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Evolution of the Lactic Acid Bacterial Community during Malt Whisky Fermentation: a Polyphasic Study

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The development of the lactic acid bacterial community in a commercial malt whisky fermentation occurred in three broad phases. Initially, bacteria were inhibited by strong yeast growth. Fluorescence microscopy and environmental scanning electron microscopy revealed, in this early stage, both cocci and rods that were at least partly derived from the wort and yeast but also stemmed from the distillery plant. Denaturing gradient gel electrophoresis (DGGE) of partial 16S rRNA genes and sequence analysis revealed cocci related to *Streptococcus thermophilus* or *Saccharococcus thermophilus*, *Lactobacillus brevis*, and *Lactobacillus fermentum*. The middle phase began 35 to 40 h after yeast inoculation and was characterized by exponential growth of lactobacilli and residual yeast metabolism. *Lactobacillus casei* or *Lactobacillus paracasei*, *L. fermentum*, and *Lactobacillus ferintoshensis* were detected in samples of fermenting wort examined by DGGE during this stage. Bacterial growth was accompanied by the accumulation of acetic and lactic acids and the metabolism of residual maltooligosaccharides. By 70 h, two new PCR bands were detected on DGGE gels, and the associated bacteria were largely responsible for the final phase of the fermentation. The bacteria were phylogenetically related to *Lactobacillus acidophilus* and *Lactobacillus delbrueckii*, and strains similar to the former had previously been recovered from malt whisky fermentations in Japan. These were probably obligately homofermentative bacteria, required malt wort for growth, and could not be cultured on normal laboratory media, such as MRS. Their metabolism during the last 20 to 30 h of fermentation was associated with yeast death and autolysis and further accumulation of lactate but no additional acetate.

Scotch malt whisky is distilled from the fermented hot-water extract of malted barley. The malted cereal is milled and infused with water (mashed) at about 63°C. After about 30 min, the first wort is removed, cooled, and pumped to the fermentation vessel. The second water, which is conducted at a higher temperature (about 75°C) to effect the maximal extraction of carbohydrate from the grist, is cooled and added to the first wort to fill the fermentation vessel. The wort is not boiled, as it is in a brewery in order to retain the activity of the soluble enzymes from the malt during the fermentation and to maximize alcohol yield. Consequently, bacteria from the malt that can survive mashing enter the fermentation, resulting in a mixed yeast-bacterial fermentation (11, 19). If large numbers of lactobacilli enter the fermentation (more than 10^6 cells/ml), they compete for nutrients with yeast cells and reduce the ethanol yield (9, 11, 20). In well-operated distilleries, however, the lactobacilli flourish after the yeast cells have reached stationary phase and grow on residual nutrients and autolysing yeast cells. This "late lactic fermentation" is encouraged by many distillers, since it is thought to have a beneficial effect on the flavor of the final spirit (13, 24).

Little is known about the composition of the bacterial community in malt whisky fermentation and its development as fermentation proceeds. Traditionally, these questions have been addressed for various natural fermentations by enumeration of bacteria on culture media followed by their identification (4, 17). Analysis of whisky fermentations in this way has revealed *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus paracasei* as the most commonly isolated bacteria, with strains of *Lactobacillus pentosus*, *Lactobacillus plantarum*, and a new species, *Lactobacillus ferintoshensis*, being less commonly encountered (23). However, culture-dependent methods may underestimate the diversity of a bacterial community, particularly in such complex environments as fermented foods and beverages (4, 5).

In this study, we have adopted a polyphasic approach by using light and electron microscopy and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified fragments of 16S ribosomal DNA (rDNA) to monitor the development of the lactic acid bacterial community throughout malt whisky fermentation. Our results reveal an underestimation of bacterial diversity by culture-dependent methods and the presence of novel lactobacilli and other taxa in malt whisky fermentation.

MATERIALS AND METHODS

Strains, media, and culture conditions. The reference strains of *Lactobacillus* used in this study were as follows: *L. acidophilus* LMG 7943 (Laboratorium voor Microbiologie, Universiteit Ghent, Ghent, Belgium), *L. amylolyticus* LA5 (Lehrstuhl für Technologie der Brauerei I, TU Munich, Germany), *L. amylovorus* NCIMB 13276 (National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom), *L. brevis* R19-113 (23), *L. buchneri* CIP 103023 (Collection de l'Institut Pasteur, Paris, France), *L. casei* 541 (M. Collins, Agriculture and Food Science Centre, Belfast, United Kingdom), *L. crispatus* CIP 105003, *L. ferintoshensis* R7-9 (23), *L. fermentum* R22-70 (23), *L. hilgardii* CIP 103006, *L. kefiri* CIP 103007, *L. paracasei* R1-69 (23), *L. pentosus* R11-128 (23), *L. plantarum* R3-72 (23), and *Lactobacillus* sp. strain Y10 (S. van Beek and F. G. Priest, Abstr. 6th Symp. Lactic Acid Bacteria Genet. Metab. Appl., p. A34, 1999). All strains were maintained in MRS medium (10) containing 30% (wt/vol) glycerol

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at −70°C and were grown at 30°C statically in MRS broth at 30°C. Modified MRS agar was prepared by using sterile distiller's wort rather than water. Distiller's wort was prepared to an original gravity of 14.5°Plato in our pilot brewery, pasteurized at 90°C for 15 min, clarified by centrifugation, and stored at −20°C. Viable counts were determined in triplicate by plating appropriately diluted fermentation samples on modified MRS agar containing 50 mg of cycloheximide (Sigma)/liter and 10 mg of sodium azide (Sigma)/liter and incubating the samples at 30°C for 24 h.

Estimation of bacterial viability. Fermentation samples were obtained from the Glenkinchie Distillery, a malt whisky distillery located in southern Scotland. They were stored at 4°C for 2 h to allow the bulk of the yeast to sediment, and 1 ml of the supernatant was clarified by centrifugation and washed twice with sterile distilled water. Viability was determined by using LIVE/DEAD *Bac*Light bacterial viability kit L-7012 (Molecular Probes, Cambridge Bioscience, Cambridge, United Kingdom), which is based on mixtures of the green fluorescence nucleic acid stain, SYTO9, that labels all cells in a population and the red fluorescence nucleic acid stain, propidium iodine, that penetrates only bacteria with damaged membranes and quenches the green stain SYTO9. Thus, in an appropriate mixture of SYTO9 and propidium iodine, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. A bacterial suspension (1 ml) was stained with 3 μl of premixed dye according to the manufacturer's instructions (http://www.molecularprobes.com), incubated at room temperature in the dark for 10 min, and immobilized on a 0.2-μm-pore-size Isopore polycarbonate filter membrane (Millipore, Watford, United Kingdom). Cells were viewed under an Axiophot microscope (Carl Zeiss, Welwyn Garden City, United Kingdom) equipped with a 50-W mercury arc lamp and Carl Zeiss filter set number 9 (excitation wavelength, 450 to 490 nm; emission wavelength, >520 nm). Photomicrographs were made with simultaneous light microscopy and epifluorescence microscopy on Fuji 1600-ASA color film. For each sample, 10 pictures were taken, each depicting 100 to 400 cells.

Environmental scanning electron microscopy (ESEM). Fermentation samples were left at 4°C for 2 h, and 15 μl of appropriately diluted material was observed by using a Philips XL30 (LaB 6) environmental scanning electron microscope (FEI UK Ltd.). The microscope was operated at about 5 torr and 5°C, with a working distance of about 7.5 mm.

Preparation of DNA and RNA. DNA from reference strains was isolated from 1 ml of late-exponential-phase culture (optical density at 600 nm, about 1.0) in MRS broth by using a PUREGENE DNA isolation kit (Philip Harris/Flowgen, Shenstone, United Kingdom) modified by the addition of 140 U of mutanolysin (Sigma)/ml to the lytic enzyme solution and incubating the cell suspension at 37°C for 45 min. Total DNA was isolated from 10-ml distillery fermentation samples which had been stored at 4°C for 2 h to allow the yeast to settle. Bacteria were collected from the supernatant by centrifugation, washed three times with distilled water, and resuspended in 1 ml of cell suspension solution from the PUREGENE DNA isolation kit. DNA was isolated as described above. The quality of the DNA was examined following electrophoresis on a 1% agarose gel in 40 mM Tris-acetate (pH 8.0)–0.1 mM disodium EDTA buffer. Nucleic acids were quantified by UV spectrometry (GeneQuant RNA/DNA calculator; Amersham Pharmacia Biotech, Buckingham, United Kingdom).

Total RNA was extracted from fermentation samples by using a PURESCRIPT RNA isolation kit (Philip Harris/Flowgen) with the same modifications as those used for DNA extraction. RNA was treated with DNase reagent and removal solution (Ambion/AMS Biotechnology Ltd., Abingdon, United Kingdom) to eliminate any genomic DNA contamination. The integrity of the RNA was checked by agarose gel electrophoresis under denaturing conditions. RNA (10 ng) was used as a template for reverse transcriptase (RT) PCR by using a RobusT RT-PCR kit (Philip Harris/Flowgen). First-strand cDNA synthesis was performed at 50°C for 45 min; inactivation of avian myeloblastosis virus RT and primer-RNA-cDNA denaturation were done at 94°C for 2 min; and second-strand cDNA synthesis and PCR amplification were accomplished during 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min, followed by a final extension cycle at 72°C for 7 min. Simultaneously, a negative control reaction without RT was performed with each RNA template (results not shown).

PCR-DGGE analysis. Purified DNA was amplified with primer pair 1 (HDA1-GC, 5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′ [the GC clamp is in boldface], and HDA2, 5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (25), spanning the V2 region of the 16S rDNA gene (positions 339 to 539 in the *Escherichia coli* gene). PCRs were performed with a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Norwalk, Conn.). The reaction mixture (50 μl) consisted of 5 μl of reaction buffer, 1 μl of a 10 mM deoxynucleoside triphosphate mixture, 3 mM $MgCl_2$, 0.1 pmol of each primer, 10 ng of genomic template DNA, and 0.5 U of Dynazyme EXT *Taq* DNA polymerase (Philip Harris/Flowgen). The amplification program was 96°C for 3 min; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; and finally 72°C for 7 min. A second pair of primers targeted to an alternative region of the 16S gene was designed from an alignment of partial and complete *Lactobacillus* 16S rRNA sequences retrieved from GenBank: *L. acidophilus* (X61138), *L. amylolyticus* (Y17361), *L. amylovorus* (M58805), *L. brevis* (AF090328), *L. buchneri* (X61139), *L. casei* (D16552), *L. crispatus* (AF257097), *L. fermentum* (AF302116), *L. farintoshensis* (AF275311), *L. hilgardii* (M58821), *L. paracasei* (D79212), *L. pentosus* (D79211), and *L. plantarum* (M58827). Primer pair 2 comprised HDA4C (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**GC GGT GGA GCA TGT GGT TTA-3′) and HDA5 (5′-CCT TCC TCC GGT TTG TCA CC-3′), covering positions 939 to 1163 in the *E. coli* gene. The PCR mixture was modified from that of primer pair 1 by reduction of the $MgCl_2$ concentration to 1.5 mM and by use of 0.2 pmol of each primer. The amplification program was 96°C for 3 min; 30 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 1 min; and an elongation step at 72°C for 7 min.

DGGE was performed with a DCode electrophoresis system (Bio-Rad, Hemel Hempstead, United Kingdom) and gels measuring 16 cm by 16 cm by 1 mm. Polyacrylamide gels (8%) were prepared and run in Tris-borate-EDTA buffer. The denaturing gradient was formed with 8% Acrylogel 2.6 (BDH, Poole, United Kingdom). PCR products were loaded onto a 40 to 55% gradient of urea and formamide and electrophoresed at a constant temperature of 60°C and a constant voltage of 50 or 60 V for 630 or 700 min, respectively. Gels were stained with ethidium bromide (0.5 mg/liter) in Tris-borate-EDTA buffer for 20 min, destained in sterile deionized water for 10 min, and viewed by UV transillumination.

DNA sequencing. DGGE bands representing unknown organisms were excised from the gels, and a very small cut of each band, previously frozen at −70°C for 4 h, was used as a template for PCR with the primer pair used for DGGE. Subsequently, about 100 ng of PCR products was used as a template for sequencing amplification with one of the primers of each pair and 5% dimethyl sulfoxide in order to overcome difficulties due to the presence of secondary structures. The following cycling profile (25 cycles) was used: 96°C for 10 s, 55°C for 5 s, and 60°C for 4 min. Energy transfer dye terminator chemistry (Big Dye Terminator kit; Applied Biosystems, Warrington, United Kingdom) was used as described by the manufacturer for labeling the fragments. The excess of dye and buffer components was removed by isopropanol precipitation. The sequencing products were separated on an ABI 310 capillary sequencing system (Applied Biosystems). The partial 16S rDNA sequences obtained were compared to the sequences in the GenBank DNA database by using the BLASTN algorithm (2).

Analytical procedures. Acetic acid and D- or L-lactic acid were assayed enzymatically by using commercially available kits (Boehringer Ingelheim Limited, Bracknell, United Kingdom). Residual sugars in fermentation samples were determined by high-pressure liquid chromatography (Waters 600E; Millipore, Bedford, Mass.). Samples were clarified by centrifugation and analyzed with an Aminex HPX-42A column (Bio-Rad) at 85°C and a flow rate of 0.5 ml of water/min. Ethanol and oligosaccharides up to maltoheptaose were detected by using a differential refractometer (Waters 410). Only late fermentation samples (>40 h) could be analyzed because the high concentrations of glucose and maltose in early samples obscured the analyses.

Nucleotide sequence accession numbers. The GenBank accession number for the almost complete 16S rRNA sequence of *Lactobacillus* sp. strain Y10 is AY029223. See Table 2 for accession numbers for DGGE bands from uncultured strains.

RESULTS

Growth of lactic acid bacteria during whisky fermentation. Bacterial growth during fermentation at the Glenkinchie Distillery occurred in three phases. A lag period lasted from the point of yeast addition (0 h) to about 35 h, during which alcoholic fermentation was maximal (Table 1). Exponential growth of bacteria ensued for the next 30 h as yeast metabolism declined. During this phase, the ethanol yield slowly reached its maximum and lactic and acetic acids accumulated at the expense of the residual sugars (Table 1). The final stationary or decline phase began at about 65 to 70 h and was characterized

TABLE 1. Fermentation products and residual sugar concentrations during the late lactic fermentation

Time (h)	g of the following product or sugar/liter[a]										
	Lactic acid	Acetic acid	Ethanol	Residual sugars[b]							
				G1	G2	G3	G4	G5	G6	G7	>G10
40	0.49	0.02	76.15	4.10	7.60	10.25	0.15	0.50	0.10	0	2.20
50	0.66	0.23	80.50	1.60	2.45	2.00	0.25	0.60	0.15	0	1.60
65	2.28	0.32	88.05	0.80	0.20	1.05	0.35	0.50	0.15	0	2.40
90	4.00	0.31	88.85	0	0	0.75	0.25	0.25	0.10	0	2.05

[a] Values are means of triplicate determinations.
[b] G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

by a large increase in the lactic acid content of the fermentation and the exhaustion of the remaining sugars.

Incubating pure cultures of lactobacilli at 70°C for 10 min reduced the viability 100- to 1,000-fold, as judged by plating on MRS agar, and the resultant cells stained red-orange with the *Bac*Light kit. These were considered dead. Fluorescence microscopy of exponential-phase cells, on the other hand, revealed bright green cells. Examination of the fermentation samples consistently revealed higher viable bacterial numbers than did counts on modified MRS agar (Fig. 1). The ratio between green and dead (red-orange) cells also varied during the fermentation. Initially, this ratio was essentially even. The wort contained numerous organisms killed or damaged by the mash conditions as well as viable lactobacilli and other cells that would be passed to the fermentation (Fig. 2A). By 15 h, some cocci derived from the wort were still present, together with some longer rods (Fig. 2B). The period at about 35 h represented a turning point in the fermentation; the yeasts were beginning to suffer heat (the malt whisky fermentation is not temperature controlled) and starvation stresses, and the bacteria were beginning to grow exponentially. During this period (35 to 70 h), cocci were lost and short rods dominated (Fig. 2C). Most (about 75%) of the bacteria at this stage produced strong green fluorescence signals (Fig. 2D). Finally, after 70 h, the number of green bacterial cells slowly decreased and red-orange cells became more numerous. During this decline phase, cells formed chains or very elongated forms (Fig. 2E and F).

ESEM avoids the drying procedures and gold coating required for scanning electron microscopy and provides a less distorted view of microbial morphology. Viewed by this procedure, the yeast cells decreased in size with time and collapsed in the later stages of the fermentation (from 60 to 100 h). At least three different morphological types of bacteria could be distinguished. After 15 h of fermentation (Fig. 3A), rods and cocci were apparent, but after 40 h, the population of rod-shaped cells increased dramatically, occasionally forming short chains (Fig. 3B). Following exponential growth of the bacteria (70 h), elongated cells were apparent (Fig. 3C). Possible physical interactions between bacteria and yeast cells, perhaps nutrient scavenging by bacteria from leaky yeast membranes, were observed in the later stages of the fermentation (Fig. 3D). Finally, the yeast cells collapsed and the bacteria began to die (Fig. 3E and F).

The experiments described above were conducted with samples taken from two fermentations, but we are confident that they represent typical fermentation conditions because it has been shown that the bacterial community in fermentations at Glenkinchie Distillery is stable over prolonged periods of several months (23) and distillery records show that these fermentations had no unusual characteristics. The DGGE results described below, however, represent typical data from several fermentations.

Community fingerprinting. Initially, we amplified 16S rRNA genes from strains representing common distillery species by using primer pair 1 (positions 339 to 539 in the *E. coli* gene). DNA fragments from *L. acidophilus* and its phylogenetic close relatives *L. amylolyticus*, *L. amylovorus*, *L. crispatus*, and *Lactobacillus* sp. strain Y10 had similar migration distances (Fig. 4A, lanes 1, 2, 3, and 5, and Fig. 4B, lane E), as did products from *L. paracasei* and *L. plantarum*. Some strains, such as those of *L. buchneri*, were distinguishable from close relatives (*L. kefiri*) because they presented two bands, with one in common (Fig. 4A, lanes 10 and 11). When DNA amplified from distillery fermentation samples was examined by using these primers, two main groups of strains were revealed. Bacteria corresponding to *L. fermentum* were present from the early stage of the fermentation but were particularly pronounced at 46 h, reaching a maximum at 70 h and then decreasing slightly. A very faint band representing the *L. acidophilus* group (comigrating with *Lactobacillus* sp. strain Y10) was present before 70 h but increased from this point on, reaching a maximum at

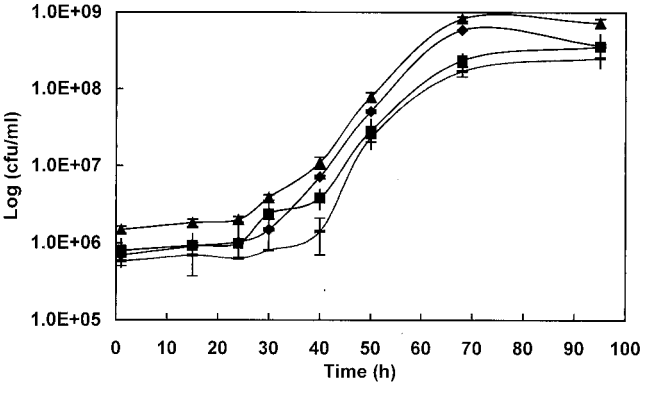

FIG. 1. Bacterial growth during Scotch whisky fermentation. Symbols: triangles, total microscopic cell count; diamonds, viable microscopic cell count; squares, stressed/dead cell count; hyphens, viable culturable cells. Each point of the microscopic counts is the mean of three samplings, and for each sample, 10 microphotographs were taken. Each point of the plate counts is the result of triplicate counts. Standard deviations are shown (error bars).

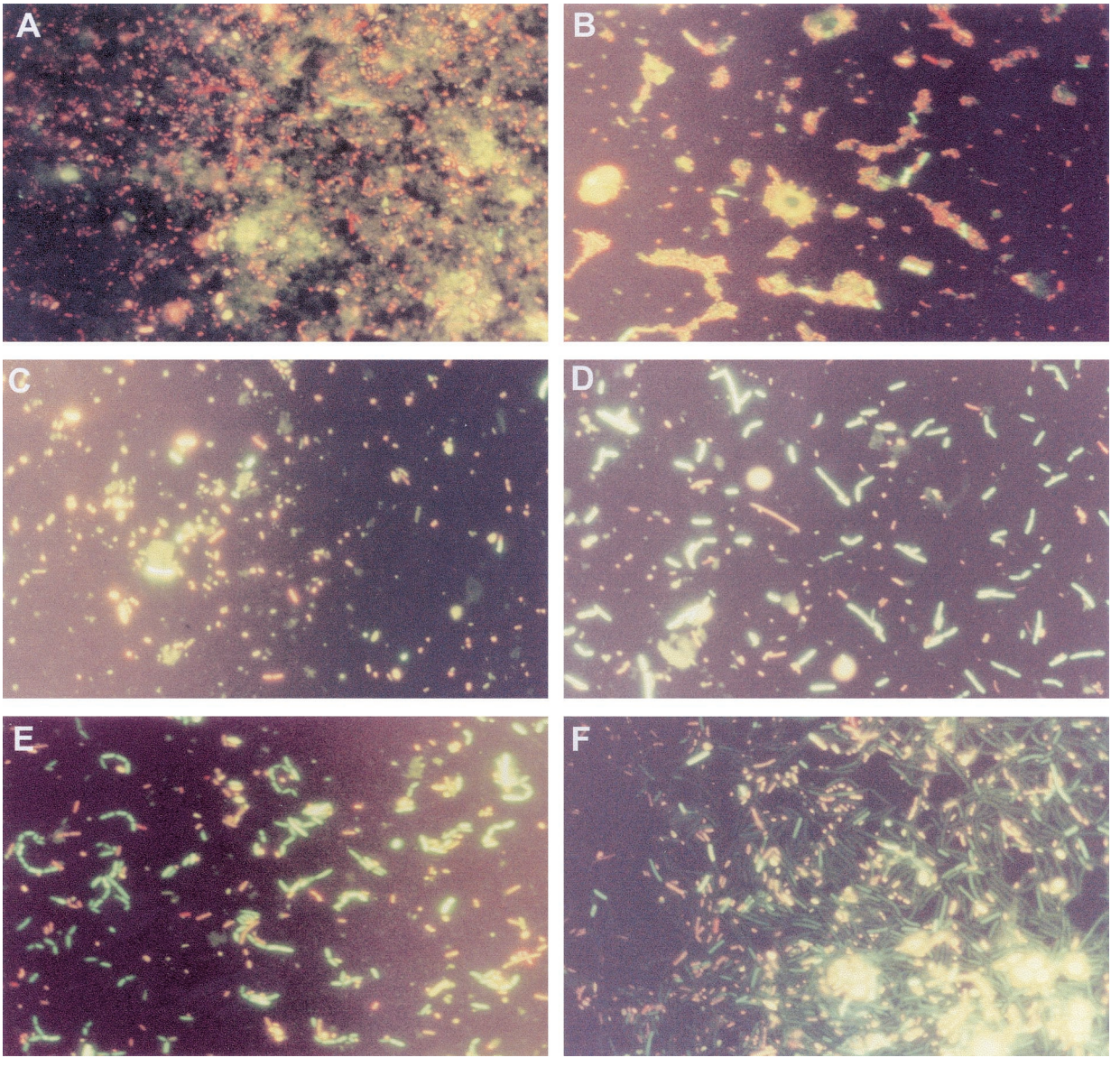

FIG. 2. Fluorescence photomicrographs of whisky fermentation samples. (A) Wort after heat treatment; (B to F) samples at 15 h (B), 40 h (C), 55 h (D), 70 h (E), and 95 h (F) after yeast addition. Live cells stain green; stressed/dead cells stain orange/red.

the very end of the fermentation. Such bacteria have been isolated from Japanese malt whisky fermentations but had not been detected previously in Scotch malt whisky fermentations (23). This band was accompanied by a second, slightly larger product, perhaps representing another species from this phylogenetic group. Other DNA fragments were less evident and corresponded to *L. plantarum* or *L. paracasei* in late fermentation samples (70 to 120 h) and to *L. brevis* from 30 to 70 h. The DNA fragment labeled "a" at 10 to 52 h could not be sequenced, and the band labeled "b" at 46 h was identified by migration distance as *L. ferintoshensis* (Table 2).

We next targeted a region of the 16S rDNA gene (nucleotides from positions 939 to 1163 of the *E. coli* gene) in which polymorphisms exist among species of the *L. acidophilus* group. Primer pair 2 gave improved discrimination of DNA fragments from species related to *L. acidophilus*, although strains of *L. amylovorus* and *L. crispatus* still migrated to the same position (Fig. 5A, lanes 7 to 10). Moreover, *L. paracasei* was now distinguished from *L. plantarum*, but strains of *L. kefiri* and *L. buchneri* (Fig. 5A, lanes 11 and 12), *L. amylolyticus* and *L. casei* (lanes 3 and 7), and *L. pentosus* and *L. plantarum* (lanes 13 and 14) were superimposed. Nevertheless, these data enabled us to analyze with different levels of discrimination the bacterial community in the fermentation (Fig. 5B). The development of the bacterial flora portrayed by these primers resembled that found earlier. Cocci were present in the early stage (labeled "a" and "b" in Fig. 5B) and declined as the middle stage began. *L. fermentum* occurred in the early stage but, on this occasion, continued into the end of the fermentation. With these primers, we recovered several bands corre-

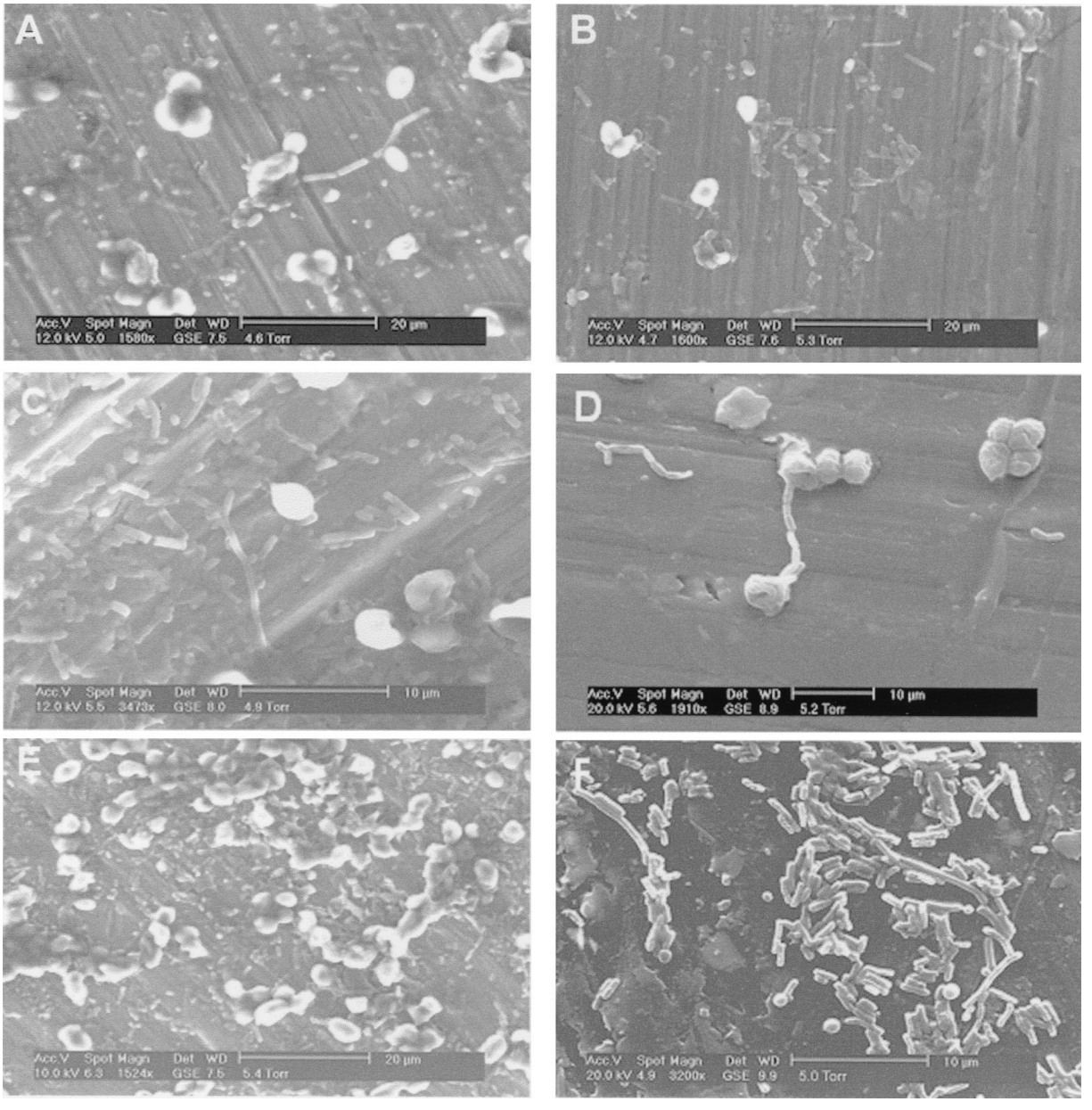

FIG. 3. Environmental scanning electron micrographs of whisky fermentation samples 20 h (A), 40 h (B), 60 h (C), 70 h (D), 95 h (E), and 100 h (F) after yeast addition.

sponding to *L. fermentum* when DNA was isolated and sequenced (labeled "c," "d," and "e" in Fig. 5B). *L. ferintoshensis* (labeled "f" in Fig. 5B) was again present in the middle stage of the fermentation, and *L. casei* or *L. paracasei* was present in the middle to later stages. An *L. acidophilus*-like bacterium again proliferated in the final stage of the fermentation, but the single band seen with primer pair 1 was now resolved into several products of different compositions. A strain corresponding to *Lactobacillus* sp. strain Y10 was identified (labeled "k" in Fig. 5B), as was a new organism which we have now isolated in a pure culture and provisionally called *Lactobacillus* sp. strain 19-2 (labeled "h" in Fig. 5B). There were other bands (labeled "l" and "m" in Fig. 5B) which we have been unable to identify with confidence due to difficulties with sequencing, but they are probably members of the *L. acidophilus* group.

On this occasion, we also examined DNA extracted from the cooled wort (prior to yeast inoculation) and from the yeast suspension before inoculation into the fermentation. The cooled wort provided a source of *L. fermentum*, *Lactobacillus* sp. strain Y10, and *Lactobacillus* sp. strain 19-2, and the major bands recovered from the yeast suspension represented *L. brevis* and *L. plantarum*.

Finally, we prepared a DGGE profile derived from total RNA extracted from the distillery fermentation and amplified by RT-PCR by using primer pair 2. Since active bacteria have higher numbers of ribosomes than dead or dormant cells, this

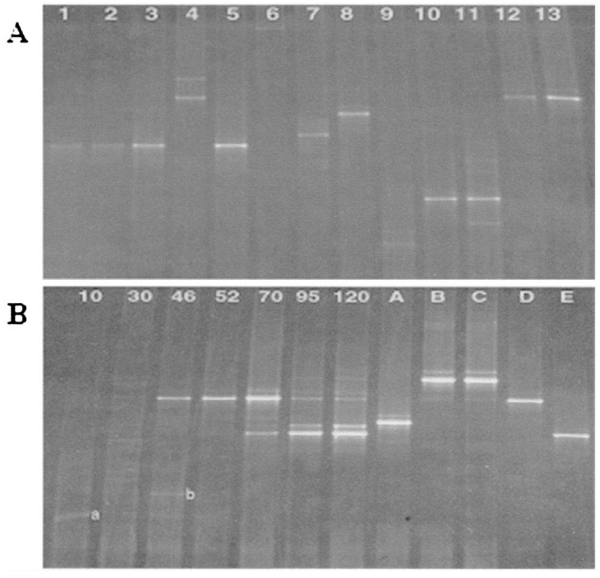

FIG. 4. DNA-based DGGE gels of *Lactobacillus* reference strains (A) and fermentation samples (B) amplified by using primer pair 1 and separated in an 8% polyacrylamide gel containing a 40 to 55% denaturing gradient. (A) Lanes: 1, *L. crispatus*; 2, *L. acidophilus*; 3, *L. amylolyticus*; 4, *L. pentosus*; 5, *L. amylovorus*; 6, *L. casei*; 7, *L. brevis*; 8, *L. fermentum*; 9, *L. ferintoshensis*; 10, *L. kefiri*; 11, *L. buchneri*; 12, *L. paracasei*; and 13, *L. plantarum*. (B) Lanes 10 to 120, DNA profiles of samples taken after 10, 30, 46, 52, 70, 95, and 120 h of fermentation. Lanes A, B, C, D, and E, DNA fragments from pure cultures of strains of *L. brevis*, *L. paracasei*, *L. plantarum*, *L. fermentum*, and *Lactobacillus* sp. strain Y10, respectively. The bands labeled "a" and "b" are described in Table 2.

procedure provided an indication of the relative activities of the major lactic acid bacteria present (Fig. 6). Cocci were evident in the wort and continued strongly until the later stage of the fermentation (85 h). The wort also appeared to be a source of *Lactobacillus* sp. strains 19-2 and Y10, *L. brevis* (labeled "j" in Fig. 6), and three different strains of *L. fermentum*. *L. brevis* and *L. fermentum* strains again flourished in the early stage, but one *L. fermentum* strain reappeared in the final sample. *Lactobacillus* sp. strain Y10 was growing particularly strongly at 85 h, and *Lactobacillus* sp. strain 19-2 was mostly represented in the earlier samples. Finally, *L. plantarum* (labeled "l" in Fig. 6) showed more activity in the early stage of the fermentation.

DISCUSSION

Many whisky distillers practice late lactic fermentation, during which lactic acid bacteria develop following yeast growth (22, 26). While gross bacterial numbers have been estimated in the past (11), this is the first time that the relative populations of the different types of bacteria present during a fermentation have been examined. Our results show that the fermentation can be divided into three phases: the period up to about 35 h from yeast inoculation, when yeast growth is rampant and bacterial growth is heavily suppressed; a second phase, from 35 h to about 70 h, when bacterial growth is exponential at the expense of exhausted yeast cells; and a final stationary or decline phase, when bacterial numbers no longer increase but lactic acid continues to accumulate (Fig. 1 and Table 1).

Microscopic analysis of the wort revealed cocci and rods as potential inocula for the initial phase. However, it is difficult to assess the viability of this population given that the heat stress encountered may lead to a false interpretation of faintly red cells as dead, while these damaged cells may recover and grow (7). Nevertheless, the subsequent observation of cocci by ESEM (Fig. 3A and B) and the recovery of cocci related to *Streptococcus thermophilus* and *Saccharococcus thermophilus* in DGGE gels indicate that these bacteria do indeed subsequently grow. The exact nature of these bacteria is obscure because they were not previously identified in distillery fermentations. Partial 16S rRNA sequence analysis identified them as either *Streptococcus thermophilus* or *Saccharococcus thermophilus*. The former was previously associated only with milk, although streptococci related to *Streptococcus bovis* have been identified as a major component in the early stages of maize fermentations (3, 4, 5). *Streptococcus thermophilus* can survive a high temperature (60°C for 30 min) and can therefore survive a distillery mash, and both fluorescence microscopy and RNA-based DGGE suggested that viable cocci entered the fermentation from the wort. The alternative identification was *Saccharococcus thermophilus* (98% similarity over 224 nucleotides), a catalase-positive coccus that metabolizes sugars to lactic acid but is phylogenetically close to the thermophilic endospore-forming bacteria (21). This bacterium was originally isolated from sugar beet, and although it could have inhabited the distillery fermentation, its low tolerance of acid pH argues against this possibility. The 98% sequence similarity with known organisms covers a relatively conserved part of the 16S rRNA gene and thus does not allow for precise phylogenetic placement, and the identification of this coccus will require either isolation in a pure culture or cloning and analysis of complete rDNA sequences.

The first phase of the fermentation is also populated by rods from the wort and yeast cells which were identified by DGGE as mainly *L. brevis*, *L. fermentum*, and some bacteria, for example, *L. ferintoshensis*, that were not apparent in the incoming ingredients and were presumably colonizing the distillery plant. Previous studies examining the distributions of lactobacilli in geographically disperse distilleries concluded that individual distilleries may generate their own flora (23). During this first phase, the cocci and rods grew slowly and showed symptoms of stress under the fluorescence microscope, presumably due to the temperature shock of mashing and the massive competition provided by the yeast cells.

However, by 35 to 40 h, the second phase of the bacterial growth pattern was beginning. This middle phase was characterized by a reduction in yeast growth (ethanol production was 86% complete at 40 h), and the sugar content of the fermentation was about 4 g of glucose/liter, 7.6 g of maltose/liter, and some residual higher dextrins. Rod-shaped bacteria of various lengths (Fig. 2C and D and Fig. 3B and C) which stained strongly with the viability stain grew during this period alongside the cocci. During the second phase the difference between the microscopic viable counts and the plate counts was minimal, suggesting that most of the bacteria could be recovered on our modified MRS agar. *L. fermentum* dominated DGGE gels from this stage on, as it does in many cereal fermentations (1,

TABLE 2. Identities of bands obtained from DGGE analysis of the bacterial community

Figure	Band	Closest relative	No. of nucleotides sequenced	% Identity[a]	Accession no.
4B	a	ND		ND	ND
	b[b]	*L. ferintoshensis*			
5B	a	*Streptococcus thermophilus* or *Saccharococcus thermophilus*	206	98	AF375001
	b	*Streptococcus thermophilus* or *Saccharococcus thermophilus*	195	97	AF375002
	c	*L. fermentum*	210	100	
	d	*L. fermentum*	208	100	
	e	*L. fermentum*	201	99	AF375003
	f[b]	*L. ferintoshensis*			
	g[b]	*L. casei, L. paracasei*, or *L. brevis*			
	h[b]	*Lactobacilus* sp. strain 19-2			
	i[b]	*L. crispatus*			
	j[b]	*L. plantarum*			
	k	*Lactobacillus* sp. strain Y10	223	100	
	l	*Lactobacillus* sp. strain Y10	212	99	AF375005
	m	*Lactobacillus* sp. strain Y10	243	99	AF375007
6	a	*Streptococcus thermophilus* or *Saccharococcus thermophilus*	206	98	Identical to AF375001
	b	ND	ND	ND	ND
	c	ND	ND	ND	ND
	d	*L. fermentum*	200	99	AF375004
	e	*L. fermentum*	220	100	
	f	*L. fermentum*	223	100	
	g	*L. fermentum*	201	99	Identical to AF375003
	h[b]	*L. ferintoshensis*			
	i[b]	*L. casei* or *L. paracasei*			
	j[b]	*L. brevis*			
	k[b]	*Lactobacillus* sp. strain 19-2			
	l[b]	*L. plantarum*			
	m	*Lactobacillus* sp. strain Y10	223	100	
	n	*Lactobacillus* sp. strain Y10	212	99	Identical to AF375005

[a] Percentage of identical nucleotides in the sequence retrieved from the DGGE gels and the sequence of the closest relative found in GenBank. ND, not determined.
[b] Band identified by comparison of migration distance with those of reference strains.

6, 14, 15, 17). Bands from neighboring locations on the gels were also identified as *L. fermentum*, reflecting the genomic diversity of this species, as previously indicated by ribotyping (23, 27) and randomly amplified polymorphic DNA analysis (16). *L. fermentum* was accompanied during the second phase by other heterofermentative species, such as *L. ferintoshensis*, and the homofermentative bacterium *L casei* or *L. paracasei*. The heterofermentative organisms were presumably responsible for the accumulation of acetic acid between 50 and 70 h as well as lactic acid (Table 1). High concentrations of acetic and lactic acids will have a detrimental effect on yeast viability and will contribute to the death of the yeast as the fermentation proceeds (20).

The final phase of the fermentation began at about 70 h as the bacteria entered stationary phase and loss of viability ensued (Fig. 1A and Fig. 2D and E). There was a tendency for the cells to form long rods and chains, presumably as a result of stress (8). This characteristic accentuated the difference between microscopic viable counts and plate counts, since the latter would underestimate viability through growth of colonies from chains of cells. The yeast cells began to collapse and die during this period (Fig. 3E and F), and there was evidence for attachment of bacteria to yeast cells (Fig. 3D). It seems likely that the release of materials through leaking yeast membranes provided valuable nutrients for the bacteria as the sugar concentration of the fermentation dropped to almost zero. Bacterial growth in this later stage was largely represented by two unknown organisms that produced superimposed bands when DGGE was performed with primer pair 1 but could be distinguished with primer pair 2. One of these bacteria is the same as *Lactobacillus* sp. strain Y10, which was isolated from Japanese malt whisky fermentations and resembles the homofermentative bacterium *L. acidophilus* (unpublished results). This is the first time that such a bacterium has been detected in Scotch whisky fermentations, although it is relatively common in Japanese distilleries. Database comparisons of the almost complete 16S rDNA sequence of the second bacterium, which we have designated *Lactobacillus* sp. strain 19-2, indicated that its closest phylogenetic relative is *Lactobacillus delbrueckii* (data not shown). Both of these bacteria have complex growth requirements based on malt wort, and neither grows in or on standard laboratory media, such as MRS broth or agar. Full descriptions of these bacteria will be given elsewhere. The growth of these organisms in the final phase of the fermentation was accompanied by a large increase in lactic acid accumulation but no further acetic acid accumulation. These findings are consistent with homofermentative metabolism.

Other traditional fermentations have also been noted to follow three-stage community changes similar to those discovered here. For example, pozol, a Mexican fermented maize dough which has been studied in detail, is initiated by a high diversity of bacteria, including various streptococci, followed at the second stage by heterofermentative lactobacilli, notably *L.*

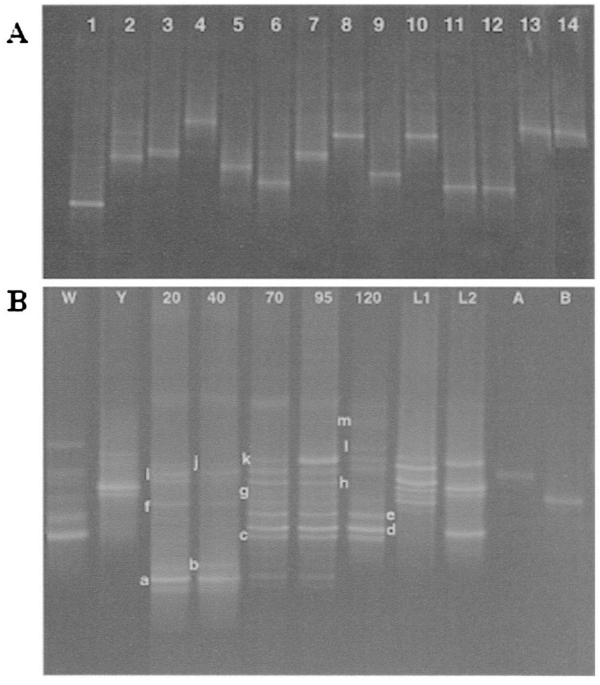

FIG. 5. DNA-based DGGE gels of *Lactobacillus* reference strains (A) and fermentation samples (B) amplified by using primer pair 2 and separated in an 8% polyacrylamide gel containing a 40 to 55% denaturing gradient. (A) Lanes: 1, *L. fermentum*; 2, *L. brevis*; 3, *L. casei*; 4, *L. paracasei*; 5, *L. hilgardii*; 6, *L. ferintoshensis*; 7, *L. amylolyticus*; 8, *L. amylovorus*; 9, *L. acidophilus*; 10, *L. crispatus*; 11, *L. kefiri*; 12, *L. buchneri*; 13, *L. pentosus*; 14, *L. plantarum*. (B) Lanes W and Y, samples taken from the wort and yeast, respectively. Lanes 20 to 120, DNA profiles of samples taken after 20, 40, 70, 95, and 120 h of fermentation. Lanes L1 and L2, reference ladders, from top: L1, strain Y10, strain 19-2, *L. paracasei*, and *L. ferintoshensis*; L2, *L. plantarum*, *L. brevis*, and *L. fermentum*. Lanes A and B, DNA fragments from pure cultures of strains of *L. crispatus* and *L. acidophilus*, respectively. The bands labeled "a" to "m" are identified in Table 2.

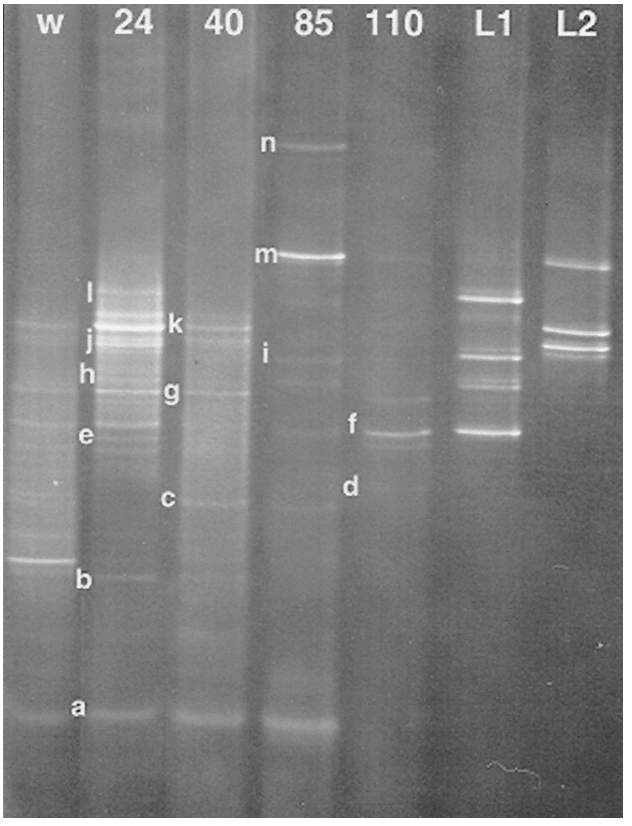

FIG. 6. RNA-based DGGE detection of *Lactobacillus* strains during whisky fermentation (from wort to 110 h) by using primer pair 2 and the conditions described in the legend to Fig. 5. Lane W, sample taken from the wort. Lanes 24 to 110, RNA profiles from samples taken after 24, 40, 85, and 110 h of fermentation. Lanes L1 and L2, DNA fragment ladders from pure cultures of strains (from top to bottom): L1, *L. plantarum*, *L. casei* or *L. paracasei*, *L. ferintoshensis*, and *L. fermentum*; L2, *Lactobacillus* sp. strain Y10, *Lactobacillus* sp. strain 19-2, and *L. brevis*. The bands labeled "a" to "n" are identified in Table 2.

fermentum, and finally homofermentative lactobacilli, in this case relatives of *L. casei* (5).

While the advantages of DGGE for the evaluation of complex community changes have been indicated in this study, the difficulty of developing a single set of primers for the differentiation of all species present has been highlighted. The close phylogenetic relationships of the lactobacilli made it impossible to differentiate all species in a single reaction. Members of the *L. acidophilus* cluster proved particularly difficult to differentiate by using primers HDA1-GC and HDA2 (25), targeted to the V2 region of the rRNA gene. The sequences of *L. acidophilus*, *L. amylolyticus*, *L. amylovorus*, *L. crispatus*, and *Lactobacillus* sp. strain Y10 are essentially identical over this region, with only seven polymorphic sites among all the strains, resulting in the failure of DGGE to resolve the PCR fragments. However, the area from positions 939 to 1163 (*E. coli* numbering) amplified by primers HDA4C and HDA5 was much smaller than the V2 region but contained 14 polymorphic sites for these bacteria and enabled discrimination. It is therefore important to substantiate DGGE gels of complex communities with multiple sets of primers directed to various parts of the rRNA gene in order to be confident that bands represent a single species.

A second drawback is that DGGE of DNA templates is not quantitative. Various ways to provide quantitative estimates involve the use of competitive quantitative PCR-based methods (12, 18) or 16S rRNA-targeted oligonucleotide probes (4). Here we simply used rRNA templates to provide a comparison with rDNA templates on the basis of the idea that actively growing cells will have large numbers of ribosomes compared with stationary-phase cells. The rRNA templates gave us a different sensitivity and, in particular, revealed the presence of *L. brevis* and *L. casei* or *L. paracasei* in the early stage of the fermentation, the continuation of the cocci well into the second stage, and the recurrence of *L. fermentum* in the final stage of the fermentation. Indeed, *L. fermentum* and *L. paracasei* were consistently recovered from late fermentation samples from various distilleries in a previous study (23).

In conclusion, we have shown that the Scotch whisky fermentation involves a changing community of bacteria starting with a diversity of cocci and rods and culminating in lactobacilli that fail to grow in or on standard laboratory media and are

probably closely related to *L. acidophilus* or *L. crispatus*. The formation of lactic and acetic acids and other metabolites might have an effect on the flavor of the final spirit. Lactic acid reacts with ethanol during distillation to produce ethyl lactate, and spirits derived from long fermentations (greater than 55 h) in which lactic acid bacteria have flourished tend to have higher ester concentrations (13). It is possible that flavor could be modified by careful attention to the balance of the various bacteria present.

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