Deoxyribonucleic Acid Homologies in the Genus *Leuconostoc*

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Six deoxyribonucleic acid (DNA)-DNA homology groups within 45 strains of the genus *Leuconostoc* were distinguishable through DNA-DNA hybridizations with four ^3H -labeled reference DNA preparations from type or reference strains of the various known named species. There is good correspondence between two of these DNA-DNA hybridization groups and the species *L. lactis* and *L. paramesenteroides*. Strains named *L. mesenteroides* are heterogeneous, comprising three different hybridization groups. One of these groups includes all the strains named *L. dextranicum* and *L. cremoris*. These results agree precisely with previous results from an immunological study of the two isofunctional enzymes, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and D-lactate dehydrogenase (EC 1.1.1.28), prepared from *L. lactis* NCDO 546 and *L. mesenteroides* NCDO 768 (ATCC 12291).

Published data on the base content and relative homogeneity of the deoxyribonucleic acids (DNAs) of the genus *Leuconostoc* are still scarce. In 1974, Garvie et al. (8) published important and more comprehensive data giving the moles percent guanine plus cytosine (G+C) of the DNA from 12 strains belonging to six species. These G+C values were rather homogeneous, ranging from 40 to 46 mol%. Before this publication, there were data from only five strains of *Leuconostoc* (13) with values of 39 to 43 mol%. During the preparation of this manuscript, Garvie reported DNA-DNA hybridization studies of strains of heterofermentative lactic acid bacteria and 16 strains of *Leuconostoc* (7). These results will be discussed and compared with our present results later in this paper.

We present here the results of DNA-DNA hybridizations of 45 strains from the six different species of *Leuconostoc* described by Garvie in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (6). Prior to our hybridization experiments, the G+C content of each strain was determined by thermal denaturation using the equation of Mandel and Marmur (11). The hybridization experiments were carried out with four purified labeled DNA preparations as references. These were from the following: (i) the type strain of *Leuconostoc paramesenteroides* NCDO 803; (ii) a suggested type strain of *Leuconostoc mesenteroides* NCDO 523 (6); (iii) a second suggested type strain of *L. mesenteroides* NCDO 768 (ATCC 12291) (6); and (iv) *Leuconostoc lactis* NCDO 546. The latter strain and *L. mesenteroides* NCDO 768 were used previously as references for a comparative

immunological study of two isofunctional enzymes in the genus *Leuconostoc*, namely their D-lactate dehydrogenase and glucose-6-phosphate dehydrogenase enzymes (9, 10). The use of the same reference strains in studies of DNA-DNA hybridizations and immunological similarities of isofunctional enzymes has distinct advantages in the study of molecular taxonomy.

MATERIALS AND METHODS

Bacterial strains and culture. The bacterial strains used are listed in Table 1. Details on these strains and cultural conditions in MRS medium (5) were described previously (10). Cells were harvested by centrifugation during the early stationary phase. They were washed twice in Tris-hydrochloride buffer [50 mM tris(hydroxymethyl)aminomethane adjusted to pH 8 with HCl] and stored at -20°C before use.

Tritium-labeled DNA from *L. lactis* NCDO 546, *L. paramesenteroides* NCDO 803, and *L. mesenteroides* NCDO 768 and NCDO 523 were obtained from cells grown at 30°C in MRS medium supplemented with 20 μCi of [^3H]thymidine per ml ([methyl- ^3H]thymidine with a specific activity of 25 Ci/mmol, purchased from CEA, France).

Extraction and purification of DNA. For labeled or unlabeled DNA, extraction was carried out by a modification of the Marmur technique (12). The suspension of cells (1 g [wet weight] per 10 ml) in saline-EDTA (0.15 M sodium chloride, 0.1 M ethylenediaminetetraacetic acid, final pH 8) was gently shaken for 1 h at 37°C with 2 mg of lysozyme per ml. Fifty micrograms of Pronase per ml was then added and allowed to react for 30 min. Lysis was obtained by the addition of sodium dodecyl sulfate (SDS) at a final concentration of 2%, followed by gentle shaking for 3 h at 37°C . Sodium perchlorate was then

TABLE 1. *Guanine plus cytosine content of Leuconostoc strains*

Strains	G+C (mol%)			
	Mandel and Marmur (11) (ρ) ^a	Garvie (7)		This paper (T_m) ^b
		(T_m) ^b	(ρ) ^a	
<i>L. lactis</i>				
NCDO 546	39.8	46.2	45.3	45.9
NCDO 533	44.4	45.3	43.4	40.7
NCDO 532				44.6
NCDO 534				43.1
NCDO 535				44.2
NCDO 536				44.8
NCDO 549				44.8
NCDO 956				40.4
NCDO 959				43.8
<i>L. cremoris</i>				
NCDO 543	38.3	42	38.5	39.6
NCDO 829	39.8			40.4
<i>L. dextranicum</i>				
NCDO 529	39.8	40.8	37.3	40.8
NCDO 812	38.8	41.1		42
NCDO 517				40
NCIB 3739				43.2
NCIB 2706				41.7
NCIB 3356				42
<i>L. mesenteroides</i>				
NCDO 768	39.8	42.7	40.8	37.8
NCDO 882	42.4			38.8
NCDO 523	37.3	41	38.7	40.4
1533	39.3			42.4
NCDO 522				42.8
NCIB 9317				38.8
NCIB 3351				39.9
CIP 54171				42.1
ATCC 10830				40.7
NCIB 8710				41
Le 512-17				44.2
NCIB 8013				41.2
NCIB 9319				42.4
V16				38.9
CIP 54168				41.7
Le 513-7				42.4
CIP 54170				41.7
Le 513-12				38.6
Le 512-13				38.6
<i>L. paramesenteroides</i>				
NCDO 803		41.5		40.6
NCDO 871		40.5	39.2	38.8
NCDO 870				40
NCDO 872				36.1
NCDO 873				38
NCDO 885				37.1
NCDO 886				37.1
NCDO 955				40.6
<i>L. oenos</i>				
ATCC 23278				42.4

^a Determination of buoyant density (ρ) in CsCl gradients, and calculation of mol% G+C from the equation $1038.47 (\rho - 1.6616)$ (11).

^b Determination of T_m by thermal denaturation and calculation of mol% G+C from the equation of Mandel and Marmur (11).

added to a final concentration of 1 M, and deproteinization was completed by adding an equal volume of Sevag solution (chloroform-isoamyl alcohol, 24:1, vol/vol) and shaking for 30 min at room temperature. The mixture was centrifuged at 5,000 rpm for 1 h. The upper phase containing the DNA was recovered.

This solution of DNA was dialyzed overnight at room temperature against 8 M urea in 0.24 M sodium phosphate buffer (pH 6.8). Further purification was achieved on hydroxylapatite (HA) (Bio-Gel HTP, Bio-Rad Laboratories) in the presence of 8 M urea, by the Britten et al. procedure (3).

Quantitative measurements of DNA were made by the method of Burton (4) with diphenylamine reagent and by spectrophotometric absorption at 260 nm. Absence of contaminating ribonucleic acid was confirmed by the orcinol technique of Ashwell (1).

Thermal denaturation and G+C content. After purification, the DNA solutions were dialyzed against 24 mM (with respect to Na^+) sodium phosphate buffer (pH 6.5). The melting temperature (T_m) of each DNA preparation was determined on a Gilford 222 A spectrophotometer by the method of Mandel and Marmur (11). The G+C content was calculated from the equation of Marmur: $\text{mol\% G+C} = 2.44 (T_m - 54.6)$.

DNA-DNA hybridizations. Labeled DNAs were dissociated by heating at 100°C for 10 min and were purified further by absorption on HA (Bio-Gel HTP, DNA grade, Bio-Rad Laboratories) equilibrated with 0.14 M sodium phosphate buffer containing 0.4% SDS (pH 6.8) and held at 60°C for 30 min. Material bound to HA under these conditions was discarded. This procedure decreases the amount of labeled DNA bound nonspecifically to HA during the hybridization experiments.

Reaction mixtures contained 150 μg of unlabeled DNA and 0.1 μg of ^3H -labeled DNA (specific radioactivities: 40,000 cpm/ μg for the DNA from NCDO 546; 180,000 cpm/ μg for the DNA from NCDO 803; 150,000 cpm/ μg for the DNA from NCDO 523; and 80,000 cpm/ μg for the DNA from NCDO 768) in 1.0 ml of 0.14 M sodium phosphate buffer (pH 6.8). Labeled DNAs were sheared by passing them several times in a 1.0-ml syringe through a 26-gauge 1/2 needle with maximum pressure applied by hand. With this procedure, the size of the DNA molecules was about 10^5 daltons (14). The DNAs were then dissociated by heating at 100°C for 10 min, put immediately into an ice bath, and incubated at 60°C for 16 h. After incubation, separation of single- and double-stranded DNA was performed on HA (DNA grade) by the batch procedure of Brenner et al. (2). Double-stranded DNA was eluted with 0.4 M potassium buffer. This buffer solution eluted double-stranded DNA better than 0.4 M sodium phosphate buffer (pH 6.8). All the supernatants from HA were precipitated with cold 10% trichloroacetic acid containing 80 μg of DNA carrier (calf thymus) and held for 1 h at 0°C. Precipitates were recovered on Whatman GF/C filters, washed once with 5% cold trichloroacetic acid and once with cold 9°C alcohol, and dried with a final wash of ether. The filters were put

into counting vials containing 10 ml of scintillation mixture [3 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene, and toluene per liter] and assayed in a Packard counter.

In each set of experiments, a control was run to make sure that the labeled fragments did not reassociate themselves; 0.1 μg of labeled DNA in 1.0 ml of 0.14 M sodium phosphate buffer (pH 6.8) was incubated under the conditions described above. About 2 to 7% of the total counts were recovered from HA with 0.4 M potassium phosphate buffer (pH 6.8). Reassociation values were corrected for this background.

RESULTS

T_m values and G+C content. The T_m values were obtained from at least three determinations and were found to be between 69.4 and 73.4°C. The calculated G+C values (moles percent) are presented in Table 1 and are in good agreement with those of Garvie et al. (8) and M. Mandel (personal communication).

In general, the highest G+C values were among strains of *L. lactis* (45.9 mol% for *L. lactis* NCDO 546) and lower values were among strains of *L. paramesenteroides* (36.1 mol% for *L. paramesenteroides* NCDO 872). Most of the strains of *L. mesenteroides* had DNA with a G+C content between 40 and 42 mol%.

DNA-DNA hybridizations. Results of the hybridization experiments are summarized in Table 2. In the DNA-DNA homologous reactions, there was 88 to 94% reassociation with *L. lactis* NCDO 546, 92 to 96% with *L. mesenteroides* NCDO 768, 93 to 95% with *L. mesenteroides* NCDO 523, and 91 to 94% with *L. paramesenteroides* NCDO 803. All reassociation values were normalized to the homologous value designated as 100%.

With labeled DNA from *L. lactis* NCDO 546, the degree of DNA reassociation was 74 to 95% for eight nonhomologous strains of the species. Low levels of reassociation of about 10% were obtained in the DNA-DNA reactions of *L. lactis* NCDO 546 and *L. paramesenteroides* or *L. oenos*. Most of the DNAs of *L. mesenteroides* hybridized at about 30% with the DNA from *L. lactis* NCDO 546, but there was a wide strain variability of 20 to 56% in the *L. mesenteroides*-*L. lactis* hybridizations, indicating a rather large array of genetic dissimilarities between *L. mesenteroides* and *L. lactis*. On the other hand, it was not possible with ^3H -labeled DNA from *L. lactis* NCDO 546 to distinguish between *L. mesenteroides*, *L. cremoris*, and *L. dextranicum*. The DNAs of *L. paramesenteroides* gave only a low degree of hybridization (6 to 13%) with this reference.

The ^3H -labeled DNA of *L. mesenteroides*

TABLE 2. Percentage of hybridization of DNAs from *Leuconostoc* strains^a

Strains	DNA-DNA hybridization (%) of [³ H]DNA from:			
	<i>L. lactis</i> NCDO 546	<i>L. mesenteroides</i> NCDO 768	<i>L. mesenteroides</i> NCDO 523	<i>L. paramesenteroides</i> NCDO 803
<i>L. lactis</i>				
NCDO 546	100	39	41	25
NCDO 956	95	61	47	9
NCDO 532	91	61		
NCDO 535	89	61		
NCDO 534	86	41		
NCDO 959	83	55		
NCDO 533	79	45	32	9
NCDO 549	79	28		
NCDO 536	74	51		
<i>L. mesenteroides</i>				
NCDO 768	36	100	46	22
NCDO 882	25	89	78	14
CIP 54171	20	40	38	
NCIB 3351	28	38	34	9
NCDO 523	28	35	100	18
NCIB 8013	30	42	99	
ATCC 10830	19	38	96	7
NCIB 8710	23	33	95	
1533	27	39	93	
CIP 54170	20	38	93	
V16	24	43	92	
NCDO 522	25	39	91	13
Le 513-7	22	37	90	
Le 512-17	29	53	89	
NCIB 9317	56	54	89	9
CIP 54168	20	44	88	8
NCIB 9319	30	40	88	
Le 513-12	23	34	87	
Le 512-13	27	43	86	
<i>L. dextranicum</i>				
NCDO 517	19	40	85	7
NCDO 529	35	38	90	19
NCIB 3739	23	45	90	
NCIB 2706	30	49	86	
NCIB 3356	29	39	89	
NCDO 812	30	42	92	5
<i>L. cremoris</i>				
NCDO 543	25	46	78	7
NCDO 829	24	45	84	8
<i>L. paramesenteroides</i>				
NCDO 803	12	17	11	100
NCDO 870	11	15	16	86
NCDO 871	12	13		86
NCDO 872	11	17		89
NCDO 873	10	18		82
NCDO 885	9	15		56
NCDO 886	12	17		60
NCDO 955	6	21		60
<i>L. oenos</i>				
ATCC 23278	8	10	11	5

^a Using four ³H-labeled DNA species from *L. lactis* NCDO 546, *L. mesenteroides* NCDO 768 and NCDO 523, and *L. paramesenteroides* NCDO 803.

NCDO 768 gave a high degree of homology with one strain only, *L. mesenteroides* NCDO 882, whereas the other *L. mesenteroides* DNAs hybridized rather poorly with a mean value of 40%. Thus, these strains of *L. mesenteroides* (CIP

54171, NCIB 3351, NCDO 523, NCIB 8013, ATCC 10830, NCIB 8710, 1533, CIP 54170, V 16, NCDO 522, Le 513-7, Le 512-17, NCIB 9317, CIP 54168, NCIB 9319, Le 513-12, and Le 512-13) cannot be distinguished by the DNA-DNA hy-

bridization technique from *L. dextranicum*, *L. cremoris*, and *L. lactis*. However, the DNAs from *L. paramesenteroides* and *L. oenos* give such low hybridization values (6 to 21%) with ³H-labeled DNA from *L. lactis* or *L. mesenteroides* that we can clearly separate them from the other groups.

The hybridization experiments with ³H-labeled DNA of the second reference strain of *L. mesenteroides*, NCDO 523, confirms that it is genetically different from *L. mesenteroides* NCDO 768. Reciprocal experiments produced 45 and 34% hybridization (Table 2). All the DNAs of *L. mesenteroides* tested except those of NCDO 768, NCIB 3351, and CIP 54171 showed a high degree of hybridization with the NCDO 523 DNA. This important similarity is found again with the DNAs of *L. cremoris* and *L. dextranicum* (78 to 92% hybridization). Lower values—in the range 32 to 47%—were found with *L. lactis* DNAs. *L. paramesenteroides* DNAs hybridized only at 11 to 16% with this NCDO 523 reference. These results distinguish clearly these two species *L. lactis* and *L. paramesenteroides* from *L. mesenteroides* NCDO 523.

The last reference DNA isolated from *L. paramesenteroides* NCDO 803 produced a low yield of hybrids with all the DNAs tested except those of *L. paramesenteroides* strains: four strains in this group exhibited a high degree of similarity, 82 to 86%, and three others produced a lower degree of hybridization, 56 to 60%.

The only strain of *L. oenos* tested (ATCC 23278) gave a very low percentage of hybridization with the four reference DNAs—5 to 11%.

DISCUSSION

The method of Britten et al. for the purification of DNA was initially applied to vegetal cells (3). This rapid method is very convenient and reliable for bacterial cells. It has the great advantage of avoiding all precipitation steps to produce a high yield of pure double-stranded DNA practically without protein and ribonucleic acid contaminants.

Four labeled reference DNAs isolated from *L. lactis* NCDO 546, *L. mesenteroides* NCDO 768 and NCDO 523, and *L. paramesenteroides* NCDO 803 were used in our hybridization experiments. The results lead to the distinction of six groups of strains: four with a high percentage of hybridization with each one respectively of the reference DNA, and two groups with a low formation of heteroduplexes with any one of the reference DNA, including *L. paramesenteroides* and *L. oenos* for which one strain only has been tested.

The DNAs of the nine strains of *L. lactis* hybridized between 74 and 100% with the refer-

ence DNA of *L. lactis* NCDO 546, whereas the highest percentage obtained with the DNA of the other strains was 56%. *L. lactis* can then be considered a well-defined species in terms of genetic homology.

A similar situation was found with *L. paramesenteroides*, whose reference DNA hybridized practically only with the DNAs of the strains of this species. Although the phenotypic differentiation between *L. mesenteroides* and *L. paramesenteroides* is based only on dextran production from sucrose, the hybridization results make it clear that *L. paramesenteroides* is indeed a separate species.

The case of *L. mesenteroides* is more complicated. At first, four strains have to be considered separately: NCDO 768, NCDO 882, NCIB 3351, and CIP 54171. The reference DNA from NCDO 768 produced a high percentage of heteroduplexes with the DNA of NCDO 882, but only in the range 38 to 54% with all other *L. mesenteroides* DNAs. On the other hand, none of these four strains hybridized at a high level with the other reference DNAs isolated from *L. mesenteroides* NCDO 523, except NCDO 882. The taxonomic position of these two groups of strains within *L. mesenteroides* is therefore unclear and requires further study, including reciprocal hybridization, to determine whether they should be considered as two distinct species.

The DNA of the *L. mesenteroides* reference strain NCDO 523 hybridized well with all the *L. mesenteroides* DNAs, except *L. mesenteroides* NCDO 768 and 882. Moreover, equivalent percentages were found with the DNAs of *L. dextranicum* and *L. cremoris*. This large group of hybridization including three nomenclatures is clearly a single homogeneous species including several biotypes. Similar results were found by Garvie (7) using the membrane filter technique.

All the above results are in remarkable agreement with our previous clustering of the genus *Leuconostoc* based on an immunological study of the D-lactate dehydrogenase and glucose-6-phosphate dehydrogenase enzymes (9, 10). In this study, our *Leuconostoc* strains were divided into six groups of identical antigenic specificity. These six immunological groups and the six groups obtained by DNA-DNA hybridization in this study contain exactly the same strains.

We previously showed that the relations between groups of identical antigenic specificity reflect the current evolutionary relationships within the genus *Leuconostoc* (9, 10). The present heterologous DNA-DNA hybridizations reflect the phylogenetic relatedness between whole genomes of strains in a comparable state

of evolution. Thus, the perfect correlation between the groups defined by these two approaches make it possible to consider the groups A to F (10) as genospecies as defined by Ravin (14). Therefore we may establish a basis for a phylogenetic taxonomy of the genus *Leuconostoc*. Thus we propose the subdivision of this genus into six genospecies. For three of them there is a good agreement with the phenospecies recognized by Garvie in *Bergey's Manual of Determinative Bacteriology* (6): *L. lactis*, *L. paramesenteroides*, and *L. oenos* corresponding respectively to the immunological groups (A), (F), and (G) determined with the antisera anti-D-lactate dehydrogenase and anti-glucose-6-phosphate dehydrogenase (9, 10). For the three other phenospecies, *L. mesenteroides*, *L. dextranicum*, and *L. cremoris*, our results suggest new rearrangements. So, it seems convenient to exclude from the species *L. mesenteroides* the four strains NCDO 768, NCDO 882, NCIB 3351, and CIP 54171. In terms of immunological identity of their isofunctional enzymes and DNA-DNA homologies, these four strains can be gathered into two genospecies: one *Leuconostoc* species corresponding to the immunological group (E) including the reference strain NCDO 768 and the strain NCDO 882; the other *Leuconostoc* species corresponding to the immunological group (B) with the reference strain CIP 54171 and the strain NCIB 3351. The other named strains of the species *L. mesenteroides* could be gathered into a group of genospecies containing all the *L. dextranicum* and *L. cremoris* strains; all the strains of immunological group (D) (anti-D-lactate dehydrogenase sera) would be included here and NCDO strain 523 might serve as the type strain.

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REPRINT REQUESTS

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