Deoxyribonucleic Acid Homology and Base Composition in Some Thermophilic Lactobacilli

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SUMMARY

Thirty-two culture collection strains of the thermophilic species of the genus *Lactobacillus* (*Lactobacillus helveticus*, *L. jugurti*, *L. bulgaricus* and *L. lactis*) and 13 field strains from natural whey cheese cultures were examined by routine physiological and serological tests. Genetic relatedness was determined by DNA-DNA molecular hybridization.

General agreement was observed between the phenotypic traits and genome homology. The close phenotypic resemblance between *Lactobacillus helveticus* and *L. jugurti* and between *L. bulgaricus* and *L. lactis* was confirmed by the genetic findings.

INTRODUCTION

On the basis of morphological, physiological and biochemical characteristics, Orla-Jensen (1919) divided the genus *Lactobacillus* into three groups: *Thermobacterium*, *Beta-bacterium* and *Streptobacterium*. These groups were further subdivided into several species that are currently accepted (Breed, Murray & Smith, 1957; Rogosa & Sharpe, 1959; Sharpe, 1961; Hansen, 1968). However, it is sometimes difficult to distinguish between lactobacilli on the basis of phenotypic characters, particularly field strains from a large diversity of habitats.

DNA-DNA hybridization may prove a useful method in classification and may reveal taxonomic differences which might otherwise be obscured by similar phenotypic characteristics. The genus *Bifidobacterium*, for example, was shown to contain several unrelated groups by DNA-DNA hybridization even though the strains resembled each other in metabolic pathways, fermentation patterns, morphology and other characteristics (Scardovi *et al.* 1971).

Recent studies by Miller, Sandine & Elliker (1971) and by Simonds, Hansen & Lakshmanan (1971) on the lactic acid bacteria showed that the extent of DNA hybridization, which reflects in part the similarity of the base sequences of the bacterial genome, is a useful technique in taxonomic studies of this group.

The present investigation deals with the extent of molecular hybridization between some thermophilic species of the genus *Lactobacillus* and some freshly isolated lactobacilli.

METHODS

Organisms. The strains used are given in Table 1. The identity of the cultures was checked by the bacteriological tests recommended by Rogosa & Sharpe (1959).

The preparation of antigens, of antisera and their absorption and technique of precipitin tests were carried out by the methods of Sharpe (1955).

Table 1. *List of strains*

Organism	Strain*	Organism	Strain*
<i>Lactobacillus lactis</i>	ATCC 11061	<i>L. jugurti</i>	ATCC 521
<i>L. lactis</i>	NIRD AH16	<i>L. jugurti</i>	NIRD WL19
<i>L. lactis</i>	NIRD L27	<i>L. jugurti</i>	NIRD J19
<i>L. lactis</i> (NCDO 270, ATCC 8000)	NIRD L1	<i>L. jugurti</i> (NCDO 101,	
<i>L. lactis</i> (NCDO 297, ATCC 10697)	NIRD L20	ATCC 7994)	NIRD J10
<i>L. lactis</i> (NCDO 280)	NIRD L3	<i>L. jugurti</i> (NCDO 481)	NIRD J14
<i>L. lactis</i>	NIRD AH40	<i>L. jugurti</i>	IMBOS29.9
<i>L. lactis</i>	NIRD L12	<i>L. jugurti</i>	IMBOS40.8
<i>L. bulgaricus</i>	NIZO 3503	<i>L. jugurti</i>	IMBOS20.7
<i>L. bulgaricus</i>	NIRD B15	<i>L. jugurti</i>	IMBOS13.8
<i>L. bulgaricus</i>	NIRD B1	<i>L. jugurti</i>	IMBOS3.1
<i>L. bulgaricus</i>	NIRD B17	Unnamed lactobacilli†	IMPC L5b
<i>L. bulgaricus</i>	ATCC 11842		IMPCM1C
<i>L. bulgaricus</i>	ATCC 12278		IMPCM5C
<i>L. helveticus</i>	NIRD H1		IMPCMt57
<i>L. helveticus</i>	NIRD H18		IMPCMCO17
<i>L. helveticus</i> (ATCC 10386)	NIRD H4		IMPCMa1
<i>L. helveticus</i>	NIRD PR3		IMPCZ18
<i>L. helveticus</i>	NIRD H5		IMPCM32
<i>L. helveticus</i>	NIRD WL7		IMPC Ld2
<i>L. helveticus</i>	NIZO 3527		IMPCMv8
<i>L. helveticus</i>	ATCC 10797		IMPCMg2
			IMPCMf1
			IMPC Lm4

* ATCC, American Type Culture Collection, Rockville, Maryland; NIRD, M. E. Sharpe, National Institute for Research in Dairying, Reading, England; NIZO, Nederlands Instituut voor Zuivelonderzoek, Ede, Holland; IMBO, Istituto di Microbiologia, Università di Bologna, Italy; IMPC, Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Piacenza, Italy.

† Recently isolated from natural whey cheese cultures.

Preparation of antisera. *Lactobacillus helveticus* NIRD H1 and *L. jugurti* ATCC 521 were used to prepare group A antisera and *L. bulgaricus* NIRD B1 and *L. lactis* NIRD L1 were used to prepare group E antisera. The organisms were grown in 10 ml of MRS (De Man, Rogosa & Sharpe, 1960) medium at 37 °C for 18 h; the bacteria were collected by centrifuging, washed twice in 0.85% saline and suspended in 10 ml saline. Suspensions were heated at 60 °C for 1 h, diluted and used for a course of seven intravenous inoculations at 4-day intervals in rabbits. The antisera were tested for potency and specificity and then stored at 4 °C with 0.01% merthiolate.

Absorption of antisera. When antisera cross-reacted with extracts of strains from other groups the antisera were absorbed with the heterologous strains using 1 vol. of packed fresh cells and 4 vol. of serum. As noted by Sharpe (1955), absorption weakened the potency of antisera so that unabsorbed antisera were used whenever possible.

Preparation of antigens. Bacteria, grown in 40 ml of MRS in which Yeastrel was replaced by tomato juice (1%, w/v), were collected by centrifuging and extracted with 2.0 ml of 0.05 N-HCl in saline in a boiling water-bath for 10 min. Extracts were neutralized, centrifuged and stored at 4 °C with 0.01% merthiolate.

Precipitin test. Extracts were layered on serum in small (2.7 mm) tubes and kept at room temperature. Ring formation, between serum and extract, was checked at intervals up to 1 h.

Growth of cultures and DNA preparation. Each strain was grown in 2 l of MRS at 37 °C for 18 h.

Labelled DNA was obtained from bacteria in the stationary phase grown in MRS medium with added [¹⁴C]uracil and [¹⁴C]adenine (each 50 µCi/l).

The bacteria were collected by centrifuging, washed twice in saline and suspended in 0.15 M-NaCl with 0.1 M-EDTA (disodium ethylene diamine tetra acetate) (pH 8.0) and then stored at -25 °C.

Bacterial lysis was carried out by the addition of 40 mg of lysozyme to suspensions, followed by incubation at 37 °C for 16 h. Then 15 mg of pronase was added and incubation continued for a further 8 h; 2.5 ml of sodium lauryl sulphate was then added and the DNA extracted and purified by the method of Marmur (1961).

All DNA preparations were stored at 4 °C in 0.1 × SSC (0.015 M-NaCl and 0.0015 M-trisodium citrate at pH 7.0) over chloroform.

DNA immobilization. Native DNA at a concentration of about 80 µg/ml in 0.1 × SSC was alkali-denatured (0.1 M-KOH final concentration for 15 min) and neutralized with 2 N-HCl at room temperature.

The denatured DNA was then diluted 1:10 with ice-cold 6 × SSC and filtered through 15 cm membrane filters (Sartorius SM 11306) that had been presoaked for 12 h in 6 × SSC. The filters were washed with 200 ml of 6 × SSC, dried overnight in air at room temperature and then cut with a paper punch into smaller disks of 10.5 mm diam. which were dried in a vacuum oven at 80 °C for 2 h and stored at 4 °C. The concentration of DNA on each stored filter ranged from 30 to 50 mg as calculated from the extinction at 260 nm of the filtrate.

DNA molecular hybridization. An annealing temperature of 65 °C was selected for DNA molecular hybridization because the melting temperatures (T_m) in 1 × SSC of all our strains ranged from 84.30 to 90.45 °C. Annealing temperatures of 25 °C below the T_m have been found to minimize adventitious pairing of bases (Marmur & Doty, 1961). The single point competition version under the conditions recommended by Johnson & Ordal (1968) was used. The filters, with DNA fixed to them, were preincubated for 4 to 6 h at 65 °C in the mixture of Denhardt (1966) made up with 2 × SSC. Each one was then transferred to a 4 ml vial containing 0.25 ml of Denhardt (1966) mixture, 1.0 to 2.0 µg of heat-denatured labelled DNA and 150 µg of heat-denatured competitor DNA in 2 × SSC. (DNA samples in 0.1 × SSC, previously sheared with a French pressure cell at about 10000 lb/in² (McCarthy & Bolton, 1963), were denatured, prior to use, by heating in a boiling water bath for 10 min.) The vials were gently shaken for 12 h at 65 °C. The filters were then washed with 2 × SSC at 65 °C, dried and counted in a Phillips model LSA 420 liquid scintillation counter using PPO plus POPOP scintillation liquid (0.1 g POPOP plus 5 g PPO in 1 l toluene).

The extent of homology was calculated by the equation:

$$\frac{\text{CPM homologous} - \text{CPM heterologous competitor system}}{\text{CPM homologous} - \text{CPM homologous competitor system}} \times 100.$$

Release of bound DNA from filters during incubation in Denhardt (1966) mixture was not taken into consideration because the temperature of incubation (65 °C) was in the range of validity of the filter method (De Ley & Tijtgat, 1970) and because the amount of DNA fixed in the absence of competitor was 20 to 30% of the input.

Determination of DNA base composition. Melting temperature values were obtained with native DNA dissolved in $1 \times \text{SSC}$; cuvettes were heated gradually with circulating water and variations in extinction at 260 nm recorded with Beckman DK 2A spectrophotometer. The base composition (% GC) was calculated from T_m by the equation of De Ley (1970):

$$\% \text{ GC} = (T_m - 69.37)/0.41.$$

RESULTS

Relations of Lactobacillus lactis and L. bulgaricus

The fermentation patterns, serological groups, moles % GC and DNA homologies are given in Table 2. Seven of the eight strains of *L. lactis* and five of the six strains of *L. bulgaricus* showed 72 to 100% homology with the reference strains of both *L. lactis* (ATCC 11061) and *L. bulgaricus* (ATCC 11842). Of these seven strains of *L. lactis* three (ATCC 11061, NIRD L20 and NIRDAH16) did not compete in the homologous system of either *L. jugurti* (ATCC 521) or *L. helveticus* (NIRDH1); *L. lactis* strains (NIRDL27 and NIRDL1) gave levels of homology of 28 and 43% respectively with *L. jugurti* (ATCC 521) but not with *L. helveticus* (NIRDH1); *L. lactis* strains (NIRDL3 and NIRDAH40) showed some homology to both *L. jugurti* (ATCC 521) and *L. helveticus* (NIRDH1).

The strain NIRDL12, which could be considered as *Lactobacillus lactis* by its fermentation pattern, showed an appreciable level of homology (46%) only with the reference strain ATCC 11842 of *L. bulgaricus*. The results with this strain in the DNA hybridization support the differences found in other characteristics: it belongs to serological group A and has a low % GC (36%).

The strains of *Lactobacillus bulgaricus*, except ATCC 12278, were highly homologous (80 to 101%) to *L. lactis* (ATCC 11061) and to *L. bulgaricus* (ATCC 11842), but did not show similarity to *L. jugurti* (ATCC 521) except for ATCC 12278 and to a lesser extent strain BI7. Their relationship to *L. helveticus* was, however, not assessed.

Lactobacillus bulgaricus ATCC 12278 displays the fermentation pattern of the species *L. bulgaricus* but belongs to serological group A and its DNA base composition of 38.2% GC is not in conformity with typical strains of this species (group E and % GC 50 to 51). Its very high homology level to *L. helveticus* (NIRDH1) and *L. jugurti* (ATCC 521) supports the opinion already expressed by Miller *et al.* (1971) that the strain should be reclassified.

Relations of Lactobacillus helveticus and L. jugurti

The results with these strains (Table 3) show that DNA preparations from all the strains behaved similarly although classified differently. The eight strains of *L. helveticus* and ten strains of *L. jugurti* all had similar DNA base compositions (36 to 38% GC) and belonged to serological group A (five strains of *L. jugurti* were not tested). The species differed in fermentation of maltose and fructose and to some extent trehalose (Table 3). The DNA of all 18 strains competed to the same high levels (85 to 102%) with DNA of the reference strains, *L. helveticus* (NIRDH1) and *L. jugurti* (ATCC 521). Low levels of homology (0 to 29%) were obtained between the DNA of *L. lactis* (ATCC 11061) and *L. bulgaricus* (ATCC 11842).

Relations of unnamed isolated thermophilic lactobacilli

The results with 13 strains of thermophilic lactobacilli, isolated from natural whey cheese cultures, are summarized in Table 4. In general the strains gave good agreement with each other in fermentation pattern, serological group and DNA base composition.

Table 2. Fermentation patterns, serology, DNA base composition and DNA homology in *Lactobacillus lactis* and *L. bulgaricus*

Bacterial strain	Fermentation of										Sero-logical group	Moles % GC	DNA homology relative to strain			
	Galac-tose	Lactose	Maltose	Salicine	Sucrose	Treha-lose	Arabi-nose	Fruc-tose	Glucose	Cello-biose			HI (<i>L. hel- veticus</i>)	521 (<i>L. jugurti</i>)	11061 (<i>L. lactis</i>)	11842 (<i>L. bul- garicus</i>)
<i>Lactobacillus lactis</i>																
ATCC 11061	+	+	+	+	+	+	—	+	+	+	E	50.4	0	2	100	83
NIRD L20	—	+	+	—	+	+	—	+	+	+	E	47.6	3	1	96	72
NIRDAHI6	+	+	+	+	+	+	—	+	+	+	E	50.3	2	0	86	83
NIRDL27	+	+	+	—	+	+	—	+	+	+	E	48.3	4	28	98	75
NIRDL1	+	+	+	+	+	+	—	+	+	+	E	50.3	1	43	100	83
NIRDL3	+	+	+	+	+	+	—	+	+	+	E	50.0	20	40	98	100
NIRDAH40	+	+	+	+	+	+	—	+	+	+	E	51.1	47	38	102	96
NIRDL12	+	+	+	+	+	+	—	+	+	+	A	36.0	7	22	0	46
<i>L. bulgaricus</i>																
ATCC 11842	—	+	—	—	—	—	—	+	+	—	N.T.*	51.1	N.T.	2	84	100
NIZO 3503	—	+	—	—	—	—	—	+	+	—	E	50.3	N.T.	N.T.	101	90
NIRDB15	—	+	—	—	—	—	—	+	+	—	E	51.3	N.T.	3	94	92
NIRDB1	—	+	—	—	—	—	—	+	+	—	E	51.4	N.T.	9	80	100
NIRDB17	—	+	—	—	—	—	—	+	+	—	E	51.1	N.T.	27	85	80
ATCC 12278	+	+	—	—	—	—	—	+	+	—	A	38.2	77	100	2	5

* Not tested.

Table 3. Fermentation patterns, serology, DNA base composition and DNA homology in *Lactobacillus helveticus* and *L. jugurti*

Bacterial strain	Fermentation of										Sero-logical group	Moles % GC	DNA homology relative to strain			
	Galac-tose	Lactose	Maltose	Salicine	Sucrose	Treha-lose	Arabi-nose	Fruc-tose	Glucose	Cello-biose			HI (L. hel-veticus)	521 (L. jugurti)	11061 (L. lactis)	11842 (L. bul-garicus)
<i>Lactobacillus helveticus</i>																
NIRDH1	+	+	+	—	—	+	—	+	+	—	A	100	99	14	3	
NIRDH18	+	+	+	—	—	—	—	+	+	—	A	93	102	9	5	
NIRDH4	+	+	+	—	—	+	—	+	+	—	A	87	98	3	2	
NIRDP3	+	+	+	—	—	—	—	+	+	—	A	84	99	16	2	
NIZO3527	+	+	+	—	—	+	—	+	+	—	A	87	103	16	4	
ATCC10797	+	+	+	—	—	—	—	+	+	—	A	86	96	29	2	
NIRDH5	+	+	+	—	—	+	—	+	+	—	A	100	102	24	5	
NIRDWL7	+	+	+	—	+	—	—	+	+	—	A	72	100	24	4	
<i>L. jugurti</i>																
ATCC 521	+	+	—	—	—	—	—	—	+	—	A	99	100	6	7	
NIRDWL19	+	+	—	—	—	—	—	+	+	—	A	100	98	29	2	
NIRDJ19	+	+	—	—	—	—	—	—	+	—	A	100	99	28	3	
NIRDJ14	+	+	—	—	—	—	—	+	+	—	A	101	100	31	5	
NIRDJ10	+	—	—	—	—	—	—	—	+	—	A	85	102	9	0	
IMBO S29.9	+	+	—	—	—	—	—	—	+	—	N.T.*	37.9	96	98	3	
IMBOS40.8	+	+	—	—	—	—	—	—	+	—	N.T.	95	101	4	5	
IMBOS20.7	+	+	—	—	—	—	—	—	+	—	N.T.	37.5	92	98	6	
IMBOS13.8	+	+	—	—	—	—	—	—	+	—	N.T.	38.2	93	91	18	
IMBOS3.1	+	+	—	—	—	—	—	—	+	—	N.T.	91	90	27	1	

* Not tested.

Table 4. Fermentation patterns, serology, DNA base composition and DNA homology of unnamed thermophilic lactobacilli isolates

Bacterial strain	Fermentation of										Sero-logical group	Moles % GC	DNA homology relative to strains			
	Galac-tose	Lactose	Maltose	Salicine	Sucrose	Treha-lose	Arabi-nose	Fruc-tose	Glucose	Cello-biose			HI (<i>L. hel-vecicus</i>)	521 (<i>L. jugurti</i>)	11061 (<i>L. lactis</i>)	11842 (<i>L. bul-garicus</i>)
IMPC15b	+	+	+	+	+	+	—	+	+	+	E	47.8	2	8	96	91
IMPCM1C	+	+	+	+	—	+	—	+	+	+	E	51.2	15	41	90	79
IMPC1d2	+	+	—	—	—	—	—	—	+	—	A	38.1	102	96	3	N.T.*
IMPCM5C	+	+	—	—	—	—	—	—	+	—	A	39.4	100	98	0	25
IMPCM157	+	+	—	—	—	—	—	—	+	—	A	39.9	83	104	23	N.T.
IMPCMv8	+	+	—	—	—	—	—	—	+	—	A	39.3	99	102	4	N.T.
IMPCMco17	+	+	—	—	—	—	—	+	+	—	A	36.4	100	96	46	7
IMPCMai	+	+	—	—	+	+	—	+	+	—	A	38.0	87	101	67	22
IMPCMg2	+	+	—	—	—	—	—	—	+	—	A	38.4	96	100	6	N.T.
IMPCM1I	+	+	—	—	—	—	—	—	+	—	A	39.4	98	90	5	N.T.
IMPCM32	+	+	+	+	—	+	—	+	+	+	E	48.3	3	5	38	36
IMPC1m4	+	+	—	—	—	—	—	—	+	—	A	38.6	103	96	2	N.T.
IMPCz18	+	+	—	—	—	—	—	+	+	—	A	49.2	58	72	12	2

* Not tested.

Eight strains (Ld2, M5C, Mt57, Mv8, MCO17, Mg2, Mr1 and Lm4) gave high levels of DNA homology with *Lactobacillus helveticus* (NIRDH1) and *L. jugurti* (ATCC 521) which might be expected from their fermentation patterns, serology (A) and % GC (36 to 39). Strains L5b and M1C were similar to *L. lactis* group in fermentation and to both *L. lactis* and *L. bulgaricus* in serology (E) and % GC (47 to 51). These similarities were supported by homology results.

Strain M32 competed to a limited extent with *Lactobacillus lactis* (ATCC 11061) and *L. bulgaricus* (ATCC 11842) although it belonged to serological group E and had a % GC of 48.

Strain Z18 did not correspond completely to any named species as it fermented fructose like *Lactobacillus bulgaricus*, but also fermented galactose like *L. jugurti*; it belonged to serological group A like *L. jugurti* and *L. helveticus* but had a % GC of 49.2.

DISCUSSION

Although limited in the number of strains, the results given indicate a close genetic similarity between *Lactobacillus lactis* and *L. bulgaricus*.

The results support the findings of Simonds *et al.* (1971), who first reported the similarity of base sequences in the DNA of *L. bulgaricus* ATCC 11842 and *L. lactis* ATCC 12315.

Miller *et al.* (1971) did not find any similarities between *Lactobacillus bulgaricus* ATCC 12278 and *L. lactis* 39-A and concluded that *L. bulgaricus* ATCC 12278 should be classified as *L. helveticus*. We found (Table 2) that *L. bulgaricus* ATCC 12278 gave 77% homology with the reference strain *L. helveticus* (NIRDH1), but 100% homology with *L. jugurti* (ATCC 521). In addition, the strain did not ferment maltose and could be classified as *L. jugurti*.

The close genetic relation of *Lactobacillus helveticus* and *L. jugurti*, as confirmed by our results, was suggested, on phenotypic grounds, by Rogosa & Sharpe (1959). Miller *et al.* (1971) found that *L. helveticus* (strain OSU) and *L. jugurti* (ATCC 521) gave only 55% homology and suggested that these two strains were distinct. However, the strain OSU is probably incorrectly classified.

Strain Z18 formed DL-lactic acid like *Lactobacillus helveticus* and *L. jugurti*, was strongly proteolytic unlike the majority of thermophilic lactobacilli (Bottazzi, 1962*b*) and presented the same amino acid composition as *L. lactis* (Bottazzi, 1962*a*). Its DNA did not give conclusive homology values with the reference strains although it seemed to be more related genetically to *L. jugurti* and *L. helveticus* than to *L. lactis* and *L. bulgaricus*. Strain Z18 might belong to a so far unrecognized group of lactobacilli.

Our results are thus in line with the current ideas on the taxonomy of the four named species studied. However, a more comprehensive taxonomic rearrangement of lactobacilli (Gasser & Sebal, 1966) requires more DNA homology studies on a much larger number of strains.

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