sucrose and 50 mg lysozyme ml^{-1} (Sigma). Mutanolysin (Sigma) dissolved in 0·1 M potassium phosphate buffer (pH 8·0) was added to 140 units ml^{-1} and the suspension was incubated at 42 °C for 2 h to effect lysis. Thereafter the published method was followed and the DNA pellet was dissolved in 500 μl 0·1 × SSC.

Table 1. Strains included in this study

Species	Ribotype/strain*	Region sequenced	No. nt/similarity to reference sequence (%)	Distillery	Area
L. brevis	R5-38			Oban	Western Highlands
	R5-86			Glengoyne	Southern Highlands
	R5-110	U1–U5	861/100	Pulteney	Northern Highlands
	R5-127			Isle of Jura	Islands
	R11-6			Glenkinchie	Lowlands
	R11-64	U1–U5	815/100	Laphroaig	Islay
	R11-83			Blair Athol	Southern Highlands
	R11-139			Glenkinchie	Lowlands
	R11-145			Glenkinchie	Lowlands
	R12-23	U1–U2	344/99	Glenkinchie	Lowlands
	R19-113	U1–U2	399/99	Dalmore	Northern Highlands
	R19-116			Dalmore	Northern Highlands
	R19-119			Lagavulin	Islay
L. fermentum	R4-75	U2–U5	474/98	Arran	Islands
	R4-105			Tobermory	Islands
	R4-89			Tamdhu	Speyside
	R8-81	U2–U5	520/99	Blair Athol	Southern Highlands
	R9-123	U2–U5	504/99	Glen Garioch	Eastern Highlands
	R10-120	U2–U5	447/99	Glen Garioch	Eastern Highlands
	R13-36	U2–U5	340/98	Oban	Western Highlands
	R21-68	U1–U5	706/99	Edradour	Southern Highlands
	R22-70	U1–U5	680/99	Edradour	Southern Highlands
	R22-130			Isle of Jura	Islands
	R23-11	U1–U5	565/99	Glenkinchie	Lowlands
	R23-90			Tamdhu	Speyside
	R24-32			Highland Park	Islands
	R24-56	U1–U5	576/99	Glenmorangie	Northern Highlands
	R25-29	U1–U5	732/99	Macallan	Speyside
	R26-99	U1–U2	323/98	Bowmore	Islay
L. paracasei	R1-2	U1–U5	941/100	Glenkinchie	Lowlands
	R1-20	U1–U5	941/100	Glenturret	Southern Highlands
	R1-25			Macallan	Speyside
	R1-31			Highland Park	Islands
	R1-37			Oban	Western Highlands
	R1-41			Oban	Western Highlands
	R1-50			Glenkinchie	Lowlands
	R1-58			Glenmorangie	Northern Highlands
	R1-69	U1–U5	899/100	Edradour	Southern Highlands
	R1-77			Knockando	Speyside
	R1-82			Blair Athol	Southern Highlands
	R1-125			Isle of Jura	Islands
	R1-135			Glenkinchie	Lowlands
	R1-44	U1–U5	941/100	Cragganmore	Speyside
	R1-106			Glen Devron	Eastern Highlands
	R14-52	U1–U5	754/100	Bunnahabhain	Islay
	R16-53	U2–U5	423/100	Bunnahabhain	Islay
	R16-76	U2–U5	399/100	Knockando	Speyside
L. pentosus	R2-65	U1–U5	899/100	Laphroaig	Islay
	R2-112			Pulteney	Northern Highlands
	R11-128	U1–U5	735/100	Isle of Jura	Islands
L. plantarum	R3-72	U1–U5	715/100	Arran	Islands
	R3-122			Glen Garioch	Eastern Highlands
Unassigned	R6-27			Macallan	Speyside
	R6-88			Glengoyne	Southern Highlands
	R6-98			Bowmore	Islay
	R7-9			Glenkinchie	Lowlands
	R7-84	U1–U8	1496/98	Blair Athol	Southern Highlands
	R15-101			Bowmore	Islay
	R15-103	U2–U5	440/97	Tobermory	Islands
	R18-39			Oban	Western Highlands

*R prefix is the ribotype assignment followed by the strain number; e.g. strain R5-38 is strain 38 assigned to ribotype 5.

Ribotype patterns were prepared using 3 μg *Hin*dIII-digested DNA, electrophoresed in 1 % agarose at a constant voltage of 50 V in Tris/acetate buffer. Southern blots were hybridized with a digoxigenin-labelled probe, derived from the amplified 16S rRNA gene of *Bacillus sphaericus* as described previously (Jahnz *et al*., 1996). For chromosomal DNA–DNA hybridization (Nielsen *et al*., 1995), the probe DNA was labelled using random-priming with digoxigenin-labelled dUTP (Roche) and hybrids were detected with a chemiluminescent substrate (CSPD, Roche) which was fixed on Kodak X-Omat film. Developed films were scanned and quantified using the NIH image analysis programme available at http://scrc.cit.nih.gov/imaging/. The density of each spot was recorded and the percentage hybridization calculated relative to the homologous reaction. Renaturation was at 62 °C (non-stringent conditions) and 72 °C (stringent) for 16 h.

Random-amplified polymorphic DNA (RAPD)-PCR was based on purified DNA (about 500 ng) or boiled cell extract as template. For the latter, a single colony on MRS agar was transferred to a microfuge tube and suspended in 15 μl 1 × PCR reaction buffer (Bioline). The cells were boiled for 10 min, transferred to ice for 10 min and centrifuged to remove cell debris. The supernatant (5 μl) was used as the template with Bioline *Taq* polymerase and the primer KS3, 5′-GGCATGACCT-3′ (Du Plessis & Dicks, 1995). The amplification programme consisted of 1 cycle at 94 °C for 5 min; 4 cycles of 94 °C for 45 s, 30 °C for 2 min, 72 °C for 1 min; 45 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min; followed by 1 cycle of 72 °C for 10 min. The reaction was carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler. PCR products were electrophoresed at 15 V for 14 h in horizontal 1·5 % (w/v) agarose gels and stained in ethidium bromide.

***In vitro* amplification and sequence determination of the 16S rRNA gene.** Primers used for PCR and 16S rDNA sequencing were those described earlier (De Silva *et al*., 1998). Products were subjected to cycle sequencing comprising 25 cycles of denaturation at 96 °C for 15 s, annealing at 50 °C for 30 s and elongation at 60 °C for 60 s. The MegaBACE ET-dye-terminator kit (Amersham Pharmacia Biotech) was used for the generation of Sanger dideoxy fragments. Excess dye-terminators were removed by ethanol precipitation, the reactions were dried and 10 μl de-ionized formamide was added to each reaction. The microtitre plate was inserted at the cathode stage in a MegaBACE 96-capillary DNA sequencing unit. Injection was performed at 3 kV for 50 s and the sequencing products were subsequently separated by electrophoreses at 5 kV for 120 min.

Phylogenetic analysis. The trace files were edited and merged into a 16S rRNA gene contig using the Sequencher assembly program (Gene Codes). The resulting primary structures were aligned manually by using the Genetic Data Environment (GDE) software (Smith, 1992). The alignment contained previously described 16S rRNA gene sequences from *Lactobacillus* species and relatives, as retrieved from GenBank and those which can be downloaded in the form of an alignment based on secondary structure models from The Ribosomal Database Project (RDP-II; Maidak *et al*., 1999). Phylogenetic calculations were performed using algorithms implemented in PHYLIP version 3.573 (Felsenstein, 1993).

Nucleotide accession numbers. The GenBank accession numbers for the 16S rRNA sequences of the reference strains used for phylogenetic calculations are given in the phylogenetic tree (Fig. 4).

RESULTS

Distribution of lactobacilli among some malt whisky distilleries

The malt whisky fermentation is rapid and yeast growth ceases 36–48 h after inoculation. As the yeast dies and autolysis ensues, lactic acid bacteria grow rapidly in the so-called 'late-lactic fermentation' (Dolan, 1974). This is encouraged by many distillers since it positively enhances whisky flavour (Geddes & Riffkin, 1989). The fermentation samples taken 48–72 h after yeast inoculation therefore typically revealed high numbers of bacteria (10^6–10^8 lactobacilli ml^{-1}) when cultured on the modified MRS agar. Colonies of distinct morphology were purified from each sample and the range of bacteria present was assessed by ribotyping. We recovered 26 distinct patterns from the 64 strains studied; some representative patterns are shown in Fig. 2. Several ribotypes were particular to a specific region or even distillery. For example, strains belonging to ribotypes 13 and 18 were unique to the Oban distillery on the west coast of the mainland while those belonging to ribotypes 9 and 10 were found only in the Glen Garioch distillery on the east coast. Similarly, strains with ribotype patterns 14 and 26 were restricted to different distilleries on the west coast island of Islay, famous for its heavily peated and phenolic/iodine flavoured whiskies, and the ribotype 24 strains were found only in the northern areas. In contrast, strains belonging to ribotype 1 were isolated from 12 distilleries widely distributed in all geographical locations while others, such as those belonging to ribotype 11, were less common but still found in geographically dispersed distilleries from the west coast islands (Islay and Jura) and the lowland region (Glenkinchie).

Ribotype patterns derived from a single 16S rRNA probe provide simple patterns with relatively few bands. They are valuable for species identification but a method with higher discriminatory power is needed to dis-

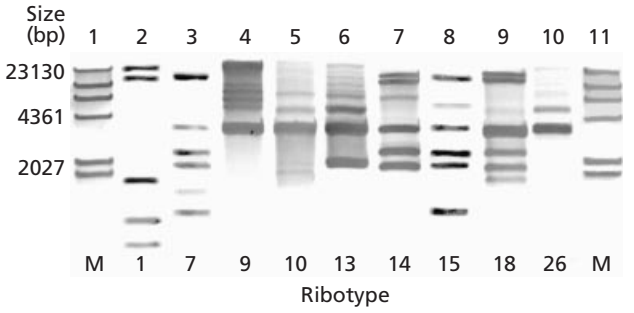

Fig. 2. Some representative ribotype patterns of lactobacilli isolated from Scotch whisky distillery fermentations. Ribotype pattern assignments are given at the base of the blot. Lanes: 1, *Hin*dIII-digested lambda DNA marker; 2, R1-69; 3, R7-84^T; 4, R9-123; 5, R10-120, 6, R13-36; 7, R14-52; 8, R15-103; 9, R18-39; 10, R26-99; 11, DNA marker. For strain identification and origin, see Table 1.

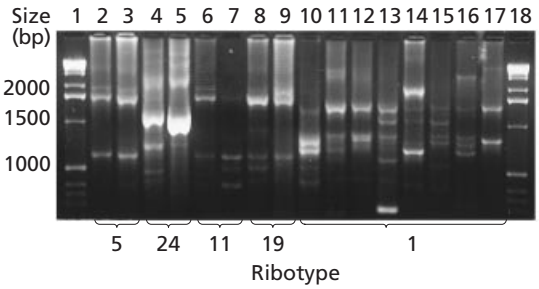

Fig. 3. Some representative RAPD patterns of lactobacilli isolated from Scotch whisky distillery fermentations. Ribotype designations are given at the bottom of the gel. Lanes: 1, 1 kb ladder; 2, R5-110; 3, R5-127; 4, R24-32; 5, R24-56; 6, R11-139; 7, R11-145; 8, R19-113; 9, R19-116; 10, R1-58; 11, R1-2; 12, R1-31; 13, R1-37; 14, R1-41; 15, R1-50; 16, R1-125; 17, R1-20; 18, 1 kb ladder.

tinguish between closely related strains (Olive & Bean, 1999). Strains within ribotype 1 could be distinguished using RAPD patterns. The results in Fig. 3 show that strains of ribotype 1 from distinct distilleries have different RAPD patterns and therefore probably represent different clonal lines of the same species. Strains of ribotypes 5, 11, 19 and 24, on the other hand, had consistent RAPD patterns within the ribotype, but this may be a reflection of the relatively few strains available for study. In summary, these results indicate that malt whisky fermentations harbour a diversity of lactobacilli, most of which are specific to individual distilleries as judged by ribotyping or RAPD.

Stability of the lactic acid bacterial flora

It was important to know if the bacteria recovered from our samplings were representative of a stable flora in the distilleries or a single 'snapshot' of the bacteria present on the sampling occasion. Late fermentation samples (about 80 h after inoculation) were taken from the Glenkinchie distillery over a 12 month period and the cultured lactic acid bacteria were characterized by ribotyping (Table 2). The *Lactobacillus* flora remained stable for 3 months until the midwinter closure period, after which strains representing ribotypes 11 and 23 were missing (fewer than 5×10^5 ml^{-1}). However, these bacteria reappeared within the next three visits to the distillery. There was also a change of malt from barley cultivar Dercado to Maud after the seventh visit and shortly after this a new strain (ribotype 22) appeared but was lost following reversion to Dercado malt. These results indicate that the lactic acid bacterial flora in a distillery is constant over prolonged periods of at least several months, but that it is influenced by the raw materials. Consequently, the strains in Table 1 are believed to represent a relatively stable situation rather than a 'snapshot' of the bacterial population present at the time of sampling.

Identification of strains and molecular phylogeny

Bacterial strains with the same ribotype are generally members of the same species (Grimont & Grimont, 1986). We therefore identified at least one member of each ribotype by partial 16S rDNA sequence analysis in which the proximal portion of the gene was compared with data for reference strains held in the relevant databases. These strains were identified as *L. brevis*, *L. fermentum*, *Lactobacillus paracasei*, *Lactobacillus pentosus* and *L. plantarum* (Table 1). *L. fermentum* and *L. paracasei* were the most frequently isolated species recovered from late fermentation samples from the distilleries. We had great difficulty obtaining good sequence data from strains of ribotypes 6 and 18, which remain unidentified (Table 1).

The difficulties in distinguishing *L. casei*, *L. paracasei* and *Lactobacillus zeae* were resolved by sequencing the U1 to U2 region of the 16S rRNA genes in which discriminatory signature codons have been identified (Mori *et al.*, 1997). This identified all the isolates as *L. paracasei* but with two variants; strains R1-20, R1-69, R14-42 and R16-76 matched perfectly with the signatures for *L. paracasei* proposed by Mori *et al.* (1997), while strains R1-1 and R1-44 contained a unique

Table 2. Occurrence of lactobacilli in fermentations at the Glenkinchie distillery during 1998

Species	Ribotype	Time (months)										
		0	0·75	1	2·5	3·25	4	4·75*	6†	7·75	8·5*	11
L. paracasei	1‡	+	+	+	+	+	+	+	+	+	+	+
L. paracasei	1‡	+	+		+					+	+	+
Lactobacillus sp.	7	+		+	+				+		+	+
L. brevis	11	+	+	+	+	+	+	+		+	+	+
L. brevis	12						+			+	+	
L. fermentum	22								+	+		
L. fermentum	23	+	+	+	+	+		+	+			+

* Change of malt (see text for details).

† Closure period for maintenance and cleaning.

‡ Isolates with the same ribotype but different colonial morphology.

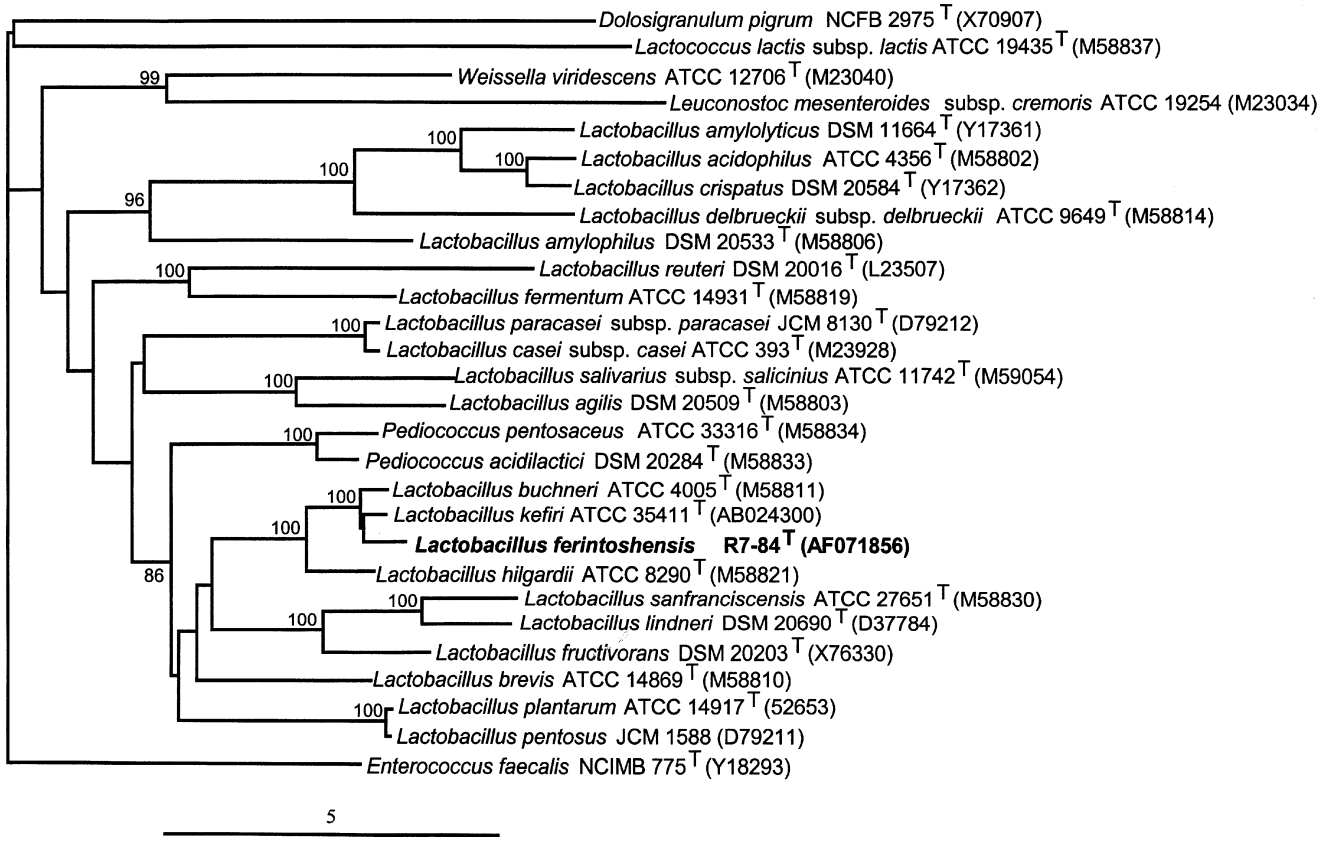

Fig. 4. A phylogenetic tree showing the evolutionary position of *L. ferintoshensis* R7-84^T among *Lactobacillus* and relatives. The tree was inferred by neighbour-joining (Saitou & Nei, 1987) from a distance matrix corrected by the method of Jukes & Cantor (1969). *Enterococcus faecalis* NCIMB 775^T was used as outgroup and bootstrap values $> 80\%$ are shown at the actual nodes. The scale bar indicates the number of substitutions per 100 nucleotide positions.

polymorphism (20% A, 80% G) in position 110 according to the numbering for *L. casei* (Mori *et al.*, 1997) but in other respects matched the *L. paracasei* signature.

Two strains, R7-84 representing ribotype 7 and R15-103 representing ribotype 15, revealed identical partial 16S rRNA sequences (Table 1) but showed a sequence similarity of only 97% to previously described species when searching the relevant databases. Therefore, the virtually complete 16S rRNA gene sequence of strain R7-84 was determined and used for deciphering evolutionary relationships to related organisms. Phylogenetic trees were constructed from a starting alignment comprising about 100 published and unpublished 16S rRNA gene sequences from members of *Lactobacillus* and relatives. Different subsets of the alignment for final phylogenetic calculations were generated by removing gaps, ambiguously aligned positions and by applying a nucleotide consistency filter of 50%, i.e. positions for which a certain nucleotide composition could not be observed in more than half of the sequences. All trees showed an overall agreement with regard to topology and a representative phylogenetic tree inferred by the neighbour-joining method from a distance matrix corrected for multiple nucleotide substitutions at single locations by the one-parameter model of Jukes & Cantor (1969) is shown in Fig. 4. The tree was computed from the resulting matrix that was corrected by only removing gapped positions. Bootstrap percentage values have been given at the nodes of the tree which were statistically supported to $\geqslant 80\%$. A close relationship was found between strain R7-84 and the obligately heterofermentative species of the genus *Lactobacillus* belonging to the *L. buchneri* group or cluster 2 (Collins *et al.*, 1991). In all trees strain R7-84 repeatedly formed a tightly held entity with *L. kefiri* ATCC 35411^T, *L. buchneri* ATCC 4005^T and *L. hilgardii* ATCC 8290^T, with 16S rDNA similarities of 98·4%, 98·1% and 97·3%, respectively.

Lactobacillus strain R7-84 represents a new species

Since the 16S rDNA sequence similarity values were above the proposed limit of 97% for species definition suggested by Stackebrandt & Goebel (1994), DNA hybridization experiments were used to evaluate the status of strain R7-84 and relatives. DNA from strain R7-84 was labelled with digoxigenin and hybridized under stringent (72 °C) and non-stringent (62 °C) conditions with DNA from related distillery isolates, as judged by RFLP patterns (Fig. 1) and phylogenetically

Table 3. DNA–DNA hybridization between DNAs from *Lactobacillus* strain R7-84 and from phylogenetically close relatives

All values are means of at least three determinations. Renaturation was conducted under stringent (72 °C) and non-stringent (62 °C) conditions.

Strain	Hybridization with DNA from strain R7-84 (%)	
	Non-stringent	Stringent
R7-84	100	100
R7-9	79	78
R15-103	73	44
R15-101	6	3
L. buchneri CIP 103023^T	65	28
L. hilgardii CIP 103006^T	32	0
L. kefiri CIP 103007^T	14	0

closely related type strains of the genus *Lactobacillus*. The results (Table 3) revealed close genomic similarity between strains R7-84 and R7-9 (79% at 62 °C renaturation temperature and 78% at 72 °C) but DNA from strain R7-84 hybridized poorly with the reference strains of *L. buchneri*, *L. hilgardii* and *L. kefiri*. In addition, one of the two ribotype 15 strains (R15-103) shared 73% and 44% hybridization with strain R7-84 at non-stringent and stringent temperatures respectively but strain R15-101 was unrelated to the other three distillery isolates and is not considered further here. These results suggested that strains R7-9, R7-84 and R15-103 should be classified in a new species.

Phenotypic characterization

The three unidentified *Lactobacillus* strains were all Gram-positive, catalase-negative rods. Cells were 3–4 μm in length and 1 μm in diameter. After incubation on modified MRS agar for 48 h, strains R7-9 and R7-84 formed shiny, creamy white colonies about 2–5 mm in diameter. Strain R15-103 formed larger (4–6 mm diameter) white colonies with irregular edges. The strains were examined for a range of biochemical and physiological characters and their distinguishing features are shown in Table 4. All isolates were heterofermentative, produced ammonia from arginine and grew at 15 °C but not at 45 °C. Strains R7-9 and R15-101 hydrolysed gelatin weakly, but starch hydrolysis was not detectable. All of the strains were resistant to discs containing 5 μg vancomycin and 2 units penicillin but were generally sensitive to the other antibiotics tested. Fermentation tests in API trays indicated that strains R7-9, R7-84 and R15-103 could be distinguished from phylogenetic relatives by their production of acid from *N*-acetylglucosamine, sucrose and trehalose, and a combination of other tests (Table 4).

DISCUSSION

Although the basic process of malt whisky production is the same throughout Scotland, plant design and local practice vary. The Scotch whisky industry therefore provides a unique opportunity to examine the effects of these minor differences on the composition of the *Lactobacillus* flora in the distillery fermentations. Most fermentation samples examined contained a mixed bacterial flora, generally comprising two or three obviously different colonial types. Representatives of six species were isolated, with *L. fermentum* and *L. paracasei* the most common (17 and 16 isolates respectively). Strains of *L. fermentum* and *L. paracasei* were recovered from 13 of the 23 distilleries, of which eight distilleries harboured both. The 11 strains of *L. brevis* were recovered from nine distilleries, with a tendency to occur in the absence of *L. fermentum* and *L. paracasei*. Other species were detected sporadically (Table 1). There was no correlation between the composition of the bacterial population and the level of peat used in the preparation of the malt (which is typically high in the Islands region), suggesting that phenolic compounds at the concentrations involved do not influence the types of bacteria present.

The recovery of *L. paracasei* from fermentations has not been reported previously, although in the past isolates may have been confused with *L. casei* which has been

Table 4. Physiological and biochemical characteristics of reference and studied strains

Species/strain	NH_3 from arginine	Lactic acid isomer(s)	Acid production from:						
			N-AG*	Aesculin	Lactose	Melibiose	Salicin	Sucrose	Trehalose
L. buchneri	+	DL	−	−	+	−	−	−	−
L. hilgardii	+	DL	−	−	+	+	−	−	−
L. kefiri	+	DL	−	+	+	+	−	−	−
R7-9	+	DL	+	+	−	−	+	+	+
R7-84	+	DL	+	+	−	−	+	+	+
R15-103	+	DL	+	+	−	+	+	+	+
R15-101	+	D	+	+	+	−	−	+	+

* N-AG, *N*-acetylglucosamine.

identified using traditional phenotypic schemes as a distillery bacterium (Bryan-Jones, 1975; Makanjuola & Springham, 1984). Indeed, much confusion surrounds *L. casei* and *L. paracasei*. A request for the name *L. paracasei* to be rejected as a synonym of *L. casei* (Dellaglio *et al*., 1991) was rejected by the Judicial Commission (Wayne, 1994). Subsequent detailed analysis of 16S rRNA sequences (Mori *et al*., 1997), ribotyping and RAPD analyses (Tynkkynen *et al*., 1999; Ward & Timmins, 1999) revealed the distinctiveness of the two species. Our results support the signature sequences for *L. paracasei* determined by Mori *et al*. (1997) with the exception that we found a polymorphism in some strains, which comprised a transition of guanine to adenine at position 110 in a minority of *rrn* operons.

Partial 16S rRNA sequence analysis is an accurate approach to identification of *Lactobacillus* species (Walter *et al*., 2000). Performing BLAST searches using between 300 and 900 nt sequence resulted in unambiguous identifications usually at the 99–100 % level although in some instances, particularly with *L. fermentum* strains, it was slightly lower (Table 1). Further 16S rDNA sequence analysis and DNA reassociation studies should reveal whether this variation is due to species heterogeneity or to the existence of subspecies or even novel species.

Ribotyping has been recommended for subspecific discrimination of lactobacilli in several studies including strains of *L. casei* and relatives (Tynkkynen *et al*., 1999), *Lactobacillus helveticus* strains from cheese (Giraffa *et al*., 2000), and various species isolated from the vagina (Zhong *et al*., 1998). Ribotyping of distillery isolates had its deficiencies in this study, largely because the range of ribotype patterns within a species varied considerably. The 16 strains of *L. fermentum* were fairly evenly distributed among 11 ribotypes, which provided for powerful discrimination. *L. fermentum* appears to be a genomically diverse species as judged by both ribotyping (Zhong *et al*., 1998) and RAPD analysis (Hayford *et al*., 1999). The 11 strains of *L. brevis* isolated from distillery fermentations were assigned to just four ribotypes, consistent with the heterogeneity detected by RAPDs in strains isolated from wine (Sohier *et al*., 1999). *L. paracasei* was the most conserved species among the distillery organisms, in that 14 strains from 12 geographically dispersed distilleries all belonged to ribotype 1. However, these ribotype 1 strains could be distinguished by RAPD (Fig. 3), which is consistent with the extensive heterogeneity detected among *L. paracasei* strains from cheese revealed by RAPD analysis (Fitzsimons *et al*., 1999). This suggests that there is no one perfect method for typing lactobacilli, and that different approaches will be most effective with different species, dependent on the genetic structure of that species. A combination of the two typing procedures was optimal and allowed us to demonstrate a wide diversity of lactobacilli in the 23 distilleries which were investigated in this study.

It was important to establish if our sampling of the various distilleries represented a stable microbial flora or a transient population. Extended sampling at Glenkinchie distillery showed that strains of *L. brevis*, *L. fermentum*, *L. paracasei* and an unidentified bacterium were generally present (Table 2). There was marked stability of these strains, as judged by ribotyping, and when particular types were not detected this may have reflected low relative numbers rather than a complete absence. A new bacterium was probably introduced with a change of malt (*L. fermentum* ribotype 22) but subsequently lost when the malt was changed back to the original cultivar. Similarly, the annual closure period accompanied by extensive cleaning and maintenance removed some types but most had returned by the final visit. Thus the distillery has a stable, resident flora that may be influenced by raw materials.

Strains of ribotype patterns 7 and 15 could not be identified as a validly described *Lactobacillus* species. The RFLPs were very similar (Fig. 2) suggesting closely related organisms. A virtually complete 16S rRNA sequence of strain R7-84 placed the organism in the *L. buchneri* cluster (Collins *et al*., 1991; Schleifer & Ludwig, 1995). *L. buchneri* and other members of this group including *L. hilgardii* (Sohier *et al*., 1999), *L. kefiri* (Kandler & Kunath, 1983) and the more distantly related *L. lindneri* (Banks, 1998) are associated with alcoholic fermentations. This is consistent with the growth of these strains in relatively high alcohol concentrations (Scotch whisky fermentations contain up to about 8 %, v/v, ethanol) indicating a possible idiosyncratic phenotypic feature of the *L. buchneri* subclade. Strains R7-84 and R7-9 had the same ribotype pattern and shared almost 80 % DNA hybridization at both stringent and non-stringent hybridization temperatures (Table 3). On the other hand, strain R15-103 is more difficult to classify. DNA hybridization at the optimal reassociation temperature with DNA from strain R7-84 (73 %) indicated that it is likely to be a member of the same species as the ribotype 7 strains. Moreover, it shared identical DNA sequence over the 440 bases of the U2 to U5 region of the 16S rRNA gene. However, the low hybridization value with strain R7-84 at stringent temperature (44 %) suggests substantial mismatching of hybrids and is inconsistent with it being included in the same species (Stackebrandt & Goebel, 1994). On balance, we suggest that it be included in the same taxon as strain R7-84 until evidence to the contrary is presented. Strain R15-101 is unusual since it has the same ribotype as strain R15-103 but appears to belong to a different species by DNA hybridization. Further hybridizations using different probe DNAs are needed to determine the taxonomic position of this strain.

The three strains were phenotypically similar to other members of the *L. buchneri* group. All were heterofermentative, produced ammonia from arginine and DL-lactic acid from glucose, features likely to have been harboured by their latest common ancestor. However, the distillery isolates could be distinguished from the other members of the phylogenetic group by a selection of sugar fermentation tests (Table 4). We therefore recommend that strains R7-9, R7-84 and R15-103 should

be included in a new species for which we propose the name *Lactobacillus ferintoshensis* after the estate in the north of Scotland that during the 18th century gave its name to one of the first brands of Scotch whisky, Ferintosh.

Description of *Lactobacillus ferintoshensis* sp. nov.

Lactobacillus ferintoshensis (fe.rin.to.shen′sis. M.L. adj. *ferintoshensis* from Ferintosh, a Scottish estate famous for its whisky).

Cells are rod-shaped and occur singly or in pairs or in short chains, 3–4 μm in length and about 1 μm wide. After 48 h incubation on modified MRS agar (MRS agar supplemented with vitamins), colonies are 2–5 mm in diameter, circular, shiny and creamy white in colour. Cells stain Gram-positive and are non-spore forming and non-motile. Catalase-negative. Grows at 15 °C but not 45 °C. Heterofermentative, producing DL-lactic acid from glucose. Acid is produced from *N*-acetyl-glucosamine, L-arabinose, galactose, glucose, fructose, maltose, melibiose, melezitose, ribose, salicin, sucrose, trehalose, D-xylose and in some cases from cellobiose, mannitol, mannose, α-methyl D-glucoside, α-methyl D-mannoside, raffinose, sorbitol, sorbose, tagatose and turanose. Acid is not produced from adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, gentiobiose, glycerol, glycogen, gluconate, inositol, inulin, 2- and 5-ketogluconate, lactose, rhamnose, L-xylose and xylitol. Aesculin but not starch is hydrolysed. Some strains hydrolyse arbutin. The type strain is positive for the variable reactions given above with the exception of acid from melezitose, melibiose, raffinose, tagatose and turanose for which it is negative. Habitat: malt whisky fermentations. The type strain is strain R7-84^T which has been deposited with the Collection de L'Institut Pasteur as CIP 106749^T.

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