Hybridization Between the Deoxyribonucleic Acids of Some Strains of Heterofermentative Lactic Acid Bacteria

ELLEN I. GARVIE

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, England

The degree of hybridization between the deoxyribonucleic acids (DNAs) of some different species of heterofermentative lactic acid bacteria was estimated. The DNAs from each of 25 strains comprising 12 species were hybridized with labeled DNAs from the type or reference strains of five species. The results suggest a very close relationship between *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, and *Leuconostoc cremoris*, whereas *Leuconostoc oenos* was found to be unrelated to other species of the genus. The results also indicate that *Lactobacillus confusus* and *Lactobacillus viridescens* are more allied to the leuconostocs than to other lactobacilli.

At one time the leuconostocs were classified as streptococci because of their morphology (1, 11), but for the past several decades leuconostocs and streptococci have been separated into two genera in the family *Streptococcaceae* (12). However, as knowledge of the leuconostocs has increased, it has become apparent that they share several properties with the heterofermentative lactobacilli and have many differences from the streptococci.

At present there are six named species of *Leuconostoc* which are generally accepted (see Table 1). *Leuconostoc oenos* alone is acidophilic and is distinct from the other five species (8). These five species have many common properties which are also found in *Lactobacillus confusus* and *Lactobacillus viridescens* (9, 23). The other species of heterofermentative lactobacilli appear to have fewer properties in common with *Lactobacillus confusus* and *Lactobacillus viridescens* than these last-named species have with the leuconostocs (9). Deoxyribonucleic acid (DNA) hybridization studies have been made in an attempt to clarify the relationships of these bacteria. The results of these studies are reported here.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. They were all obtained from the National Collection of Dairy Organisms (NCDO). Several had been included in earlier work (6-10). Bacteria were prepared for use as previously described (10). The strains of *Lactobacillus brevis* and *Lactobacillus buchneri* were grown in MRSB (18) at 30 C, and the strains of *Lactobacillus fermentum* were grown at 37 C.

Purification of DNA. Cells were grown for extraction of DNA in medium 1 (a yeast-glucose-peptone broth, pH 6.5); cells of *Leuconostoc oenos* were grown in a modified medium 1 at pH 4.8 (8). Cells were

harvested in the late logarithmic or early stationary phase of growth and were washed twice in water. *Escherichia coli* cells were grown on nutrient agar overnight at 37 C. The cells were washed off the agar with water and then given a second wash in water.

The wet, packed cells were frozen at -20 C and were stored at this temperature at least overnight and often for several days. The frozen cells were prepared for use by covering them with a small amount of water and allowing them to thaw at room temperature. Cells of lactic acid bacteria were lysed overnight at 37 C with lysozyme and Pronase in 3% 4-amino salicylate which was dissolved in standard saline citrate (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate). The components of the lysing solution are shown in Table 2. These were added in the order shown in the table. Cells of *Escherichia coli* were not treated with lysozyme and Pronase but were suspended in 3% 4-amino salicylate in SSC.

The lysed cells and the *Escherichia coli* cells were treated at 60 C for 10 min with 0.5% isopropyl naphthalene sulfonate (15) by adding 2 ml of a 6.25% solution to each 25 ml of lysate or cell suspension.

The viscous solutions were deproteinized by shaking for 15 min in the cold with phenol water (10) saturated with SSC. A volume of chloroform-isoamyl alcohol (24:1) equal to a quarter of the aqueous volume was added to help separate the layers on centrifuging (3). After centrifuging, the viscous aqueous upper layer containing DNA was removed and deproteinized by shaking for 15 min with chloroform-isoamyl alcohol (24:1). The layers in this mixture were separated by centrifugation, and the DNA in the aqueous upper layer was precipitated with 66% ethanol and collected on a spooling rod.

Additional purification followed the method of Young and Spizizen (24). The spooled DNA was dispersed in SSC and treated with ribonuclease (final concentration, 50 µg/ml of DNA solution) for 30 min at 37 C, and then with Pronase (final concentration, 1.0 mg/ml of DNA solution) for 60 min at 37 C. The solution was deproteinized with 0.2% sodium deoxycholate in 2 M NaCl. After 60 min of incubation at 37 C, the solutions were held overnight at 4

TABLE 1. *Bacterial strains used*

Species	NCDO no.	Additional information
<i>Leuconostoc oenos</i>	1674 1707	Type strain
<i>Leuconostoc mesenteroides</i>	523 797 768	Reference strain B523 NCIB 9317 Strain 39
<i>Leuconostoc dextranicum</i>	529 812 1589 1590	Reference strain NCDO 516 Does not form dextran Does not form dextran
<i>Leuconostoc cremoris</i>	543 828	Reference strain
<i>Leuconostoc lactis</i>	546 533 2037	Type strain
<i>Leuconostoc paramesenteroides</i>	803 871	Type strain
<i>Lactobacillus viridescens</i>	403 1655	S41A S38A; type strain
<i>Lactobacillus confusus</i>	1586 930 1975	ATCC 10881; type strain <i>Lactobacillus coprophilus</i>
<i>Lactobacillus vermiforme</i>	1965	
<i>Lactobacillus brevis</i>	1749	Type strain
<i>Lactobacillus buchneri</i>	110	Neotype strain
<i>Lactobacillus fermentum</i>	1750	Neotype strain
<i>Escherichia coli</i>	1984	K-12; NCTC 10538

C. The DNA was separated from impurities by centrifugation and was precipitated with 66% ethanol. This procedure was repeated but with the Pronase treatment omitted. Finally, the DNA was twice precipitated with isopropyl alcohol (19). DNA was washed in ethanol and was finally dissolved in 0.1× SSC (SSC diluted 1:9 with water).

After each precipitation, at least 24 h were allowed for the subsequent solubilization of the DNA. This process was helped by occasionally warming the DNA in a water bath at 45 to 50 C, a temperature well below that at which thermal denaturation starts. As far as possible, sterile glassware and solutions were used during preparation to minimize the risk of bacterial growth during the long solubilization stages. This method of isolating DNA is discussed later.

Testing for purity. DNA solutions were tested for protein by the sensitive method of Lowry et al. (17),

for ribonucleic acid (RNA) by the orcinol test for pentose (22), and for DNA concentration by the diphenylamine test of Burton (2). In addition, melting curves were plotted and hyperchromicity was calculated.

Labeled DNA. A 100-μCi amount of [methyl-³H]thymidine (Amersham Radiobiochemicals) was added to each 1,000 ml of medium. Cells were grown and harvested. The DNA was isolated as described.

Hybridization of DNA. The method used was based on the membrane-filter method of Denhardt (5). Filters were loaded with heat-denatured, unlabeled DNA as described by De Ley and Tutgat (4) using 25-mm-diameter membrane filters (Millipore Corp.). The difference in optical density (260 nm) of the DNA solution before and after filtration together with that of the rinse was used to calculate the DNA on each filter. The calculation assumed that 10 μg of heated DNA per ml gave an optical density at 260 nm (OD₂₆₀) of 0.280.

The filters were washed with 10 ml of 6× SSC and allowed to dry in air for 2 to 3 h. They were then heated overnight at 60 C in a vacuum desiccator over P₂O₅. The dried filters were stored at 4 C in the vacuum desiccator.

The DNA-loaded filters were cut into quarters and marked with a pencil for identification. Three separate quarters were placed in each 5.0-ml conical flask containing 1.4 ml of 2× SSC-30% dimethyl sulfoxide (Me₂SO) (4). At least one of these quarters contained DNA homologous with the labeled DNA to be added. One flask in every batch contained a filter quarter saturated with 6× SSC but without any DNA.

Labeled DNA was diluted in 0.1× SSC. It was sheared by passing 10 times through a hypodermic syringe (25G × 5/8) and denatured by holding at the temperature of boiling water for 10 min and then plunging into ice-cooled water. The solutions were then stored frozen at -10 C. For use, they were diluted in 0.1× SSC, and 0.1 ml was added to each flask containing the filter quarters. The concentration of DNA was adjusted so that the disintegrations per minute of the filter quarter containing homologous DNA were between 1,000 and 2,000.

After the labeled DNA was added, the flasks were manually shaken to mix the solutions and then shaken overnight in a water bath at 45 C.

The filter quarters were transferred to scintillation vials and washed three times in 5.0 ml of 2×

TABLE 2. *Suspension used for lysing heterofermentative lactic acid bacteria*

Substance	Concentration	Solute	Pretreatment	Amount in lysing solution ^a
Wet-packed cells				1.0 g
Water				4.5 ml
Pronase	40 mg/ml	EDTA-NaCl (pH 7.0) ^b	Incubate 1 h, 37 C	0.25 ml
Lysozyme	2.5 mg/ml	Water		2.0 ml
Concentrated saline citrate ^c				0.75 ml
4-Amino salicylate	12%	SSC		2.5 ml

^a Total volume (excluding cells), 10.0 ml.

^b EDTA, 0.1 M; NaCl, 0.15 M.

^c Concentrated saline citrate is SSC concentrated 10-fold.

SSC; 10.0 ml of polyphosphor [methoxyethanol, 600 ml; anisole, 400 ml; 2,5-diphenyloxazole, 7 g; 1,4-bis-(5-phenyloxazolyl)benzene, 6.3 g; naphthalene, 100 g] was then added to the vials. The coating from the filter dissolved, leaving the transparent center part, which was then broken on a Whirlimix (Fisons Scientific Apparatus). Counts were made on a Phillips liquid scintillation counter (LSA 4500/01).

The reasons for using direct hybridization of DNA are given in the Discussion.

RESULTS

Purity of DNA. The protein concentration (wt/wt DNA) was less than 10% and, in most cases, it was less than 5%. RNA proved more difficult to remove than protein, and it was necessary to precipitate twice with isopropyl alcohol to lower the concentration of RNA to between 5 and 10%.

The hyperchromicity of DNA with a moles percent guanine plus cytosine (mol% G+C) of 38 to 50 is anticipated to be 0.42 to 0.39. The DNA used in the hybridization experiments had a hyperchromicity of 0.37 to 0.39. This was slightly lower than expected and was probably due to the protein, RNA, and other impurities absorbing at 260 nm.

Testing the hybridization method. De Ley and Tutgat (4) have shown that $2\times$ SSC plus 30% Me_2SO is a satisfactory solution for DNA/DNA hybridization experiments using membrane filters. Preliminary trials comparing this solution with the preincubation mix of Denhardt (5) and with the formamide used by Kourilsky et al. (16) showed that the first solution was slightly more efficient than the others in preventing DNA from washing off the filters and in preventing labeled DNA from adhering to blank filters. Kourilsky et al. (16) demonstrated that several filters could be tested in a single container. Preliminary experiments not only confirmed these observations but also showed that the variation between two filters in the same flask was no greater than that between two filters in separate flasks. This technique has the advantages of economizing on the amount of labeled DNA used and of allowing a direct comparison of the DNA from different bacteria.

Loading of filters. The loaded filters were calculated to bear between 30 and 40 μg of DNA/cm². De Ley and Tutgat (4) give a figure of 32 μg of DNA/cm² for a saturated PHWP filter.

Labeled DNA. Preparations of labeled DNA were tested for contaminating protein and RNA. The counts per microgram were also estimated and were between 3,000 and 8,000 dpm for the leuconostocs. *Lactobacillus confusus*

was more efficient than the leuconostocs at incorporating thymidine and had a count of about 20,000 dpm/ μg .

Relationships between lactic acid bacteria. Table 3 shows the degree of DNA/DNA hybridization between the various strains examined. Initially, labeled DNA was prepared from four strains, which were selected for the following reasons. The reference strains of *Leuconostoc mesenteroides* and the type strain of *Lactobacillus confusus* were chosen because these species cause difficulty in classification. *Lactobacillus viridescens* was used because the G+C content of its DNA is intermediate between that of *Lactobacillus confusus* and that of *Leuconostoc mesenteroides* (10). In this instance, the type strain (NCDO 1655) of *Lactobacillus viridescens* was not used because it usually gives a low yield of DNA and is troublesome to handle. *Lactobacillus viridescens* NCDO 403, one of the original Niven strains, was chosen because it was known to give a higher yield of DNA and generally to behave better in preparation than the type strain. The second *Leuconostoc* species selected was *Leuconostoc lactis*, which has DNA with a higher G+C content than other species of *Leuconostoc* (10). Here again the type strain of *Leuconostoc lactis*, NCDO 533, was not used, but NCDO 546 was selected because it is an easier strain with which to work.

After it was found that *Leuconostoc paramesenteroides* DNA failed to hybridize with *Leuconostoc mesenteroides* DNA, labeled DNA was prepared from *Leuconostoc paramesenteroides* NCDO 803, the type strain. Experiments could then be made reversing the labeled and unlabeled DNA, and the relationship of these two species could then be determined.

Hybridization of non-dextran-forming strains of leuconostocs. Two strains, NCDO 1589 and NCDO 1590, were included. They do not form dextran from sucrose but had been considered to belong to *Leuconostoc dextranicum* because phenotypically they differed from strains of the non-dextran-forming species *Leuconostoc paramesenteroides* (Table 4). The identity of NCDO 1589 and 1590 was supported by the results of DNA/DNA hybridization (Table 3).

DISCUSSION

Many methods have been described for the preparation of bacterial DNA and, of these, the one described in detail by Marmur (19) is most often used. However, for the heterofermentative lactic acid bacteria, this method gave a low yield of DNA, and the product contained pro-

TABLE 3. Percent hybridization between the deoxyribonucleic acids of some heterofermentative lactic acid bacteria

Bacteria	NCDO no.	Hybridization (%) of labeled DNA from:									
		<i>Leuconostoc mesenteroides</i> 523		<i>Leuconostoc lactis</i> 546		<i>Leuconostoc paramesenteroides</i> 803		<i>Lactobacillus viridescens</i> 403		<i>Lactobacillus confusus</i> 1586	
		Mean ^a	±SEM ^b	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
<i>Leuconostoc oenos</i>	1674	15	7.1	11	6.6			0.5	3.3	4	3.7
	1707	14	4.8	2	2.2			1	1.2	-5	4.1
<i>Leuconostoc mesenteroides</i>	523 ^c	100		36	6.8	9	3.4	13	3.7	13	1.5
	768	48	3.8	38	3.6	10	9.6	14	2.3	15	1.9
	797	80	4.2	32	6.5	12	6.7	26	1.7	17	0.4
<i>Leuconostoc dextranum</i>	529	110	9.3	22	9.0			20	7.7	16	3.1
	812	84	19.9	25	1.5			12	0.7	10	1.5
	1589	104	3.6	30	5.7	15	4.9	26	1.5	17	0.1
	1590	48	6.4	32	5.0	20	0.3	34	0.02	18	2.6
<i>Leuconostoc cremoris</i>	543	66	10.3	29	6.0			12	2.3	13	2.3
	828	105	17.1	35	5.7			21	6.0	13	0.5
<i>Leuconostoc lactis</i>	546 ^c	49	3.3	100		46	10.3	26	5.9	18	2.3
	533	58	1.4	85	4.0			20	9.5	18	2.4
	2037	39	7.6	90	27.4	37	9.5	49	0.2	31	14.1
<i>Leuconostoc paramesenteroides</i>	803 ^c	6	1.8	13	1.2	100		19	2.0	28	3.3
	871	19	6.8	14	2.4	87	8.1	13	2.8	26	2.8
<i>Lactobacillus viridescens</i>	403 ^c	23	3.2	21	3.3	35	10.0	100		50	8.8
	1655	12	1.7	18	1.6			90	2.4	32	2.7
<i>Lactobacillus confusus</i>	1586 ^c	15	2.1	15	1.5	46	5.8	39	9.3	100	
	930	20	6.1	12	4.3			29	5.7	82	5.4
	1975	14	3.8	20	1.4			64	2.5	86	10.7
<i>Lactobacillus veriforme</i>	1965	22	4.5	17	4.0			9	1.9	10	2.3
<i>Lactobacillus brevis</i>	1749	-2	6.0	-7	2.2			10	3.8	8	0.9
<i>Lactobacillus buchneri</i>	110	-5	11.1	3	4.0	10		6	9.0	11	6.4
<i>Lactobacillus fermentum</i>	1750	1	5.5	7	5.8			2	2.1	5	3.0
<i>Escherichia coli</i>	1984	4	2.1	2	0.01			2	1.0	-4	8.2

^a Mean of duplicate tests on several different days.^b SEM, Standard error of the mean.^c Strains from which labeled DNA was prepared.

tein and RNA in concentrations which were unacceptable for DNA/DNA hybridization studies. De Ley and Tutgat (4) found it necessary to modify the Marmur technique. A number of published procedures were tried, and the composite method described here was found to be the most satisfactory. Difficulties with the Marmur technique were encountered from the start, for the gram-positive lactic acid bacteria may not lyse with lysozyme when the cells are suspended in ethylenediaminetetraacetic acid (EDTA) saline at pH 8.0. The cells were ren-

dered sensitive to lysozyme by first freezing and thawing them and then allowing lysozyme in dilute aqueous solution to come into contact with the cells which were suspended in water (20). Salt (see Table 1) was then added, and the pH was adjusted to 7.0, for lysozyme is almost inactive at pH 8.0. EDTA was replaced by 4-amino salicylate as it not only chelates but also releases protein from DNA (14). Finally, Pronase and lysozyme were used together, as it was found that the former did not interfere with lysis by lysozyme.

TABLE 4. Comparison of the characteristics of *Leuconostoc dextranicum*, *Leuconostoc mesenteroides*, and *Leuconostoc paramesenteroides* and of some individual strains of *Leuconostoc*

Character	Leuconostoc dextranicum					Leuconostoc para-mesenteroides — ^a	Leuconostoc mesenteroides			Leuconostoc cremoris	
	— ^a	529 ^b	812 ^b	1589 ^b	1590 ^b		— ^a	523 ^b	768 ^b	543 ^b	828 ^b
Growth at:											
10 C	+	+	+	+	+	+	+	+	+	+	
37.5 C	20 ^c	+	+	—	—	14	28	+	—	—	
39 C	10	—	+	—	—	13	21	+	—	—	
Reaction in yeast glucose litmus milk ^d	ACR or ACRG	ACR	AC-RG	A	ACR	A, ACR, some G	AC-RG	AC-RG	ACr	A	
Hydrolysis of arginine	—	—	—	—	—	—	—	—	—	—	
Dextran formation	+	+	+	—	—	—	+	+	—	—	
Acid from:											
Arabinose	—	—	—	—	—	16	+	+	—	—	
Xylose	16	—	+	—	Slow	5	24	+	—	—	
Fructose	19	+	+	+	Slow	16	30	+	—	—	
Galactose	16	—	+	+	+	+	29	+	+	+	
Glucose	+	+	+	+	+	+	+	+	+	+	
Sorbitose	—	—	—	—	—	—	—	—	—	—	
Cellobiose	3	—	—	—	—	7 (slow)	19	+	—	—	
Lactose	12	—	—	—	—	5 (slow)	16	+	+	+	
Maltose	19	—	+	+	+	16	29	+	+	+	
Melibiose	15	—	+	+	+	15	25	+	—	—	
Sucrose	20	+	+	+	+	+	+	+	—	—	
Trehalose	+	+	+	+	+	+	+	+	—	—	
Raffinose	9	—	+	+	+	7	16	+	—	—	
Glycerol	—	—	—	—	—	—	—	—	—	—	
Mannitol	7 (slow)	—	—	Slow	—	7 (slow)	11	—	—	—	
Sorbitol	—	—	—	—	—	—	—	—	—	—	
Aesculin	8	—	—	—	—	7	29	—	—	—	
Salicin	3	—	—	—	—	—	24	—	—	—	
Hydrolysis of aesculin	8	—	—	—	—	7 (slow)	29	+	—	—	

^a Taken from reference 6. Number of strains examined: *Leuconostoc dextranicum*, 21; *Leuconostoc paramesenteroides*, 17; *Leuconostoc mesenteroides*, 31.^b NCDO number.^c Number of strains giving a positive reaction.^d Abbreviations: A, acid; C, clot; G, gas; R, reduction; r, slight reduction.

Some of the strains used were slow growing and gave low cell yields, and other strains, although growing well, gave a low yield of DNA. Therefore, it was advisable to select a method of hybridization which did not require large amounts of DNA. Competitive DNA hybridization techniques are favored by several workers, but these techniques require large amounts of DNA if the action of the homologous DNA is to be effectively swamped. De Ley and Tutgat (4) found that 700 μ g of DNA was necessary to counteract the effect of 2 μ g of labeled homologous DNA. In addition, competitive techniques exclude the direct comparison of two DNA preparations in the same solution. Therefore, a non-competitive filter method was adopted.

Phenotypically, four of the five non-acidophilic species of *Leuconostoc* form an apparently homologous group in which it is difficult to find clear divisions. The fifth species, *Leuconostoc cremoris*, is characterized by its ability to grow and form acid on a limited range of carbohydrates. However, estimates of the melting temperatures (T_m) of the DNAs of the *Leuconostoc* species (10) showed that the T_m of the DNA of *Leuconostoc lactis* was 1 to 2 degrees higher than those of the other species, including *Leuconostoc oenos*, the acidophilic species which has many distinguishing features. The hybridization studies have shown four genetic groups: *Leuconostoc lactis* and *Leuconostoc oenos* form two of these groups; a third comprises the three species *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, and *Leuconostoc cremoris*, whereas the fourth group contains *Leuconostoc paramesenteroides*. However, there are anomalies within these groups: NCDO 768, which phenotypically has no distinguishing features from other strains of *Leuconostoc mesenteroides*, appears to be genetically different because only 48% hybridization occurred between the DNA of NCDO 768 and that of the reference strain NCDO 523. The significance of this genetic variation cannot be assessed on the limited information available. The properties of both strains of *Leuconostoc mesenteroides* are shown in Table 4. There are no characteristics of NCDO 768 which would identify it as belonging to a separate species, and DNA/DNA hybridization cannot be used as the sole criterion for species differentiation. The high relationship between *Leuconostoc cremoris* and *Leuconostoc mesenteroides* is also difficult to interpret. The characters of NCDO 543 and NCDO 828 are also shown in Table 4. Both strains can be clearly separated from *Leuconostoc mesenteroides*. *Leuconostoc cremoris*

and *Streptococcus cremoris* are the main bacteria of dairy starters, and no wild source of either is known. It may be that *Leuconostoc cremoris* has evolved from *Leuconostoc mesenteroides* or from *Leuconostoc dextranicum* in the special environment of the creamery. The results suggest that both *Leuconostoc dextranicum* and *Leuconostoc cremoris* may be subspecies of *Leuconostoc mesenteroides* rather than separate species. Only further results will clarify the taxonomic position of these bacteria.

The properties of NCDO 1589 and NCDO 1590 given in Table 4 suggest that these strains should belong to the same species. The DNA hybridization studies show that NCDO 1589 clearly belongs to the "mesenteroides/dextranicum" group. On the other hand, NCDO 1590 DNA gave only 48% hybridization with the DNA of *Leuconostoc mesenteroides*. From these results, it is concluded that NCDO 1589 and NCDO 1590 are genetically different despite their phenotypic similarities.

Other difficulties in the classification of the leuconostocs arise because, until recently, *Leuconostoc paramesenteroides* was regarded as a non-dextran-forming variant of *Leuconostoc mesenteroides*, but these organisms are now seen to be distinct genospecies. However, Pederson and Albury (21) found that dextran production from sucrose was an unstable character. If strains of either species gained or lost the ability to form dextran, the change might affect their genetic type only slightly, but phenotypic studies could place such strains in the wrong species. Genetic studies on a much larger number of strains are necessary to sort out and resolve these problems.

The results of this study have shown that the strains of *Lactobacillus confusus* and *Lactobacillus viridescens* examined are not closely related to the leuconostocs and, furthermore, that they are closer to each other than to any of the other strains examined. Although *Lactobacillus confusus* and *Lactobacillus viridescens* show a distant relationship to the non-acidophilic leuconostocs, an even more distant relationship is shown to the other species of heterofermentative lactobacilli. Similarly, the relationship between the acidophilic and non-acidophilic leuconostocs is more distant than that between the latter and *Lactobacillus confusus* or *Lactobacillus viridescens*.

The presently used generic dividing lines, which are based on morphology, cannot be justified by the results obtained here. It has already been suggested that all of the heterofermentative lactic acid bacteria should be in a single genus (9, 23), and this still appears to be

an attractive arrangement. The alternative is to divide these bacteria into several genera, some of which would then only contain a single species. This is not appealing at present, for the relationships between the different species of the heterofermentative lactobacilli are not known.

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REPRINT REQUESTS

Address reprint requests to: Dr. Ellen I. Garvie, National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, England.

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