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Using thermal denaturation methodology, the nucleic acid base composition of deoxyribonucleic acid (DNA) isolated from type, neotype and stock strains of Lactobacillus species was determined. The genus was found to be heterogeneous in base composition, ranging from 33.1 to 50.0 moles % guanine plus cytosine (GC).

Genus members fell into three groups based on DNA GC content. Group I included species with a GC content between 33.1 and 39.7 moles %: L. salivarius, L. jensenii, L. acidophillus, L. piscium sp. nov. (fish isolate), L. helveticus, and L. sanfrancisco (sourdough bread isolate). Group II included species with a GC content between 44.0 and 48.0 moles %: L. plantarum, L. rhamnosus (human isolate) and L. casei var. casei. Group III included organisms with a GC content between 48.4 and 50.0 moles %: L. leichmanii and L. fermenti. Several well known Lactobacillus species

were given varietal status.

These three groups were further divided into homofermentative and heterofermentative subgroups leading to the formation of six groups. Speciation of group members was determined from DNA-DNA hybridization results as follows:

Homofermentative group I - L. salivarius, L. jensenii, L. acidophillus, L. helveticus, and L. piscium sp. nov.

Heterofermentative group I - L. sanfrancisco

Homofermentative group II - L. plantarum, L. casei and L. rhamnosus (formerly L. casei var. rhamnosus)

Heterofermentative group II - L. brevis and L. viridescens.

Homofermentative group III - L. leichmanii, including the varieties lactis, bulgaricus and delbrueckii

Heteromentative group III - L. fermenti, including the cellobiosus variety

This classification, while differing significantly from the traditional taxonomic system for these organisms based on biochemical and serological properties, nevertheless constitutes a firm basis for species identification. This is substantiated from these homology values:

1. With L. salivarius 11742 as reference organism, the following homology values for the indicated organisms were obtained: L. helveticus 15009, 10%; L. jensenii 25258, 0%,

L. acidophilus 4356, 9%.

2. With L. piscium as the reference organism, the following homology values for the indicated organisms were obtained: L. piscium LRPKI-70, 92%, L. salivarius 11742, 45%; L. jensenii 25258, 22%; L. helveticus 8018, 16%; L. jugurt 521, 32%; Erysipelotrix insidiosa 19414, 14%.

3. With L. helveticus 15009 as reference organism, the following homology values for the indicated organisms were obtained: L. jugurt 521, 79%; L. jensenii 25258, 0%; L. acidophilus 4356, 0%; L. salivarius 11742, 9%; L. sanfrancisco WRRL-L, 0%; L. piscium 0%;

4. With L. sanfrancisco WRRL B as reference organism, the following homology values for the indicated organisms were obtained: L. sanfrancisco WRRL C, 89%; L. sanfracisco WRRL L, 95%; L. sanfrancisco WRRL T95%; L. acidophilus 4536, 23%; L. salivarius 11742, 12%; L. jugurt 521, 6%; L. helveticus 8014, 32%; L. brevis NCDO 473, 39%.

5. With L. plantarum 14917 as the reference organism, the following homology values for the indicated organisms were obtained: L. casei 393, 17%; L. casei 7469, 0%; L. brevis NCDO 473, 0%; L. lactis 12315, 0%; L. fermenti NCDO 215, 2%; S. inulinus, 0%; L. helveticus 15009, 6%; Lactobacillus MSH, 0%; Lactobacillus Sardo 6, 84%; Lactobacillus DS46F, 88%.

6. With L. casei 393 as the reference organism, the following homology values for the indicated organisms were obtained: L. casei 7469, 14%; L. plantarum 14917, 43%; L. brevis NCDO 473, 16%; L. fermenti NCDO 215, 4%; L. helveticus 15009, 17%; L. salivarius 11742, 10%; S. inulinus, 21%; L. lactis 12315, 4%; Lactobacillus Sardo 6, 36%; Lactobacillus MSH, 41%; Lactobacillus DS46F, 24%.

7. With L. lactis 12315 as the reference organism, the following homology values for the indicated organisms were obtained: L. bulgaricus 11842, 78%; L. delbrueckii 9649, 85%; L. leichmanii 4797, 78%; L. fermenti, NCDO 215, 11%; L. cellobiosus NCDO 927, 8%; L. helveticus 15009, 30%.

8. With L. fermenti NCDO 215 as the reference organism the following homology values for the indicated organisms were obtained: L. cellobiosus NCDO 927, 83%; L. delbrueckii 9649, 0%; L. leichmanii 4797, 0%; L. lactis 12315, 6%; L. bulgaricus 11842, 11%; L. helveticus 15009, 21%.

These homology values were obtained from experiments done at the stringent hybridization conditions of T_m - 15 C.

Genome size determinations also were carried out and two findings emerged: one, lactobacilli have approximately half the genetic capability of Escherichia coli and two, Sporolactobacillus inulinus has a genome which is as large as E. coli and similar to

members of the Bacillus genus.

Cesium chloride density gradient centrifugation and agarose gel electrophoresis revealed the presence of supercoiled (plasmid) DNA in L. rhamnosus MSH. The plasmid was approximately one million daltons in molecular weight.

DNA hybridization studies of a Lactobacillus associated with fish kidney disease revealed that it is a new member of the genus. It was assigned the name L. piscium.

Deoxyribonucleic Acid Hybridization and Plasmid Studies in the Genus *Lactobacillus*

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INTRODUCTION

While members of *Lactobacillus* genus are widely distributed in nature and found wherever protein breakdown products, carbohydrates and vitamins occur, they have received relatively little research attention. They are highly fastidious from a nutritional standpoint and are well known for their importance in maintenance of a normal microbial flora of the intestine. They also have received attention for possible involvement in dental caries and avitaminoses in certain individuals and in rare instances they have been implicated in disease. However, they are most well known for their role in initiating or contributing to certain food fermentations. Therefore, the use of well-defined and differentiated species becomes an absolute necessity.

Orla-Jensen (1919) divided the genus into three subgenera, the *Thermobacterium*, *Streptobacterium* and *Betabacterium*. They were divided on the basis of nutritional and biochemical properties. These subgenera have been substantiated by additional physiological characters (Davis, 1960; Rogosa and Sharpe, 1959; Rogosa, 1970), serological studies (Sharpe, 1955; Sharpe and Wheater, 1957), numerical taxonomic analyses (Seyfried, 1968; Hayashi and Mimura,

1968; Barre, 1969), infra-red spectroscopy (Goulden and Sharpe, 1958) and chromatographic analyses (Cummins and Harris, 1956; Baddiley and Davison, 1961).

The rationale of bacterial taxonomy by numerical analysis with the aid of computers is that the vast number of observations made will outweigh inevitable bias by assigning a value of one for every character irrespective of its importance. While this is theoretically possible, it is difficult from a practical standpoint.

The most ideal classification approach would be to determine the total potential of a bacterium by decoding the genetic material, but present technology has not yet attained the sophistication necessary to decode the deoxyribonucleic acid (DNA). In other words, there is no method available to correlate phenotypic characters of an organism with purine-pyrimidine base sequences of the genetic material. The best alternative would be a detailed comparative analysis of the genetic material for size, composition and homology. This has been the objective of the present study of the Lactobacillus genus.

Methods of DNA base composition analyses are now recognized to be of great value in microbial taxonomy. The DNA base composition of the Lactobacillus genus has been studied by several investigators (Cantoni et al., 1965; Gasser and Sebald, 1966). Also, a comparative analysis of moles % guanine plus cytosine (GC) content

data determined by chemical analysis and buoyant density centrifugation along with published data from thermal denaturation profiles has been reported by Gasser and Mandel (1968), Mandel et al. (1970) and Delay (1970).

Miller studied Lactobacillus taxonomy using DNA-RNA hybridization. Since it is known that only a portion of the total genome is transcribed at any particular moment and that one class of RNA molecules (messenger type) is unstable, doubt is cast on acceptance of DNA-RNA hybridization results for taxonomic conclusions. Therefore DNA-DNA hybridization studies were conducted in the present research to determine genetic relationships between type, neotype and recently isolated species of the genus Lactobacillus. While this work was in progress, DNA-DNA hybridization results involving a few type species of the genus were reported (Simonds, 1971; Mattenzzi, 1972; Dellaglio et al., 1973; Johnson, 1973).

Also included in the present study were genome size determinations to get a measure of the total genetic capability of the organisms. The hybridization and genome size data were supplemented with DNA GC content determinations to evaluate the systematics of the genus Lactobacillus.

In addition to recognized type strains of lactobacilli, three other lactobacilli were extensively studied. One, Lactobacillus sanfrancisco, responsible for the souring reaction in fermented

San Francisco type bread dough, was shown to be a new species by DNA-DNA hybridization. The other two were originally isolated from lesions in man and fish (pathogens); these also were studied by nucleic acid hybridization and in addition were examined for and found, in one case, to contain plasmid DNA. While plasmid DNA has been noted in streptococci, this is apparently the first report of plasmids in lactobacilli.

REVIEW OF LITERATURE

General Taxonomy

Oral-Jensen (1919) divided the genus Lactobacillus into three subgenera (actually three genera), Thermobacterium, Streptobacterium and Betabacterium, on the basis of sugar fermentations, temperature range of growth, type of lactate produced and nitrogen requirement. Thermobacteria and streptobacteria were homofermentative but they differed in their temperature range of growth. All the homofermentative species produced lactic acid exclusively as the end product of sugar fermentation. Heterofermentative species, on the other hand, produced lactate, acetate, acetaldehyde, ethanol and carbon dioxide as the end products of sugar fermentation; they were assigned to the subgenus Betabacterium.

These three groups were consolidated by further studies of Rogosa and Sharpe (1959) and Sharpe (1962) subsequent to the review of literature on Lactobacillus taxonomy published by Briggs and Briggs (1954). Davis (1960) attempted to use additional characters like production of gas from citrate, reactions on blood agar, serological grouping and habitat in a more detailed classification; his data were in close agreement with those of Rogosa et al. (1953). Taxonomic reviews of the genus also have been published by Wheater (1955) and Briggs (1953).

Serological classification of the Lactobacillus genus was first attempted by Williams (1948). Orland (1950), Miller and Hansen (1957), Sharpe (1955) and Efthymiou and Hansen (1962) also attempted to use serology for the classification of these bacteria. Irregular cross reactions, autoagglutinations of the antigen suspensions and a low degree of immunogenicity combine to limit agglutination reactions as a tool for classification.

Precipitin reactions (Sharpe, 1955) on the other hand, are more consistent with the classical taxonomic picture. In their report, Sharpe and Wheater (1957) classified Lactobacillus organisms into six serological groups, designated A through F.

Phage typing of the genus has been reported by Coetzee, de Klerk and Sacks (1960). Also, Goulden and Sharpe (1958) have shown that infra-red absorption patterns are in general agreement with Orla-Jensen's grouping of the genus into three subgenera.

Seyfried (1968), using computer methodology, has classified the genus into three groups, which also correspond to the three subgenera described by Orla-Jensen. However, numerical taxonomy of lactobacilli isolated from wines (Barre, 1969) revealed that some organisms were non-typable or not representative of any known species. Hayashi et al. (1968) using adansonian analysis evaluated the relationship of tribes of Streptococceae, Lactobacilleae and Propionbacterieae.

Paper chromatography has been used by Cummins and Harris (1956) to examine hydrolysates of cell walls isolated from several strains of lactobacilli. The amino acids present were characteristic of the genus and relative proportions of sugars and hexosamine were used to differentiate the individual species; results however were equivocal. The same technique was used by Baddiley and Davison (1961) to investigate the occurrence of teichoic acids in cell walls and both glycerol and ribitol teichoic acids were found; the presence and type of teichoic acid in the wall correlated reasonably well with the serological behavior of lactobacilli and provided a useful means of classification. Cheeseman et al. (1957) and Cheeseman and Silva (1959) applied paper chromatography to differentiate and classify species of lactobacilli; they found that they could differentiate species in this manner and that the classification resulting was identical to the physiological classification. Williams (1971), investigating cell wall and enzyme composition, recommended division of each subgenus into two on the basis of cell wall amino acid analysis, carbohydrate enzyme assays, electrophoretic forms of enzymes and GC content of the DNA.

Rogosa (1970) has substantiated the division of the genus into the three Orla-Jensen subgenera by the following characters: ribose fermentation, gas production from gluconate, type of lactate produced and thiamine requirement. He has also shown that only

homofermentative strains possess aldolase. In addition, classification of the subgenera was amplified by classical fermentation patterns, vitamin requirements, serological reactions and growth temperatures.

Genetic Relatedness

The DNA base composition of the genus Lactobacillus has been extensively studied by several workers (Cantoni et al., 1965; Gasser and Sebald, 1966; Gasser and Mandel, 1968; Miller et al., 1970; Simonds et al., 1971; Mattenzzi, 1972; Dellaglio et al., 1973; Sriranganathan et al., 1973). With a close correlation now established between thermal denaturation data, buoyant density in cesium chloride and GC content (Delay, 1969; Mandel et al., 1970), the substantial differences observed previously by the workers in the field of Lactobacillus taxonomy have been reduced and values from different laboratories are now more consistent.

Gasser and Sebald (1966) divided the genus into three groups on the basis of DNA base composition: Group I included species with a GC content between 33 and 37.9 moles percent; group II included species with a GC content of 42 through 47 moles percent; group III included species with a GC content between 47 and 53 moles percent. This grouping seems reasonable and was substantiated by Miller et al. (1970).

McCarthy and Bolten (1963) investigated DNA competition

experiments to measure quantitatively the genetic relatedness among organisms. This was further studied by Hoyer et al. (1964) and the procedure was established as a tool for use in a molecular approach to systematics. Though the procedure was established very recently, it has been extensively used by scores of workers to evaluate genetic relatedness in organisms. The following articles cited will illustrate application of the technique to Lactobacillus taxonomy.

The first attempt was made by Miller et al. (1971) from our laboratory. They used DNA-RNA hybridization. The results showed that there was extensive homology among physiologically closely related species like L. lactis and L. leichmanii, L. cellobiosus and L. fermenti and a lower amount of homology between L. helveticus and L. jugurt. Simonds et al. (1971) showed that L. bulgaricus and L. lactis were genetically highly related to each other. Dellaglio et al. (1973), studying a large number of thermophilic Lactobacillus species, confirmed the observations of Simonds et al. and also showed that L. helveticus and L. jugurt had high genetic homology, in agreement with their phenotypic similarities.

A high degree of homology has been shown between L. casei var. casei and L. casei var. alactosus by Johnson (1973). He also showed that L. casei var. casei and L. casei var. rhamnosus had only 30 to 40% homology.

Genome size provides one measure of the total genetic

capability of an organism. Wetmur and Davison (1968) studied genome size by measuring the kinetics of renaturation of DNA. They showed that the rate of renaturation of fully denatured DNA revealed second order reaction kinetics. The reaction rate increased as the temperature decreased below the T_m, reaching a broad flat maximum at 15 to 30 C below the T_m and then increasing with a further decrease in temperature. This was further confirmed by Britten and Kohne (1968).

Gillis et al. (1970) described a new method for the determination of molecular weight of bacterial DNA. They used the renaturation kinetics for DNA of known molecular weight as the standard and compared it with the test DNA. Leth Bak et al. (1970) showed that the genome size of DNA can be calculated from the second order reaction rate (K_2) or from relative measurement of $Cot_{0.5}$, which is the product of the initial concentration of single stranded DNA sheared to a standard length and the half time for the completion of the renaturation reaction (Britten and Kohne, 1968).

Delay et al. (1970) established a new method to measure genetic relatedness among bacteria. The method was based on renaturation rate determinations for DNA from different species and their mixtures. Seidler and Mandel (1971) established a correction factor of 18% for every mole percent GC difference from the standard used, thereby providing more accurate application of the method. They

also showed that when no similarity existed between two organisms, the observed $Cot_{0.5}$ of the mixture was the sum of the independently determined $Cot_{0.5}$ values. Lack of additivity was correlated with similarity in polynucleotide sequences of the reassociating DNA molecules.

With the increasing number of reports of new Lactobacillus species (Rogosa et al., 1953; Gasser et al., 1970; Dakin and Radwell, 1971; Sharpe et al., 1972; Carr and Davies, 1970 and 1972; Sharpe et al., 1973; Stetter and Kandler, 1973) and with the increasing number of organisms described as either intermediates or non-typable, it was decided to approach Lactobacillus taxonomy at the molecular level by DNA-DNA hybridization, GC content and genome size determinations. The objective of the project was to determine homology patterns within the genus for use in clarification of speciation.

Extrachromosomal DNA

Once the existence of extrachromosomal genetic material (plasmids and episomes) was established (Watanabe, 1963) it gradually was shown that the physiochemical properties of these closed, circular DNA molecules differ in several respects from those of linear or circular DNA containing one or more single strand sciccions (Vinograd et al., 1965). For example, resistance to renaturation (Vinograd and Lebowitz, 1966), sedimentation velocity in neutral and

alkaline solutions and buoyant density in alkaline solution were all enhanced in the closed, circular extrachromosomal molecules. These effects are due to the topological requirement that the number of interstrand cross-overs must remain constant in the closed molecules (Radloff et al., 1967). Below are cited some of the papers that have used these special characters of plasmid DNA for their differential isolation.

Radloff et al. (1967) described an isolation method based on the buoyant behavior of closed circular DNA in the presence of intercalating dyes. Bauer and Vinograd (1968) reported on the effect of interaction of intercalative dyes with closed circular and nicked circular DNA, noting that mixed molecules migrated more slowly than the intact circular types. Bazaral and Helinski (1968) described an extensive but simple procedure for the isolation of plasmid DNA from Escherichia coli using a Brij lysate technique while Silver and Falkow (1970) detailed a procedure for handling the frractions from the gradients. An extensive review on the subject has been written by Clowes (1972) in which he describes the different methods of isolation and characterization of bacterial plasmids. Hirt (1967) was the first to use differential (preferential) precipitation of the higher molecular weight cellular DNA in the presence of sodium lauryl sulfate and a high concentration of sodium chloride for the separation of polyoma DNA from cellular DNA. Modification of the

Hirt procedure for isolation of bacterial plasmid DNA was described by Guerry et al. (personal communication, 1974). In the present work this method was only slightly modified to affect lysis of lactobacilli.

The existence of plasmids that code for bacterial toxins and R factors in gram positive bacteria (Warren et al., 1974; Courvalin et al., 1972; Dunny et al., 1973) has been shown. Lactobacilli isolated from lesions in animals and man may have plasmids that code for pathogenic determinatnts observed occasionally in this non-pathogenic genus.

MATERIALS AND METHODS

Organisms and Media

Representative strains of type and neotype species of the genus Lactobacillus used in this study are listed in Table 1, along with all the other strains used in the study and their source. Their identity was confirmed according to the criteria established by the taxonomic subcommittee on lactobacilli and closely related organisms (Hansen, 1968). The enzymatic method of Mattson (1965) was used to determine the type of lactic acid produced. In addition API plates (Analytab Products, Inc., 516 Mineola Ave., Carle Place, N. Y.) were used for the determination of their biochemical properties.

The known lactobacilli were grown in MRS broth (DeMan et al., 1960) at their optimum growth temperature of 32 or 37 C. Strains of L. sanfrancisco were grown in the SD broth (Kline and Sugihara, 1971) and incubated in an air atmosphere at 30 C. Lactobacillus strains B2-70 and LRPKI-70 (later identified as L. piscium sp. nov.) were grown in brain heart infusion broth. Cells for DNA extraction were washed twice with distilled water and then with saline-ethylenediaminetetraacetic acid (0.15 M NaCl plus 0.1 M EDTA, pH 8.0). They were stored frozen until used. To confirm that the four strains of L. sanfrancisco exhibited the same phenotypic properties described by Kline and Sugihara (1971), cultural studies were

Table 1. Organisms used, their strain number and origin.

Organism	Strain Number	Origin or Source
L. helveticus	ATCC 15009	American Type Culture Collection (ATCC)
L. helveticus	ATCC 8018	Oregon State University (OSU)
L. acidophilus	ATCC 4356	ATCC
L. jugurt	ATCC 521	OSU
L. jensenii	ATCC 25258	ATCC
L. salivarius	ATCC 11742	OSU
L. sanfrancisco	B	Western Regional Research Laboratory (WRRL)
L. sanfrancisco	C	WRRL
L. sanfrancisco	L	WRRL
L. sanfrancisco	T	WRRL
Lactobacillus piscium	B2-70	OSU
Lactobacillus piscium	LRPKI-70	OSU
Erysipelothrix insidiosa	ATCC 19414	ATCC
L. casei var. casei	ATCC 393	ATCC
L. casei var. rhamnosus	ATCC 7469	OSU
L. plantarum*	MSH	Mount Sinai Hospital, N. Y.
Lactobacillus	DS46F	Microlife Technics
L. lactis	ATCC 12315	ATCC
L. leichmanii	ATCC 4797	OSU
L. delbrueckii	ATCC 9649	OSU
L. bulgaricus	ATCC 11842	OSU
Lactobacillus	Sardo-6	Microlife Technics
L. brevis	NCDO 473	National Collection of Dairy Organisms (NCDO)
L. fermenti F1	NCDO 215	OSU
L. cellobiosus G1	NCDO 927	OSU
Escherichia coli	ATCC K-12	OSU
E. coli	WP 2	OSU
E. coli	CSH 52	OSU
E. coli	CSH 45	OSU
E. coli	CSH 25	OSU

*Identified as L. rhamnosus in the present study.

made. Methods of these authors and those described in the Manual of Microbiological Methods (Conn, 1957 ed.) were used. E. coli was grown in nutrient broth with incubation at 37 C for 24 h.

Electron Microscopy

Two types of cultures were prepared for electron microscopy.

(a) Actively growing cultures in broth were transferred into fresh broth and incubated at their optimum growth temperature. Strains of L. sanfrancisco were grown in SD broth under 50 to 60% CO_2 tension for 18 to 20 h (to stationary growth phase). Cells were harvested by centrifugation at 15,000 x g for 30 min and washed twice in dilute potassium phosphate buffer, pH 7.0. These cells were resuspended in the same buffer to give an approximate concentration of 10^6 cells/ml.

(b) Physiologically active cells were obtained by a transfer of 1% (vol/vol) of cells from a mature culture in broth into fresh medium with incubation for 20 min at their optimum growth temperature. These cells were treated similarly to those of stationary phase cells. The cells were not fixed unless otherwise stated. When fixed, 1% gluteraldehyde in the 0.01 M phosphate buffer was used. After fixation, the cells were washed several times and placed on Formvar-coated (300 mesh) grids and stained with buffered 2% phosphotungstic acid. After drying for a few seconds, the cell suspension was

blot-dried with chromatographic paper strips and then observed under a Phillips EM2 electron microscope.

For thin sections, early stationary growth phase cells were fixed in 5% gluteraldehyde in 0.01 M phosphate buffer pH 7.0. The cells were embedded in 2% melted ion-agar (Difco) and washed in phosphate buffer for 2 to 3 h. The embedded cells were post-fixed with 1% osmium tetroxide in the 0.01 M phosphate buffer for 6 to 7 h. Samples were dehydrated in increasing concentrations of acetone (30, 50 and 70%) for 10 min. The cells were stained with a saturated solution of uranyl acetate in 70% acetone for 4 h and dehydrated in 100% acetone with three changes of acetone every 15 min. After dehydration, the embedded cells were mixed in a plastic (Araldite 6005 and EPON 815):acetone (2:1) mixture (Mollenhauer, 1964). The plastic was allowed to penetrate for 1.5 to 2 h at 25 C. Sections of the order of 70 to 80 nm were cut in a microtome with a diamond knife and observed under the electron microscope.

DNA Preparation and Labeling

DNA extraction was carried out by using a modified technique of Marmur (1961). As it is difficult to lyse gram positive organisms, especially the lactobacilli, a dual enzyme system (Miller, 1970) of Lytase (20% vol/vol; Baltimore Biological Laboratory) or lytic factor from Strptomyces albidoflavus (5% vol/vol; Schuster et al., 1968)

plus lysozyme (2 to 4 mg/ ml; Sigma Chemical Co.) was used. The suspension was incubated at 37 C and checked for lysis every 30 min by a spot test (One drop of cell suspension plus one drop of 25% sodium lauryl sulfate). Complete lysis was indicated by clearing and an increase in viscosity. Deproteinizations (initial and the one after ribonuclease treatment) were carried out with saline-EDTA-equilibrated liquid phenol (redistilled) at pH 7 to 8. Other deproteinizations (a minimum of four) were carried out with Sevag solution (chloroform:isoamylalcohol, 24:1).

The DNA concentration of solutions was estimated from the absorption of ultraviolet light at 260 nm. An optical density of 1.0/ ml was assumed to be equivalent to a DNA concentration of approximately 50 μg/ml. Reference strains were grown in appropriate broth containing 1 uCi/ml of ^{3}H-6-thymidine to stationary growth phase at their optimum growth temperature. Cells were harvested, washed twice, and used for the extraction of DNA. The specific activity of the labeled DNA samples, as determined in a dual channel Beckman Tri-Carb or Nuclear Chicago liquid scintillation spectrometer, are listed in Table 2.

The DNA base composition was determined using T_m data and the equation of Mandel *et al.* (1970). *E. coli* K-12 or WP2 DNA was included in each melting as the internal standard. The difference between the T_m of the test organism and that of *E. coli* (ΔT_m) was

used for the actual calculations of the base compositions.

Table 2. Specific activities of reference DNA.

Organisms DNA	Specific activity CPM/μg	Strain number
L. salivarius	2403	ATCC 11742
L. piscium	4660	B2-70
L. helveticus	2772	ATCC 15009
L. sanfrancisco	4600	WRRL St B
L. plantarum	3691	ATCC 14917
L. casei	3727	ATCC 393
L. lactis	5565	ATCC 12315
L. fermenti F1	2760	NCDO 215

Loading Membrane Filters

Unlabeled (cold) reference DNA was loaded onto the membrane filters (B-6; Schleicher and Schuell Co.) by the method of Gillespie and Spiegelman (1965). The DNA in single strength standard saline citrate (1xSSC; 0.15 M NaCl plus 0.015 M $Na_3C_6H_5O_7$, pH 7.0±0.2) was diluted with 0.1xSSC to a DNA concentration of 50 μg/ml. The DNA was denatured by adding 1 N NaOH to a final concentration of 0.1 N and incubated for 10 min at 25 C. The solution was then diluted with cold (5 C) 6xSSC to give a final DNA concentration of 10 μg/ml. The alkali was neutralized with 2N NaH_2PO_4 and the

denatured DNA was gravity-loaded onto membrane filters (pre-washed with 6xSSC) by using slight suction. Then the filters were washed with 200 ml of 6xSSC. The filters were dried over-night at 25 C and then incubated at 80 C for 2 to 3 h in a vacuum oven. Small filters (6 mm in diameter) were punched out and stored in a vacuum dessicator at 4 C until used.

Hybridization Experiments

DNA homologies were estimated by DNA competition (Hoyer et al., 1964; McCarthy and Bolten, 1963). DNA samples were dialysed three times against 6xSSC containing 40% formamide (Okanishi and Gregory, 1970) at 4 C for 18 to 24 h. Samples were sheared in a French pressure cell at a pressure of 10,000 to 15,000 lb/in^2. Just before use, the DNA samples were denatured by immersion in a slow-boiling water for 10 min and immediately quenched in an ice bath to stop reassociation. The 6 mm filters were incubated at 25 or 15 C below the T_m in 6xSSC containing 40% formamide, 1 μg of tritium-labeled reference DNA and 300 μg of the competitor DNA. Each filter contained 12 to 15 μg of unlabeled reference DNA. In the experiments in which the samples were incubated 25 C below the T_m, the final volume of hybridization solution was 1.0 ml, while in the T_m minus 15 C experiments, the final volume was 0.5 ml. The samples were incubated for 18 h in a water bath with intermittent shaking.

After the incubation, the filters were washed in three to five changes of 2xSSC at the hybridization temperature and dried at 55 C overnight before counting in a liquid scintillation spectrometer. Homology or similarity values were calculated by the amount of depression caused by heterologous DNA divided by the amount of depression by the homologous competitor multiplied by 100.

Lytic Factor from Streptomyces albidoflavus

S. albidoflavus was grown in the culture medium of Tabata and Terui (1962) containing 0.3% crude yeast cell wall at 30 C for 70 h on a shaker. The composition of the medium was potassium phosphate dibasic, 0.2%; yeast extract, 0.05%; magnesium sulfate, 0.1% ($MgSO_4 \cdot 7H_2O$); Peptone, 0.1%; potassium chloride, 0.05%; glucose, 1%; sodium chloride, 0.05% and sodium nitrate, 0.13%. At the end of this period, the cells were filtered and centrifuged out. The lytic enzymes in the supernatant were precipitated at 70% ammonium sulfate by storing overnight. The precipitate was centrifuged and dissolved in one-twentieth of the initial volume in 0.1 M sodium phosphate buffer at pH 6.0. The enzyme solution was extensively dialysed against the same buffer. Then the enzyme was stored frozen in 10 ml quantities until used.

The crude yeast cell wall was prepared as follows. Commercially available autolysed yeast (Amber-BYF, 300 Series, Amber

labs, 3456 N. Buffum St., Milwaukee, Wis.) was washed twice with 0.5 N sodium hydroxide. Then the cell wall was neutralized with acetic acid and washed with water until there was no pH change. Finally it was washed with ethanol and used in the medium.

Optical Method of Hybridization

$Cot_{0.5}$ Determinations

$Cot_{0.5}$ values were determined according to the procedures of Seidler and Mandel (1971). Purified samples of DNA in 0.1xSSC at 5 to 8 O.D./ml concentration were sheared twice in a French pressure cell at 15,000 lb/in^2. Then the samples were dialyzed against 200 to 300 volumes of 3xSSC plus 20% dimethylsulfoxide (DMSO). This was done to reduce the actual time of renaturation and to lower the temperature of renaturation (person communication - Ramon J. Seidler). The samples of DNA were filtered through a prewashed (with 3xSSC plus 20% DMSO) 0.45 μm millipore filter. Concentration of DNA in solution was determined by measuring the optical density at 260 nm.

The sheared DNA samples, typically 55 to 65 μg/ml were placed in glass-stoppered cuvettes with 5 to 6 drops of mineral oil to reduce evaporation. The DNA samples were heat denatured in an automatic recording spectrophotometer (Gilford Instruments, Inc.).

The full scale on the recorder was set at 0.36 x the optical density at 260 nm at room temperature. The base lines were set using offset controls. The temperature was then increased to T_m - 25 C. After attaining equilibirium in temperature between the chamber and the Haake circulating water bath, the base lines were accurately set. The samples were denatured by increasing the temperature to about 12 C above the T_m.

The denaturing temperature was maintained for 6 to 8 min, until the hyperchromic shift at 260 nm, and then the temperature in the Haake was rapidly reduced by pumping out 700 to 1000 ml of the hot ethylene glycol and replacing it with ethylene glycol at minus 20 C, simultaneously bringing down the temperature on the thermostat control to T_m - 30 C. After 6 to 8 min, the temperature was increased to T_m - 22 C and optical density readings were recorded every 2 min for the next 30 min; then they were recorded every 15 min and the actual time was written on the chart. The measurements were continued until 60 to 65% renaturation occurred. The renaturation data were plotted as the log Cot versus percent reassociation (Britten and Khone, 1963).

Genome sizes were estimated from the proportionality relationship between the $Cot_{0.5}$ of a standard of known genome size and the $Cot_{0.5}$ of the unknown. They were also corrected for the GC content according to Seidler and Mandel (1971). E. coli B was used

as the primary standard with 2.2×10^9 daltons as the genome size of the standard.

Hybridization by Optical Renaturation

This was conducted by using equal amounts of the two test DNA samples to give a final concentration of 55 to 65 μg/ml. The $Cot_{0.5}$ values were determined as described before. When no similarity exists between the two organisms, the observed $Cot_{0.5}$ of the mixture is the sum of the two independently determined $Cot_{0.5}$ values. Lack of additivity was correlated with similarities in the polynucleotide sequence of the reassociating DNA molecules (Seidler and Mandel, 1971).

Isolation and Characterization of Plasmid DNA

L. plantarum MSH

Plasmids are known in other organisms to code for such properties as toxin production and drug resistance. This particular L. plantarum (actually a strain of L. rhamnosus) was isolated from a case of endocarditis in man by Axelrod et al. (1973). Therefore it was thought worthwhile to determine whether or not a plasmid(s) could be demonstrated in this organism, or among any members of the genus Lactobacillus. The presence of such an element in the

genus has apparently not been reported.

Plasmid DNA Isolation

Plasmid DNA extraction was carried out by a modified procedure of Guerry et al. (1974), which is a modification of the method of Hirt (1967). L. plantarum MSH was grown in MRS broth supplemented with 0.5 μg/ml of thymine, 250 μg/ml of desoxyadenosine and 10 uCi/ml of ^{3}H-6-thymidine. Cells were harvested by centrifugation and resuspended in 25% sucrose in 0.05 M Tris [(hydroxymethyl) amino-methane Tris], pH 8.0, at the rate of 1 ml for every 25 ml of broth. Lysozyme (Sigma; 5 mg/ml in 0.25 M Tris pH 8.0) was added to a final concentration of 1 mg/ml. The suspension was placed in an ice bath for 5 min. Ethylenediaminetetraacetate (EDTA, 0.25 M pH 8.0) was added at the rate of 33.33% to the cell suspension and iced for an additional 5 min. Lytic factor (5% vol/vol) or Lytase (BBL, 20% vol/vol) was added and incubated at 37 C for an hour. Complete lysis was accomplished by the addition of sodium lauryl sulfate to a final concentration of 1%. Following cellular lysis, 5 M sodium chloride was added with gentle mixing to a final concentration of 1 M. The lysates were stored at 4 C overnight, after which they were centrifuged at 17,000xg for 30 min. The supernatant was decanted and used in running the sucrose and cesium chloride-ethidium bromide gradients and agarose gel electrophoresis. Sometimes they were stored

over two drops of chloroform.

Sucrose Gradient

After storage overnight, the lysate was centrifuged at 17,000 x g for 30 min. Samples (100 µl) of the supernatant were mixed with an equal volume of TE buffer (0.01 M Tris plus 0.001 M EDTA, pH 8.1) to lower the salt concentration and layered onto a 5 to 20% (wt/vol) linear sucrose gradient. The sucrose gradient contained 0.5 M NaCl, 0.01 M potassium phosphate, pH 7.0. Bacteriophage lambda DNA was added as a sedimentation marker (30×10^6 daltons; 34S). Gradients were centrifuged at 36,000 rpm for 3 h in a SW50L rotor of a Beckman L2-65 preparative ultracentrifuge. After centrifugation, three-drop fractions were collected from a hole punctured in the bottom of the tube and the fractions were counted as described elsewhere.

Alkaline Sucrose Gradient

Samples (100 µl) of the supernatant fluid were layered onto 5 to 20% (wt/vol) linear sucrose gradient containing 1 M NaCl, 0.3 M sodium hydroxide; ^{14}C-lambda DNA was added as a sedimentation marker. Gradients were centrifuged at 36,000 rpm for 3 h in a SW50L swinging bucket rotor of Beckman L2-65 preparative ultracentrifuge. After centrifugation, five drop fractions were collected from a hole punctured in the bottom and the fractions were counted

as described in the next paragraph.

Preparation of Radioactive Samples

Radioactive samples were precipitated with an equal volume of 10% trichloroacetic acid containing 50 μg of thymine. When the samples contained free DNA, carrier DNA was first added. The precipitates were collected onto 25 mm diameter membrane filters with a pore size of 0.45 μm. The filters were prewashed with 10% trichloroacetic acid plus thymine to saturate the thymine adsorption sites; they were then washed successively with 5% trichloroacetic acid and 70% ethanol. Then the filters were dried at 55 C for 2 h and counted in a liquid scintillation counter after placing the filters in glass vials containing 10 ml of scintillation fluid with the following composition: 2, 5-diphenyloxazole, 5.0 g; 1, 4-bis-(2-(5-phenyl-oxazolyl))-benzene 0.1 g and toluene 1 liter.

Cesium Chloride-Ethidium Bromide (CsCl-EtBr) Density Gradients

The supernatant fluid was mixed with CsCl and a solution of ethidium bromide (700 μg/ml, in 0.1 M sodium phosphate buffer pH 7.0) to attain a mean density of 1.677 g/c.c., having a refractive index of 1.4007. When the solution was made, it contained 100 μg/ml of ethidium bromide. Then the samples were centrifuged in a

SW50L swinging bucket rotor of Beckman L2-65 preparative ultracentrifuge at 36, 000 rpm for 36 h. Five-drop fractions were collected from a hole punctured in the bottom of the tube and analyzed for radioactivity or absorbancy at 260 nm. Once established, the band under UV light was collected separately and dialyzed against 0. 1xSSC extensively. The DNA was stored at 4 C over a drop of chloroform until used.

Agarose Gel Electrophoresis of the Plasmid DNA

Gel electrophoresis was carried out according to the procedure of Sharp et al. (1973). The agarose gel contained 0. 7% agarose (Sigma electrophoresis grade) in 3 E buffer (Tris 0. 12 M; sodium acetate 0. 06 M; and EDTA 0. 003 M pH 7. 2). After melting the agarose, it was maintained at 50 C for an hour before using. The gel was poured into plexiglass tubes of 295 mm length with a 6 mm diameter bore. Care was taken to see that there was no air bubble in the tube and the gel was poured at a single stretch. Once poured, the top was covered with wax paper with holes. Then the tubes were inverted and the small cork was removed. The space created on the top of the tube would function as the sample reservoir inside the cathode buffer tank. Then both the anode and cathode tanks were filled with 3 E buffer. A 100 µl of the sample was mixed with 100 µl of glycerine and a drop of 16% sucrose containing 0. 05% bromophenol

blue. This was slowly pipetted onto the reservoir, with care taken to insure there was no spilling of the sample into the buffer. Once all the samples were ready, electrophoresis was carried for 6 h at 5 mA/gel. The blue dye acted as a visual marker, to follow the migration. After the electrophoresis, the gel was carefully blown out of the plexiglass tube into a trough of 3 E buffer containing 0.5 μg/ml of ethidium bromide. The gel was incubated at 4 C for an hour and then visualized under a UV source for the fluorescent bands. The gels were photographed using Polaroid type 55 P/N film. Then the bands were cut to the nearest inch and fractionated (10 fractions to an inch) into scintillation vials. The gel samples were dissolved in 100 μl of 8 M sodium-perchlorate (Dean et al., 1973). Once the gel was totally dissolved (2 h), 15 ml of scintillation fluid (Triton-X-100 plus POP, POPOP cocktail; 1:2) was added and the sample counted in a liquid scintillation counter for 10 to 20 min. The settings were adjusted to attain 2% spill of ^{3}H in the ^{14}C channel and 20% spill of ^{14}C in the ^{3}H channel. The data were corrected for the spillage before plotting.

Radioactive Lambda DNA Preparation

E. coli CSH 45 [d(lac)(Lambda C 1857 S7) trp R] carrying the heat-inducible prophage lambda was grown in L. B. broth (Miller, 1972 ed.) overnight. Twenty ml of overnight broth culture were

inoculated into a 2-liter flask containing 200 ml of L. B. broth with 1 uCi/ml of 2-^{14}C-thymine. The culture was incubated at 32 C on a shaker for 30 to 60 min. After the initial incubation, the flask was transferred to a shaker water bath at 43-44 C for 20 to 30 min. Then the culture was put on a shaker at 37 C for the next 3 h. The cells then were extracted by centrifugation at 5, 000xg for 20 min. The supernatant was saved for further concentration and extraction of lambda DNA. The pellet was resuspended in 10 ml of SM buffer (NaCl 0.1 M; $MgSO_4$ 0.001 M; Tris-HCl 0.02 M; and gelatin 0.01%, pH 7.5). After adding 1 ml of chloroform, the suspension was agitated at 37 C for 10 min to lyse the cells. The lysate was treated with 10 to 20 µl of DNase solution (1 mg/ml in distilled water) and incubated at 37 C on a shaker. The sample was stored overnight at 4 C. The DNase treatment was repeated the next day and then the sample subjected to DNA extraction as described elsewhere.

Concentration of Phage and Extraction of DNA from the Supernatant

The supernatant was filter-sterilized with a 0.45 µm millipore filter to eliminate whole cells of E. coli. The phage was concentrated by a modified procedure of Yamamoto et al. (1970). Sodium chloride was added to a final concentration of 1 M. Once the salt dissolved, Union Carbide polyethylene glycol 4000 (PEG) was added to the final

concentration of 10%. PEG was dissolved slowly by stirring. Then the sample was stored overnight at 4 C. The precipitate formed was recovered by centrifugation at 6, 000 rpm for 45 min. The pellet was washed with 30 ml of SM buffer and resuspended in 4 to 8 ml of SM buffer. A serial dilution of the lysate as well as the concentrated pellet from the supernatant were subjected to agar overlay technique to determine the actual plaque-forming units using E. coli CSH 25 as the indicator strain; this strain has the suppressor mutation for the S7 amber mutation in the lambda of CSH 45.

Both the lysate and supernatant concentrate were subjected to three deproteinizations using an equal volume of neutralized phenol (saturated with 0.1 M tris pH 7.6) and rolled on a roller for 20, 15, and 5 min respectively. The top aqueous layer was treated with RNase for 30 min at 37 C. Additional deproteinizations with phenol and Sevag solutions were conducted before dialyzing the sample against 0.1xSSC overnight with three changes. The DNA was stored over a drop of chloroform at 4 C.

Curing of L. plantarum MSH with Different Concentrations of Ethidium Bromide

The cells were incubated in broth containing ethidium bromide from $1x10^{-6}$ M to $5x10^{-4}$ M. The growth was followed by optical density determinations at 640 nm. At the concentration of $5x10^{-4}$ M

ethidium bromide, cellular toxicity was observed, while at the rest of the concentrations no toxicity was observed. All the cured samples were subjected to the following analysis: (a) plasmid DNA extraction and identification, (b) animal inoculation tests, (c) biochemical tests.

Animal Inoculation Tests for LD_{50} Determination

L. plantarum MSH was grown in broth to stationary growth phase (18 h). Culture dilutions ($1x10^{0}$, $5x10^{-1}$ and $5x10^{-2}$) were inoculated intra-peritoneally into young mice weighing 40-45 grams. For each dilution triplicate samples were used. As control, L. plantarum ATCC 14917 was used with similar dilutions, as well as sterile MRS broth. Filtered supernatant and autoclaved culture were used to check for the presence of exotoxin and endotoxin respectively. The mice were observed over a period of 14 days before discarding. Dead mice were subjected to post-mortem and the heart blood was cultured under asceptic conditions. The internal organs were observed for gross lesions. The recovered organisms were subjected to an array of biochemical tests to determine their identity. Similarly, another set of mice was inoculated with the same dilutions of plasmid-cured (ethidium bromide treated) L. plantarum MSH. Bacteria in all the dilutions used in animal inoculation tests were enumerated by the agar pour plate method. Approximate LD_{50}'s were determined by probit analysis.

RESULTS

Biochemical characteristics of lactobacilli used in this study are listed in Tables 3, 4, and 5, where they are grouped according to GC content. Lactobacilli are gram positive, non-motile, non-sporeforming rods. They ferment glucose and do not produce catalase. They are microaerophilic and do not reduce nitrate. In addition to the characteristics listed in the tables, most strains did not ferment L(-) sorbose, inulin, starch, glycogen, inositol, erythrytol, adonitol, methyl xyloside, dulcitol or amylose. All of the organisms produced a hazy zone of α hemolysis on sheep blood agar plates incubated under microaerophilic conditions. All except the fish isolates B2-70 and LRPKI-70 did not grow on blood agar plates containing 0.05% potassium tellurite. Generally they were not resistant to nine antibiotics tested (Table 22), however L. sanfrancisco (B, C, L and T) did not grow on the test plates. Cultures were generally resistant to streptomycin and kanamycin. Some of the organisms were also resistant to gentamicin and furadantin.

In addition, the four sourdough isolates (L. sanfrancisco strains B, C, L, and T) required fresh yeast extractives for growth. Berg (1974) has shown that the active ingredient in the fresh yeast extract is a small peptide made up of 5 different amino acids with about 9 residues in all. These strains produced acetate, ethanol, and

Table 3. Biochemical properties of lactobacilli belonging to the low GC group.

ATCC or NCDO no.	Lactate	Gas from glucose	Gas from gluconate	Aldolase	Ribose	NH_3 from arginine	Serological group*	15 C Growth	45 C Growth	Esculin	Amygdalin	Cellobiose	Lactose	Maltose	Mannitol	Melibiose	Melizitose	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Galactose	α-methyl-D-glucoside
11742	DL	-	-	+	-	-	x	-	+	+	-	-	±	+	+	±	-	+	-	+	+	+	+	±	-
25258	D(-)	-	-	+	-	+		-	+	+	+	+	-	+	-	-	-	-	-	+	±	+	+	±	-
4356	DL	-	-	+	-	-	?	-	+	+	±	+	±	±	-	±	-	±	-	+	-	+	±	±	-
B2-70	L(+)	-	-	+	+	+		+	-	±	+	+	+	+	±	-	-	-	-	+	+	+	+	±	+
LRPK170	L(+)	-	-	+	+	+		+	-	±	+	+	+	+	±	-	-	-	-	+	±	+	+	-	+
8018	DL	-	-	+	-	-	A	-	+	-	-	-	+		-	-	-	-	-	-	-	-	-	+	-
15009	DL	-	-	+	-	-	A	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	±	+	-
521	DL	-	-	+	-	-	A	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
B	DL							+	-					+											
T	DL							+	-					+											
L	DL							+	-					+											
C	L(+)							+	-					+											

+ = positive; - = negative; ± = doubtful; $\overline{+}$ = more negative than positive; NA = no acid; Lys = Lysine *Data from Rogosa (1970) **Data from Williams (1971)

Vitamin requirement data are from published reports Rogosa and Sharpe (1959)

Table 3. (Continued)

ATCC or NCDO no.	Mannose	Fructose	Dextrin	Glycerol	α-methyl-D-mannoside	Growth in % NaCl	Growth in % Teepol	Cell wall composition**	Arbutine	Pyruvic acid V. P.	O. N. P. G.	d-Arabinose	l-Arabinose	d-Xylose	l-Xylose	N-acetyl glucosamine	Thiamine	Riboflavin	Pyridoxal	Folic acid	Vit B_{12}	Thymidine	Pantothenate	Acid in milk (%)	Nicotinic acid
11742	+	+	±	-	-	6	.2	Lys	±	+	+	-	-	-	-	±	-	+	-	+	-	-	+	1.4	
25258	+	+	-	-	-	0	-		+	+	-	-	-	-	-	-	-	-	+	+	-		+		
4356	+	±	-	±	-	3	.1	Lys	-	-	+	-	-	-	-	-	-	+	±	+	±	-	+	2	
B2-70	+	+	+	±	+	3	.3		+	-	-	-	-	-	-	-								NA	
LRPKH-70	+	+	+	±	+	3	.3		+	-	-	-	-	-	-	-								NA	
8018	+	-	-	-	-	6	.1	Lys	-	-	-	-	-	-	-	-	-	+	+	-	-	-		2	+
15009	+	±	-	-	-	3	.1	Lys	-	-	+	-	-	-	-	+	-	+	+	-	-	-	+	2	+
521	+	+	-	-	-	2	.1	Lys	-	+	-	-	-	-	-	-	-	+	+	-	-	-		2	

Table 4. Biochemical properties of lactobacilli belonging to the medium GC group.

ATCC or NCDO no.	Lactate	Gas from glucose	Gas from gluconate	Aldolase	Ribose	NH_3 from arginine	Serological group	15 C Growth	45 C Growth	Esculin	Amygdalin	Cellobiose	Lactose	Maltose	Mannitol	Melibiose	Melizitose
14917	DL	-	+	+	+	-	D	+	-	+	+	+	+	+	+	+	+
8014	DL	-	+	+	+	-	D	+	-	+	+	+	+	+	+	+	+
DS46F	DL	-	+	+	+	-		+		-	+	+	+	+	+	+	+
Sardo-6	DL	-	+	+	+	-		-	±	+	+	+	±	+	+	-	+
M. S. H.		—	+	+	+	-		+	-	+	+	+	+	+	+	+	+
7469	L(+)	-	+	+	±	-	BC	+	+	+	+	+	+	+	+	-	+
393	L(+)	-	+	+	±	-	C	+	+	+	+	+	+	±	+	-	+
473	DL	+	+	-	+	+	E	+	-	+	-	-	-	+	-	+	±

+ = positive, - = negative, ± = doubtful; ± = more negative than positive; DAP=diamino pimelic acid; Lys = lysine. *Data from Rogosa (1970) **Data from Williams (1971). Vitamin requirement data from published reports (Rogosa and Sharpe, 1959).

Table 4. (Continued)

ATCC or NCDO no.	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Galactose	α-methyl-D-glucose	Mannose	Fructose	Dextrin	Glycerol	α-methyl-D-mannoside	Growth in % NaCl	Growth in % Teepol	Cell wall composition	Arbutine
14917	+	-	+	+	+	+	+	±	+	+	±	-	+	9	.3	DAP Lys	+
8014	+	-	-	+	+	+	+	+	+	+	-	-	+	9	.3		+
DS46F	+	-	-	+	+	+	+	±	+	+	±	-	+	6	.3		+
Sardo-6	-	±	+	+	+	+	±	±	+	+	+	+	-	9			-
M. S. H.	-	+	+	+	-	+	+	+	+	+	-	-	-	6	.3		+
7469	±	+	+	+	±	+	+	+	+	+	±	±	-	9	.1	Lys	+
393	-	-	+	-	±	+	+	-	+	+	-	-	-	9	.2	Lys	+
473	-	-	-	-	-	-	+	+	-	+	-	-	-	6	.3	Lys	+

Table 4. (Continued)

ATCC or NCDO no.	Pyruvic acid V.P.	O.N.P.G.	d-Arabinose	l-Arabinose	d-Xylose	l-Xylose	N-acetyl glucosamine	Thiamine	Riboflavin	Pyridoxol	Folic acid	Vit B_{12}	Thymidine	Pantothenate	Acid in milk (%)	Sorbitol	Sorbose
14917	-	+	+	+	-	-	+	-	±	-	-	-	-	+	<1	-	-
8014	+		-	+	+	-	+	-	±	-	-			+		-	-
DS46F	-		-	-	-	-	+									-	-
Sardo-6	-	+		-	-	-	+								<1	-	-
M. S. H.	+	-	-	-	-	-	+									±	-
7469	+	+	+	-	-	-	+	-	+	+	+			+	2	±	+
393	+	+	-	-	-	-	+	-	+	+	+	-	-	+	2	-	-
473	-	+	-	+	+	-	+	+	-	-	+	-	-			-	

Table 5. Biochemical properties of lactobacilli belonging to the high GC group.

ATCC or NCDO no.	Lactate	Gas from glucose	Gas from gluconate	Aldolase	Ribose	NH_3 from arginine	Serological group *	15 C Growth	45 C Growth	Esculin	Amygdalin	Cellobiose	Lactose	Maltose	Mannitol	Melibiose	Melizitose
12315	D(-)	-	-	+	-	-	E	-	+	±	-	±	±	±	-	-	-
11842	D(-)	-	-	+	-	-	E	-	+	-	-	-	+	-	-	-	-
9649	D(-)	-	-	+	-	±		-	+	-	-	-	-	$\overline{+}$	-	-	-
4797	D(-)	-	-	+	-	±	-	-	+	+	±	±	+	+	-	-	-
215	DL	+	+	-	+	+	F	-	+	-	-	-	+	+	-	+	-
927	DL	+	+	-	+	+		±	-	$\overline{+}$	$\overline{+}$	$\overline{+}$	-	+	+	-	-

+ = positive; - = negative; ± = doubtful; $\overline{+}$ = more negative than positive; Lys = lysine; Or = Ornithine; * Data from Rogosa (1970)
** Data from Williams (1971).
Vitamin requirement data from published reports Rogosa and Sharpe, 1959). Blank = not done. NA = no acid.

Table 5. (Continued)

ATCC or NCDO no.	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Galactose	-methyl-D-glucose	Mannose	Fructose	Dextrin	Glycerol	-methyl-D-mannoside	Growth in % NaCl	Growth in % Teepol	Cell wall composition**	Arbutine
12315	±	-	+	-	+	±	+	-	+	+	-	-	-	4	.1	Lys	-
11842	-	-	-	-	-	-	-	-	±	∓	-	±	-	2	-	Lys	-
9649	-	-	-	-	+	-	±	-	+|	+	-	-	-	4	.1	Lys	-
4797	-	+|	+|	-	+	+	±	±	+	+	+|	+|	-	2	.1	Lys	+
215	+	-	-	-	+|	-	+	-	-	+|	-	-	-	-	-	Or	-
927	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	Or	-

Table 5. (Continued)

ATCC or NCDO no.	Pyruvic acid V.P.	O.N.P.G.	d-Arabinose	l-Arabinose	d-Xylose	l-Xylose	N-acetyl glucosamine	Thiamine	Riboflavin	Pyridoxol	Folic acid	Vit B_{12}	Thymidine	Pantothenate	Acid in milk (%)	Nicotinic acid
12315	-	-	-	-	-	-	+	-	+	-	-	+|	-	+	2	+
11842	-	±	-	-	-	-	-	-	+	-	-	-	-	+	2	+
9649	-	-	+|	+|	-	-	-	-	+	-	-	-	+		NA	
4797	±	+	-	-	-	-	±	-	-	-	+	+	-	+	1	+
215	+	+	-	-	-	-	-	+	-	-	-	-	-			
927	-	+	-	+|	+	+	-	+	-	-	-	-	-			

CO_2 in significant amounts and were thus judged to be heterofermentative organisms.

Tables 3, 4 and 5 also show that the type and neotype species behaved as expected. Only a few characters have displayed variability. Note that the three organisms DS46F, Sardo-6 and MSH typed as medium GC organisms. Biochemically they were members of the subgenus Streptobacterium.

Of all the nine organisms that were subjected to typing by the conventional biochemical tests, only one (Sardo-6) remained true to its classification. The other six could not be typed, suggesting that they may be new species. The last two could only be typed by the DNA hybridization method.

Lactobacilli are gram positive organisms with a cell wall 22 to 35 nm thick (Fig. 4 and 8). The cells were approximately 1x1 to 3 μm in size as seen in Figures one through ten. Each cell typically possessed one to three large internal membraneous structures or mesosomes located at the plane of cell division (Fig. 1, 4, 5 and 8) or at a terminal position (Fig. 1, 2, 5, 6 and 10) and usually exhibited structural anomalies at the membrane wall interface. Hurst and Stubbs (1969) suggested that these cell surface structures are artifacts resulting from the discontinuous pulling away of the cell membrane from the cell wall during the air-drying process of specimen preparation. This feature is readily seen in Figures 2, 5 and 10.

Figure 1. Negatively-stained preparation of L. sanfrancisco strain B cells in stationary phase of growth (x 12, 350).

Figure 2. Negatively-stained preparation of L. sanfrancisco strain T cells in stationary phase of growth (x 31, 000).

Figure 3. Negatively-stained preparation of glutaraldehyde fixed L. sanfrancisco strain B cells in stationary phase of growth (x 12, 350).

Figure 4. Thin section of L. sanfrancisco strain L in stationary phase of growth (x 41, 200).

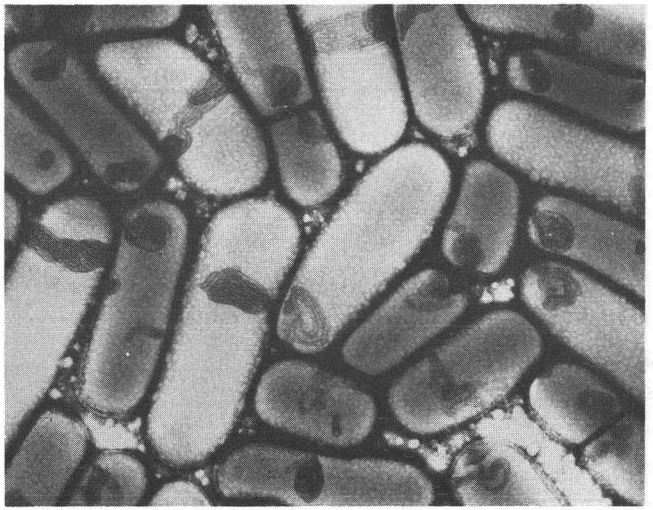

Figure 1

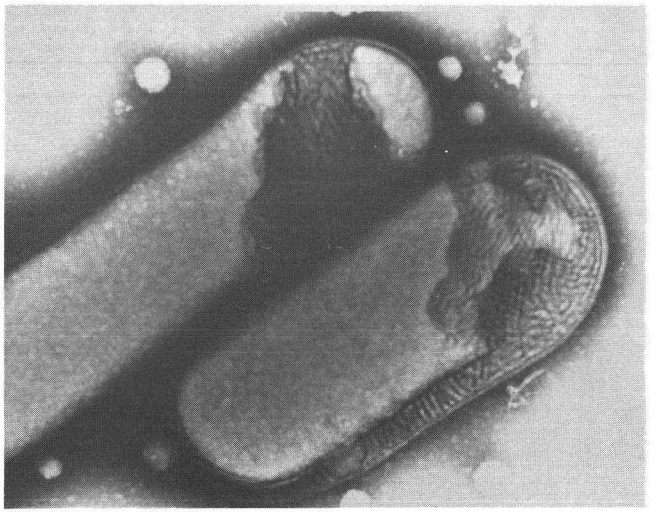

Figure 2

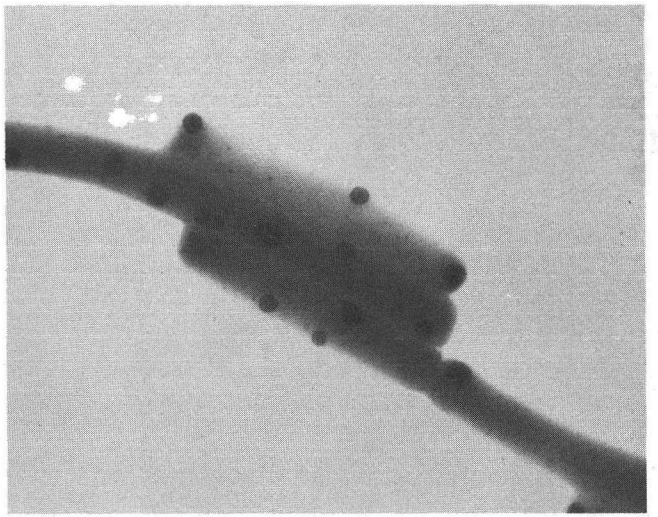

Figure 3

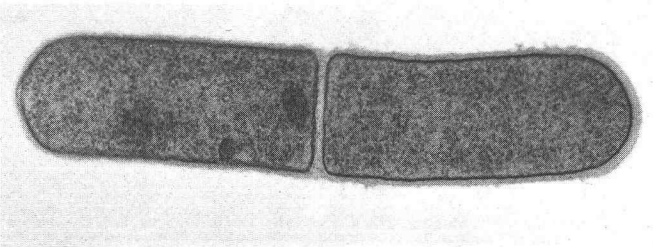

Figure 4

Figure 5. Negatively-stained preparation of Lactobacillus MSH in stationary phase of growth (x 31, 000).

Figure 6. Negatively-stained preparation of glutaraldehyde fixed Lactobacillus MSH in stationary phase of growth (x 25, 900).

Figure 7. Negatively-stained preparation of Lactobacillus MSH in the process of phage liberation (x 50, 500).

Figure 8. Thin section of Lactobacillus MSH in stationary phase of growth (x 41, 200).

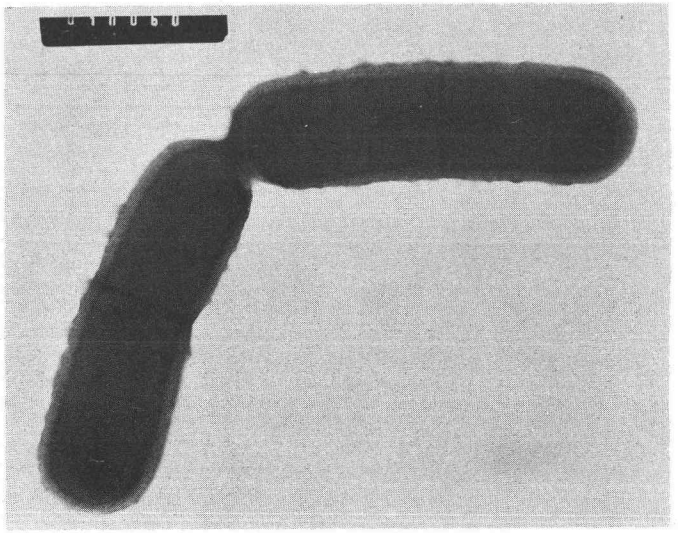

Figure 5

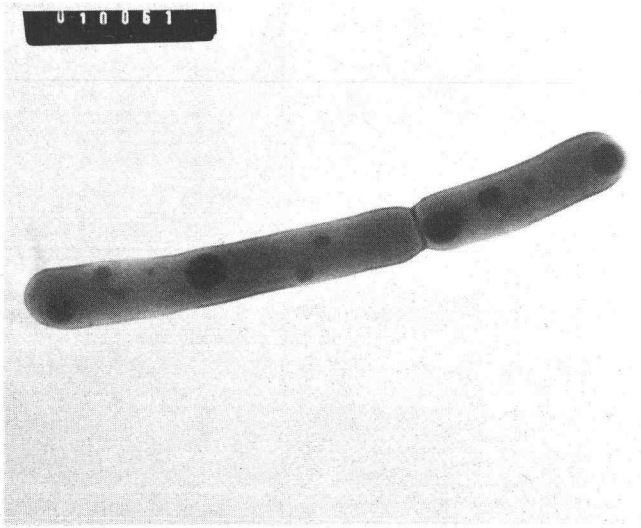

Figure 6

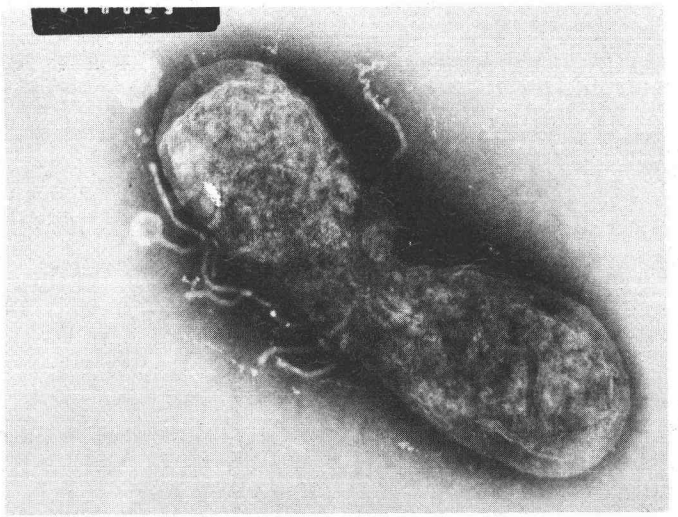

Figure 7

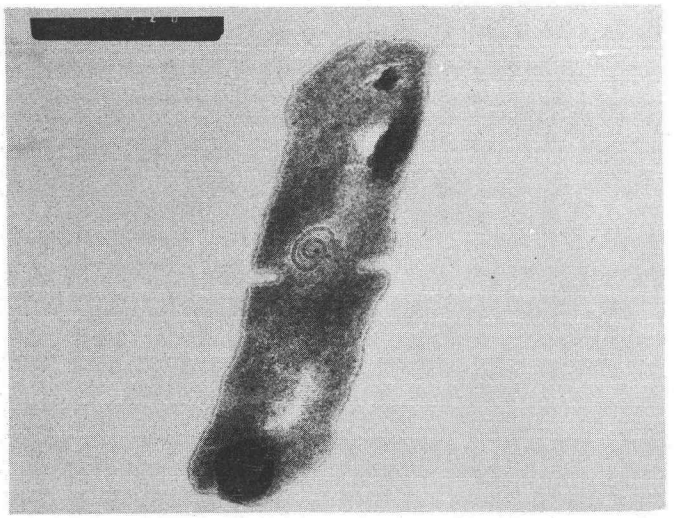

Figure 8

When glutaraldehyde fixation was employed, no plasmolysis was observed (Fig. 3 and 5). The cell wall of these preparations does not stand out and the only obvious structures are the mesosomes. In Figure 6 there are additional large dark structures; these are probably lipid inclusions.

Figures 4 and 8 show thin sections of lactobacilli from different sources and the typical thick cell wall characteristic of all gram positive organisms is evident. The mesosomes are located at the plane of cell division suggesting that they probably are involved in the division process.

The electron dense circular spots seen in Figure 8 are probably lipid or lipoprotein inclusions. They appear as well organized spherical particles, 0.2 μ in diameter. Figure 9 represents an early lag (20 min) phase culture of Lactobacillus piscium B2-70. Note that all membrane structures are absent.

In Tables 6, 7 and 8, the moles % GC content of DNA from all the organisms studied are tabulated as calculated from thermal denaturation curves (Fig. 13). In every T_m determination, E. coli DNA was included as the internal standard. The difference between the T_m of E. coli and the T_m of the test DNA (ΔT_m) was used for calculating the GC content according to the equation of Mandel et al. (1970). The three tables represent the low, medium and high GC groups of organisms. These GC values agree with published reportes within

Figure 9. Negatively-stained preparation of Lactobacillus piscium B2-70 in early log phase of growth (x 41, 200).

Figure 10. Negatively-stained preparation of Lactobacillus piscium LRPKI-70 in stationary phase of growth (x 31, 000).

Figure 11. Ethidium bromide stained agarose gels run for 6 h at 5 mA/gel with, top to bottom:

a) Supernatant from cured Lactobacillus MSH.
b) Purified supernatant from uncured MSH.
c) Supernatant from uncured Lactobacillus MSH.
d) Bacteriophage lambda as marker for the gels.

Figure 12. Electron photomicrograph of circular plasmid DNA prepared using the Kleinschmidt technique (x 80, 000).

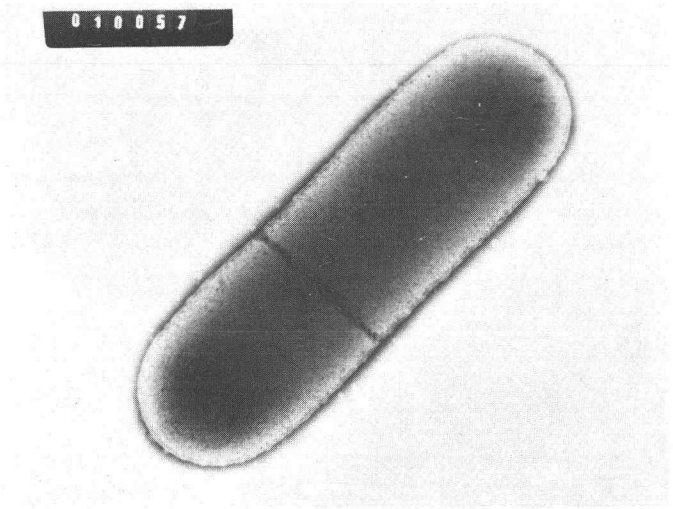

Figure 9

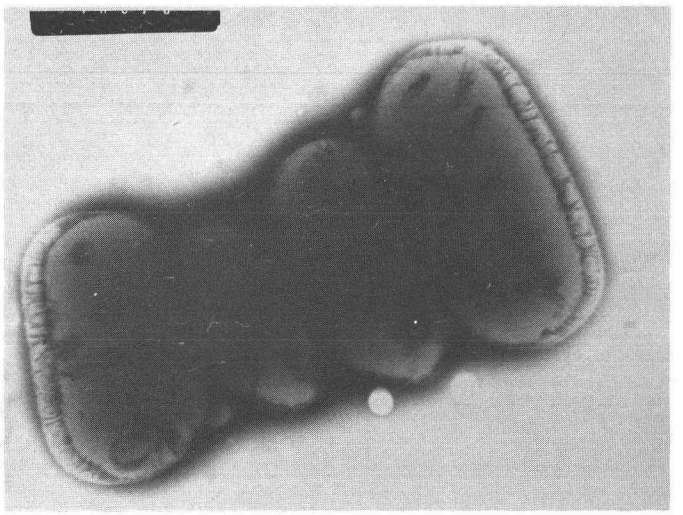

Figure 10

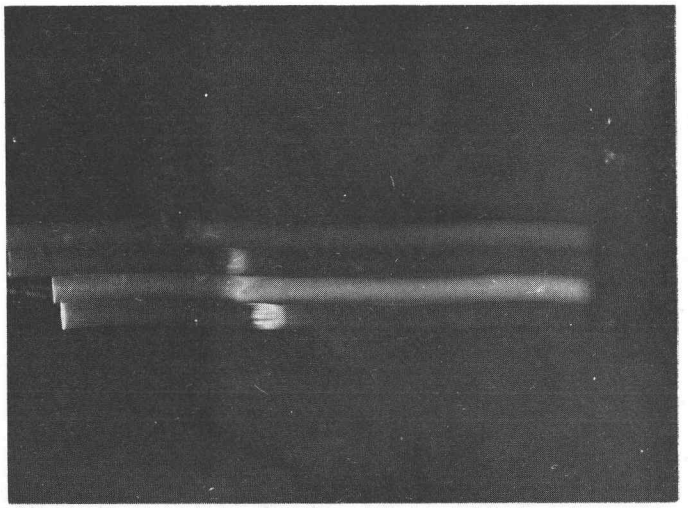

Figure 11

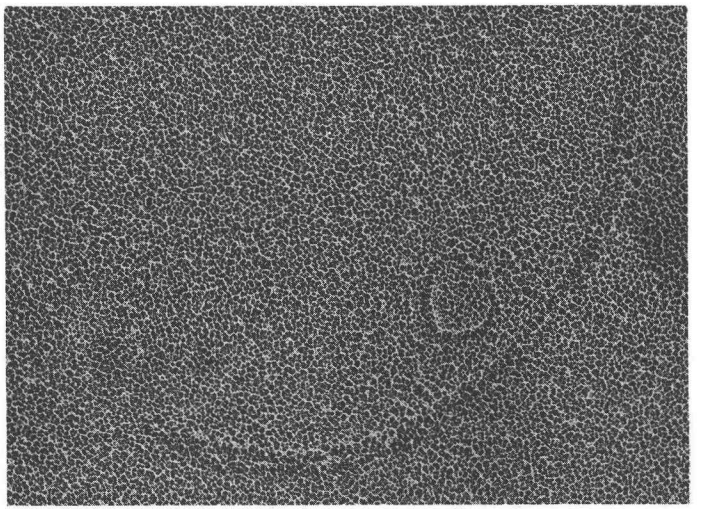

Figure 12

Table 6. Thermal denaturation data and percent moles guanine plus cytosine values for lactobacilli belonging to the low GC group.

Organism	$\bar{x}\Delta T_m$	Moles % GC[a]
L. salivarius 11742	9.0±0.3	33.1
L. jensenii 25258	8.9±0.4	33.3
L. acidophilus 4356	7.9±0.1	35.3
E. rhusiopathiae 19414	7.5±0.0	36.1
L. piscium B2-70	7.5±0.0	36.1
L. piscium LRPKI-70	7.4±0.1	36.3
L. helveticus 8018	7.0±0.0	37.1
L. helveticus 15009	6.9±0.1	37.3
L. jugurt 521	6.7±0.7	37.7
L. sanfrancisco B	6.5±0.3	38.1
L. sanfrancisco T	6.4±0.2	38.3
L. sanfrancisco L	6.2±0.4	38.7
L. sanfrancisco C	5.7±0.1	39.7

[a]Calculated by using the equation of Mandel et al. (1970):

$$\% \text{ G+C}_x = \% \text{ G+C}_{\text{E. coli}} + 0.0199\,(T_{m_x} - T_{m_{\text{E. coli}}}) \times 100.$$

Table 7. Thermal denaturation data and percent moles guanine plus cytosine values for lactobacilli belonging to the medium GC group.

Organism	$\bar{x}\Delta T_m$	Moles % GC[a]
L. plantarum 14917	3.5±0.1	44.0
Lactobacillus DS46F	3.5± 0.7	44.0
S. inulinus	2.7±0.0	45.6
Lactobacillus Sardo-6	2.6± 0.1	45.8
L. brevis 473	2.6±0.0	45.8
Lactobacillus MSH	2.4± 0.2	46.2
L. casei 7469	2.0±0.0	47.0
L. casei 393	1.5±0.3	48.0

[a]Calculated by using the equation of Mandel et al. (1970); see Table 6 for equation.

Table 8. Thermal denaturation data and percent moles guanine plus cytosine values for lactobacilli belonging to the high GC group.

Organism	$\bar{x}$ T_m	Moles % GC[a]
L. delbrueckii 9649	1.3±0.1	48.4
L. lactis 12315	1.0±0.2	49.0
L. bulgaricus 11842	1.0±0.0	49.0
L. leichmanii 4797	0.9±0.1	49.2
L. fermenti 215	0.6±0.1	49.8
L. cellobiosus 927	0.5±0.1	50.0
E. coli K-12	74.5±0.5[b]	51.0
E. coli WP-2	74.3±0.5[b]	51.0
E. coli CSH-52	74.7±0.3[b]	51.0

[a] Calculated by using the equation of Mandel et al. (1970); see Table 6 for equation.

[b] T_m values determined experimentally for E. coli and used to calculate $\bar{x}$ T_m.

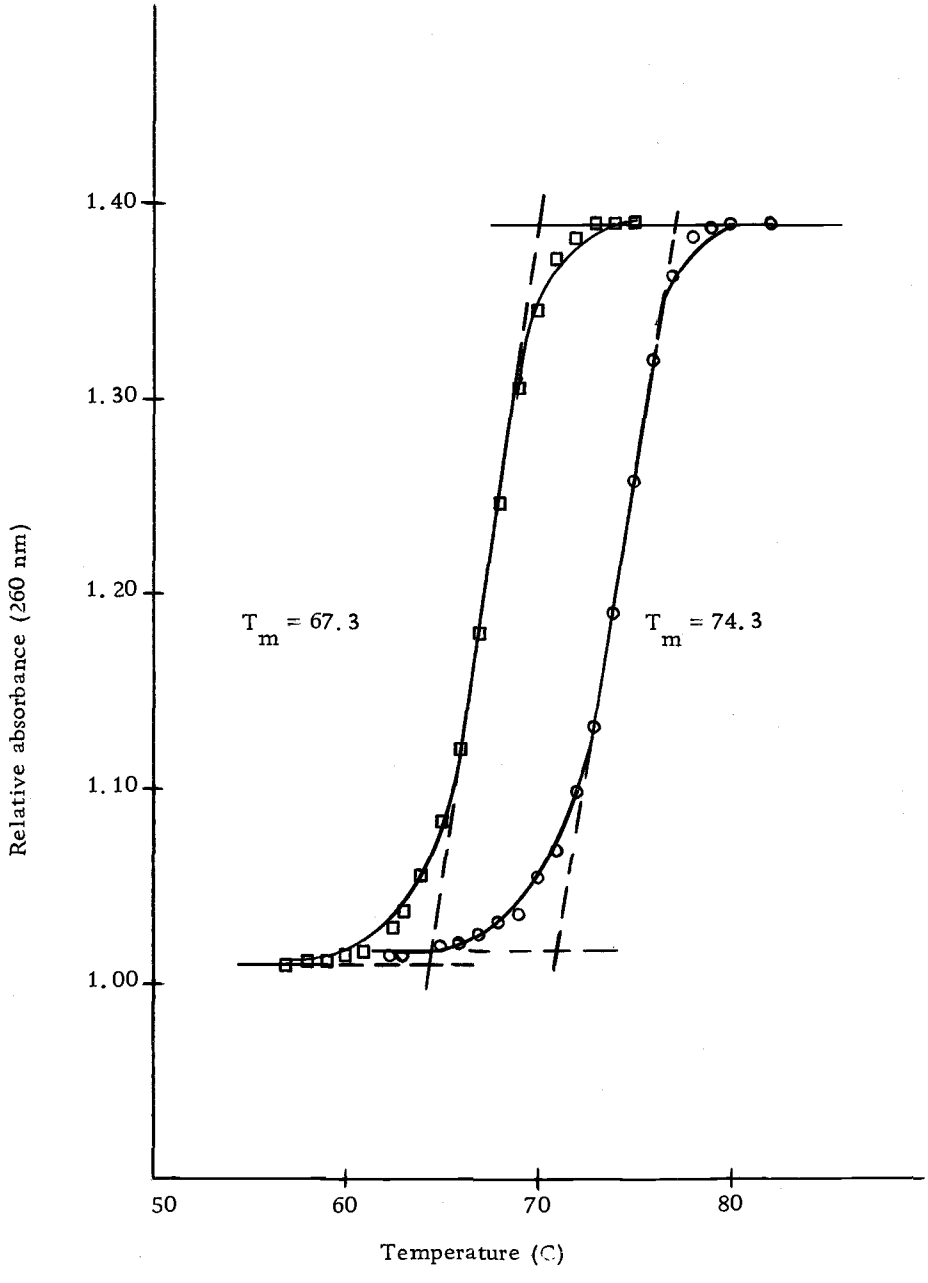

Figure 13. Absorbance vs. temperature denaturation profile for L. helveticus and E. coli K-12.

experimental error.

Results of renaturation experiments to determine the genome sizes are reported in Tables 9, 10 and 11. For the most precise estimate of the genome sizes, a correction of 18% was applied (Seidler and Mandel, 1971), depending upon the difference in GC content between the test DNA and that of E. coli DNA. The genome sizes of all lactobacilli indicated that they possess only half the genetic capability of E. coli. This is in agreement with their complex nutritional requirements. As can be seen, S. inulinus appeared to have a genome as large as the sporeforming bacilli (Leth Bak et al. (1970). This suggested that this organism is not a member of the genus Lactobacillus.

Preliminary experiments were conducted with DNA from all reference organisms to determine the exact T_m in the hybridization buffer. This was done to determine the precise influence of the denaturant formamide on the T_m by melting the reference DNA in 6xSSC containing 40% (vol/vol) formamide; it facilitated the determination of exact hybridization temperatures at either T_m - 25 or T_m - 15 C. The 6xSSC with 40% formamide lowered the T_m of the DNA samples so that hybridizations could be carried out at fairly low temperatures (between 49 and 56 C).

Table 9. $Cot_{0.5}$ and genome sizes of lactobacilli belonging to the low GC group.

Organism	$Cot_{0.5}$	Observed	Corrected[a]
		Genome size x 10^6 Daltons	
L. salivarius	0.76±0.1	1520	1030
L. jensenii	0.54±0.0	1080	740
L. acidophilus	0.51±0.1	1020	732
E. insidiosa	0.37±0.0	740	540
L. piscium B2-70	0.56±0.1	1120	820
L. helveticus	0.68±0.0	1360	1030
L. sanfrancisco	0.68±0.0	1360	1060

[a]The genome sizes were corrected for their GC content using Seidler and Mandel's equation (1971):

$$G = (2,200 \times 10^6 \, X/Y)\, 1 - (\Delta GC_{51} \times 0.018)$$

where X = the observed $Cot_{0.5}$ of E. coli; Y = the observed $Cot_{0.5}$ of the test DNA; ΔGC_{51} = the % difference in GC between the sample and E. coli DNA.

Table 10. $Cot_{0.5}$ and genome sizes of lactobacilli belonging to the medium GC group.

Organism	$Cot_{0.5}$	Observed	Corrected[a]
		Genome sixe x 10^6 Daltons	
L. plantarum	0.78±0.00	1560	1360
Lactobacillus DS46F	0.51±0.00	1020	890
S. inulinus	1.36±0.01	2720	2460
L. brevis	0.66±0.04	1320	1200
Lactobacillus MSH	0.58±0.00	1160	1060
L. casei 393	0.81±0.06	1620	1530
L. casei 7469	0.69±0.10	1380	1280

[a]The genome size was determined and corrected using Seidler and Mandel's equation (1971), see Table 9.

Table 11. $Cot_{0.5}$ and genome sizes of lactobacilli belonging to the high GC group.

Organism	$Cot_{0.5}$	Observed	Corrected[a]
		Genome size x 10^6 Daltons	
L. lactis	0.49±0.06	980	950
L. bulgaricus	0.34±0.02	680	660
L. fermenti	0.54±0.05	1080	1060
L. cellobiosus	0.44±0.01	880	860
E. coli	1.10±0.05	2200	2200

[a]The genome size was determined and corrected using Seidler and Mandel's equation (1971); see Table 9.

Experiments were also conducted to determine the amount of leaching occurring during the incubation period at the hybridization temperatures used. To measure this, the tritium-labelled DNA was loaded onto membrane filters which were then incubated under the exact conditions used for hybridization. Results in Table 12 indicated that leaching was negligible under these conditions. These findings agreed with the observations of DeLey and Tijtgat (1970)

and Okanishi and Gregory (1970).

Table 12. Leaching effect of hybridization temperature on DNA loaded on membrane filters.

Sample	Before the Experiment (CPM)	Sample	After the Experiment (CPM)
A	4870	A_1	4450
B	3650	B_1	3950
C	3610	C_1	3720
	Average = 4042		Average = 4040

To evaluate the specific nature of the hybrid material formed at T_m - 25 and T_m - 15 C, thermal elution experiments were conducted. Figure 14 shows that the thermal elution midpoint of the renatured DNA and the T_m of native DNA differ by 5 C in the T_m - 25 C plot, whereas there was a difference of only 2.6 C in the T_m - 15 C experiment. This suggested that the duplex formed at the more stringent temperature of T_m - 15 C contained a smaller amount of mispaired bases compared to that of T_m - 25 C hybrids. This is in support of the observations of others (Brenner et al., 1972; Johnson and Ordal, 1968 and Palleroni et al., 1972) on the influence of temperature on the percent of mismatched base pairs formed in the renatured DNA. Therefore DNA form only two reference organisms, L. sanfrancisco strain B and Lactobacillus piscium B2-70,

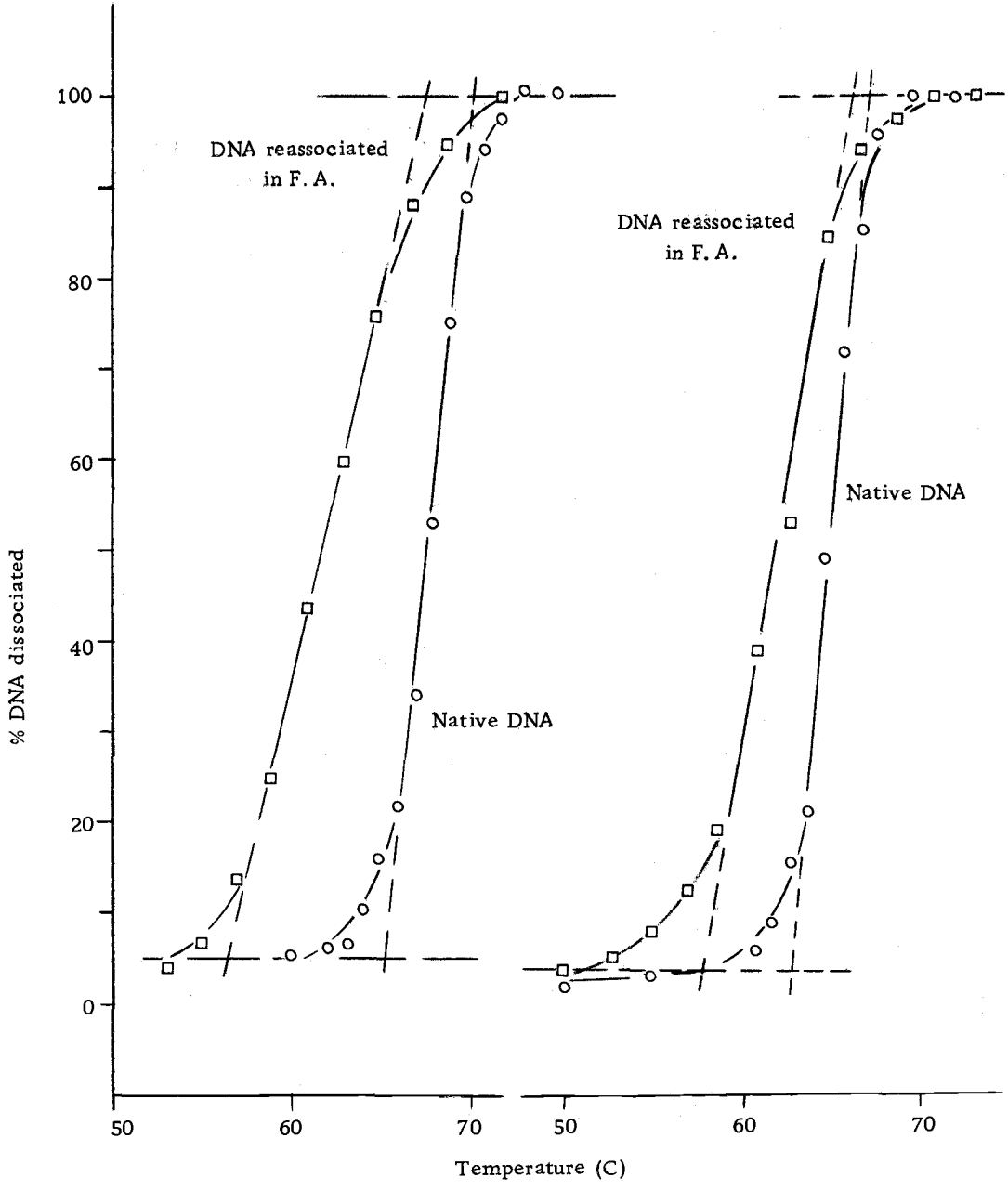

Figure 14. Thermal melting curves of L. sanfrancisco strain B DNA in the native and reassociated state (T_m - 25 C, left; T_m - 15 C, right).

were subjected to both T_m - 25 and T_m - 15 C hybridization experiments. The rest of the experiments were conducted at the more stringent conditions of hybridization. In addition % homology data at T_m - 25 C for at least a few of the reference organisms are already published by other workers in the field (Simonds et al., 1971; Dellaglio et al., 1973; and Johnson, 1973).

Results of the homology experiments are shown in Tables 13 through 20. In Table 13 the hybridization data for DNA from L. sanfrancisco strains C, L, and T showed 93, 98, and 93% homology respectively at T_m - 25 C. When hybridization was carried out at T_m - 15 C the values were 89, 95, and 95% homology with the reference strain B. This suggested that all four sourdough strains were essentially identical. This also was supported by the cultural, biochemical and cytological characters. Competition by the homofermentative species of the low GC group of organisms was minimal and at T_m - 25 C may have been non-specific. This is supported by the still lower degree of competition obtained in the T_m - 15 C experiments. L. brevis, a heterofermentative organism, showed about 40% homology, which is slightly higher than observed with the homofermentative organisms.

Attention is called to the two organisms in Table 1. Lactobacillus B2-70 and LRPH1-70. It should be noted that they were isolated from fish and recent findings (R. Holt, personal

Table 13. Hybridization data obtained with reference L. sanfrancisco B at T_m - 25 and T_m - 15 C.

Organism	T_m - 25		T_m - 15	
	Depression (CPM)	% Homology	Depression (CPM)	% Homology
L. sanfrancisco B	1025	100	3937	100
L. sanfrancisco C	952	93	3493	89
L. sanfrancisco L	1007	98	3744	95
L. sanfrancisco T	956	93	3749	95
L. acidophilus	240	23	907	23
L. salivarius	310	30	476	12
L. jugurt	377	37	234	6
L. helveticus	352	35	1247	32
L. brevis	--	--	1541	39

communication) indicate that these organisms are only nominally pathogenic for fish. Originally they were isolated along with a Corynebacterium from kidney lesions in trout. Whether or not they are invasive pathogens is still open to question; rather it seems that they can cause disease under conditions of stress or subsequent to infection by other more invasive organisms.

Homology data with the fish isolate Lactobacillus piscium B2-70 as the reference bacterium is tabulated in Table 14. The hybridization data for an independent isolate LRPKI-70 showed 96 and 92% homology at T_m - 25 C and T_m - 15 C respectively. This indicated that the two isolates, though from two different hatcheries, were identical. This is in agreement with the cultural, biochemical and cytological characters. In general the data indicated that this isolate had approximately 40 to 45% relationship with lactobacilli of the low GC group. Consistent lower homology values at T_m - 15 C showed that this more stringent temperature condition of hybridization was more suitable for species identification.

The fish isolates type as homofermentative streptobacteria, except they hydrolyze arginine (Table 3). In addition the isolates are resistant to 0.05% potassium tellurite, suggesting a relationship to Corynebacterium species. To evaluate this, E. insidiosa was included in the experiments; B2-70 had only 14% homology with E. insidiosa.

Table 14. Hybricization data obtained with reference fish isolate B2-70 at T_m - 25 and T_m - 15 C.

Organism	T_m - 25		T_m - 15	
	Depression (CPM)	% Homology	Depression (CPM)	% Homology
Lactobacillus B2-70	875	100	766	100
Lactobacillus LRPKI-70	841	96	703	92
L. salivarius	394	45	346	45
L. jensenii	275	31	165	22
L. acidophilus	384	44	363	47
L. helveticus 8018	346	40	122	16
L. jugurt	377	43	245	32
E. insidiosa	122	14	112	14

Both known species of streptobacteria have a GC content of 44 and 48 moles %, whereas the fish isolates belong to the low GC group with 36.0 moles % GC. Theoretically they cannot have any homology with the two known species of streptobacteria. This suggests that they probably are new species. Also, their 40 to 45% genetic relationship with the low GC organisms and their 14% homology with E. insidiosa indicate that these organisms are more closely related to members of the genus Lactobacillus than to members of the genus Erysipalothrix.

Table 15 shows the data from hybridization experiments with L. salivarius 11742 as the reference. It may be seen that L. salivarius has negligible genetic relationship with L. helveticus, L. jensenii or L. acidophillus 4356. This shows that L. salivarius is a well differentiated species.

L. helveticus 15009 with 37 moles % GC was the reference strain for the data in Table 16. This bacterium revealed a very high degree of relationship or homology with L. jugurt 521, consistent with a published report (Dellaglio et al., 1973). Biochemically the only difference between L. helveticus and L. jugurt is that the latter does not ferment maltose and is variable in the ability to ferment trehalose. L. helveticus weakly ferment trehalose and fructose. L. jugurt appears therefore to be a mutant strain of L. helveticus. Sharpe (1971) has indicated that she was not able to differentiate between a

Table 15. Hybridization data obtained with reference L. salivarius 11742 at T_m - 15 C.

Organism	Depression (CPM)	% Homology
L. salivarius	1214	100
L. helveticus	126	10
L. jensenii	0	0
L. acidophilus	108	9

Table 16. Hybridization data obtained with reference L. helveticus 15009 at T_m - 15 C.

Organism	Depression (CPM)	% Homology
L. helveticus	878	100
L. jugurt	690	79
L. jensenii	0	0
L. acidophilus	0	0
L. salivarius	81	9
L. sanfrancisco	0	0
L. piscium B2-70	0	0

maltose negative L. helveticus and L. jugurt, so they must be considered as one and the same species with L. jugurt as a maltose negative L. helveticus. L. helveticus does not show any homology with any of the species in the low GC group of organisms. It shows only 9% homology with L. salivarius, which is very similar to the 10% homology seen when L. salivarius was the reference.

Hybridization data with L. plantarum 14917 of the medium GC group (44% GC) as the reference are listed in Table 17. This organism shows about 20% homology with L. casei var. casei and none with L. casei var. rhamnosus. It also shows no or negligible homology with L. brevis, L. fermenti, S. inulinus or L. helveticus. L. plantarum 14917 does not have any homology with L. plantarum MSH, suggesting that the latter has been misnamed by the previous authors (Axelrod et al., 1973). The reference shows a very high genetic relationship with lactobacillus Sardo-6. This confirms the biochemical and cultural identification and that Lactobacillus Sardo-6 is L. plantarum. Similarly Lactobacillus DS46F is L. plantarum, showing 88% homology with L. plantarum 14917.

Experiments with L. casei var. casei 393 as the reference (Table 18) show that it has only 14% homology with L. casei var. rhamnosus. This indicates that L. casei var. rhamnosus is a new species rather than a variety. This value of 14% is lower compared to the report from another laboratory (Johnson, 1973), but the

Table 17. Hybridization data obtained with reference L. plantarum 14917 at T_m - 15 C.

Organism	Depression (CPM)	% Homology
L. plantarum	1835	100
L. casei 393	311	17
L. casei 7469	0	0
L. brevis	0	0
L. lactis	0	0
L. fermenti	40	2
S. inulinus	0	0
L. helveticus	119	6
Lactobacillis MSH	0	0
Lactobacillis Sardo-6	1534	84
Lactobacillis DS46F	1615[a]	88[a]

[a]Normalized cpm from another set of experiments

Table 18. Hybridization data obtained with reference L. casei 393 at T_m - 15 C.

Organism	Depression (CPM)	%
L. casei 393	1987	100
L. casei 7469	281	14
L. plantarum	862	43
L. brevis	322	16
L. fermenti	73	4
L. helveticus	343	17
L. salivarius	195	10
S. inulinus	416	21
L. lactis	81	4
Lactobacillis Sardo-6	710	36
Lactobacillis MSH	819	41
Lactobacillis DS46F	485	24

present experiments were conducted at more stringent condition of T_m - 15 C. Also, the results of present homology experiments are in agreement with the biochemical pattern. These organisms differ in their capacity to ferment six sugars and in their antigenic structure. In addition L. casei var. rhamnosus has a variable capacity to ferment three more sugars (Table 4).

L. casei var. casei has about 40% homology with L. plantarum 14917 and L. plantarum MSH. Lactobacillus Sardo-6, which showed 84% homology with L. plantarum 14917, revealed about 36% homology with L. casei var. casei. Similarly Lactobacillus DS46F showed 24% homology with L. casei var. casei. Lactobacillus DS46F showed 88% genetic relationship with L. plantarum 14917. These experiments show that the two known species from the subgenus Streptobacterium have 20 to 40% homology. This supports the grouping of the two under the subgenus. L. plantarum MSH showed about 40% homology with strain 393 and it did not show any homology with L. plantarum 14917, suggesting that the organism has been misnamed as L. plantarum. It probably belongs to the L. casei group.

Table 19 lists the hybridization results with L. lactis 12315 as the reference strain. It belongs to the high GC group and its GC content is 49 moles %. It shows a consistently high homology of 78, 85 and 78% with L. bulgaricus 11842, L. delbrueckii 9649 and L. leichmanii 4797, respectively. This suggests that the four

organisms are a single species rather than four species. This is also evident from their biochemical patterns and cultural characteristics. They all produce D(-) lactate, have the same cell wall composition, are homofermentative and two of them have the same cell antigens (the other two have not been determined - Table 4). They differ only in their ability to ferment sugars and in their vitamin requirements. All the four species have identical GC contents within the limits of experimental error. L. lactis shows a negligible relationship (11 and 8%) to L. fermenti and L. cellobiosus and only 30% homology with L. helveticus.

Table 19. Hybridization data obtained with reference L. lactis 12315 at T_m - 15 C.

Organism	Depression (CPM)	% Homology
L. lactis	1974	100
L. bulgaricus	1530	78
L. delbrueckii	1692	85
L. leichmanii	1536	78
L. fermenti	213	11
L. cellobiosus	162	8
L. helveticus	590	30

Table 20 shows the results of hybridization experiments with L. fermenti 215 as the reference strain. It belongs to the high GC group with 50.0 moles % GC content. L. fermenti has 83% homology with L. cellobiosus 927. Biochemically both appear similar except

for their sugar fermentations. They should be considered as varieties of one species. In fact some L. cellobiosus strains (e.g., ATCC 11739) are known not to ferment cellobiose and therefore this species does not seem justified. When one examines large numbers of strains of this species, even the sugar fermentation patterns become inconsistent. L. fermenti showed no relationship with L. delbrueckii or L. leichmanii and very low homology of 6 and 11% with L. lactis and L. bulgaricus respectively.

L. helveticus was included in all the hybridization experiments to get a measure of relatedness over the entire genus, irrespective of GC content. As can be seen it showed a homology of 10 to 30% with different references. This supports the concept that all lactobacilli belong to a single genus.

To reaffirm the results from the membrane method of hybridization, an independent set of experiments was conducted using renaturation kinetics to determine homology values for some of the organisms used in the previous method. The procedures were identical to those described by Seidler and Mandel (1971) except for the buffer. Instead of the standard saline citrate (SSC), a buffer of 3xSSC containing 20% dimethylsulfoxide was used (personal communication, R.J. Seidler). This lowered the thermal denaturation point as well as reduced the renaturation reaction time.

Table 20. Hybridization data obtained with reference L. fermenti NCDO 215 at T_m - 15 C.

Organism	Depression (CPM)	% Homology
L. fermenti	1242	100
L. cellobiosus	1034	83
L. delbrueckii	0	0
L. leichmanii	0	0
L. lactis	76	6
L. bulgaricus	135	11
L. helveticus	257	21

The results of these experiments are recorded in Table 21. Lactobacillus piscium B2-70 showed about 27% homology with L. salivarius 11742 as compared to 45% obtained by the membrane method. The renaturation reaction is plotted in Figure 15. The results agree in almost all cases within experimental error. Lactobacillus B2-70 had about 24% genetic relationship with E. insidiosa 19414 as against the 14% observed in the membrane procedure (Fig. 16). It should be noted that results of the membrane procedure are obtained at a more stringent incubation temperature of T_m - 15 C, whereas the renaturations were conducted at T_m - 25 C. This should explain some of the higher values obtained by the renaturation method.

Lactobacillus MSH gave 85% homology with L. casei var. rhamnosus 7469 (which itself appears to be a new species), suggesting

Table 21. Homology data from renaturation experiments.

Expt. no.	Organism by strain no.	$Cot_{0.5}$	Percent homology from renaturation method[a]	Percent homology from membrane method
1	B2-70 (0.56) + 11742 (0.76)	1.14	27	45
2	B2-70 (0.56) + 19414 (0.37)	0.82	24	14
3	DS46F (0.51) + 7469 (0.69)	0.98	27	ND
4	7469 (0.69) + 14917 (0.78)	1.6	0	0
5	7469 (0.69) + 393 (0.81)	1.8	0	0
6	MSH (0.58) + 393 (0.81)	1.18	31	41
7	393 (0.81) + 14917 (0.78)	1.42	22	17
8	MSH (0.58) + 14917 (0.78)	1.3	9	0
9	7469 (0.69) + MSH (0.58)	0.73	85	ND

[a] Calculated using the Seidler and Mandel's equation #3 (1971).

$$\% \text{ homology} = \left\{1 - \left(\frac{(\text{obs. } Cot_{0.5} \text{ mix} + (Cot_{0.5}\ 100 - Cot_{0.5}\ 0)}{Cot_{0.5}\ 100}\right)\right\} \times 100$$

Individual $Cot_{0.5}$ (mole sec liter^{-1}) values are given in parentheses.

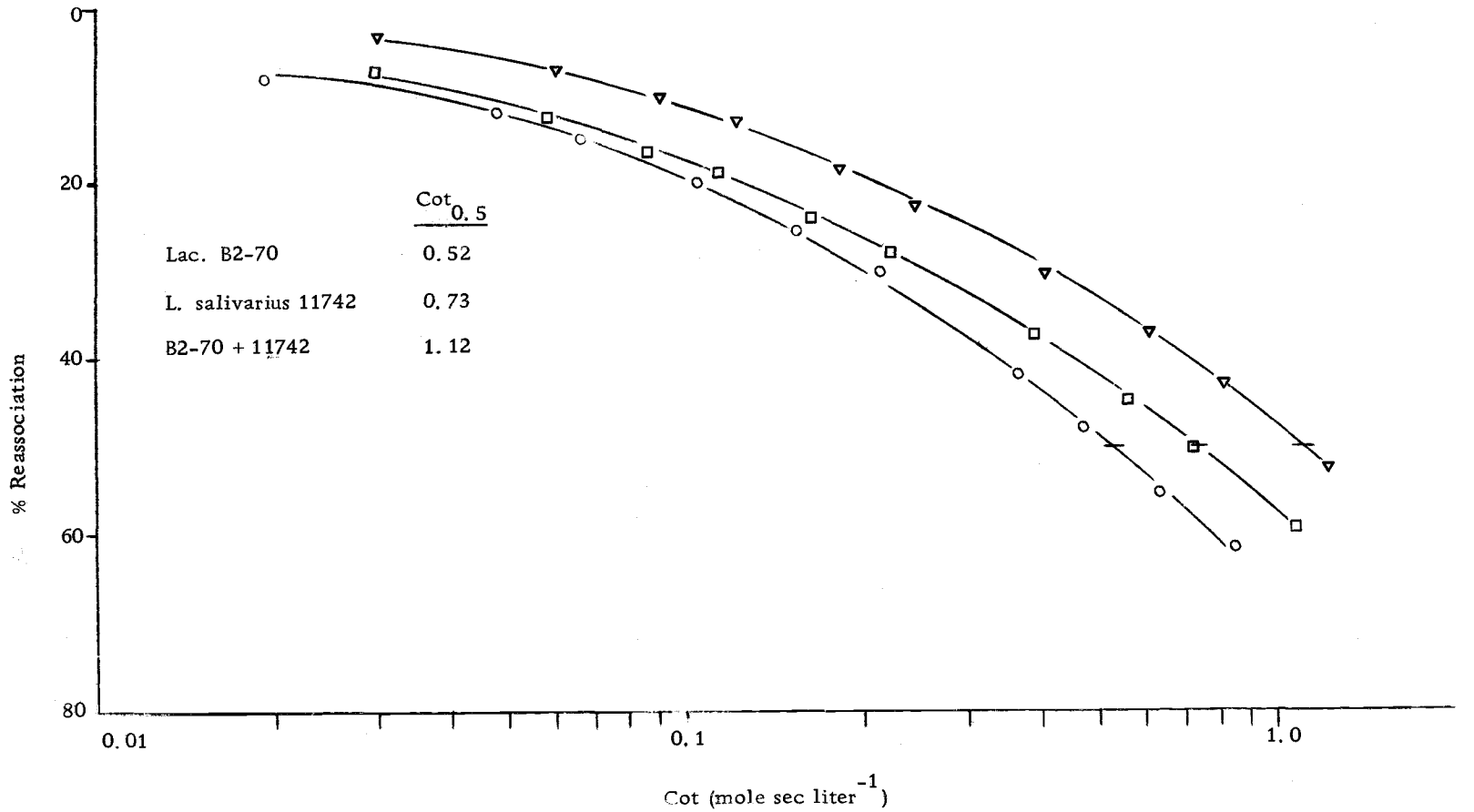

Figure 15. Cot (mole sec liter $^{-1}$) vs. % reassociation for (O) Lactobacillus B2-70, (□) L. salivarius 11742, and (△) a mixture of the two.

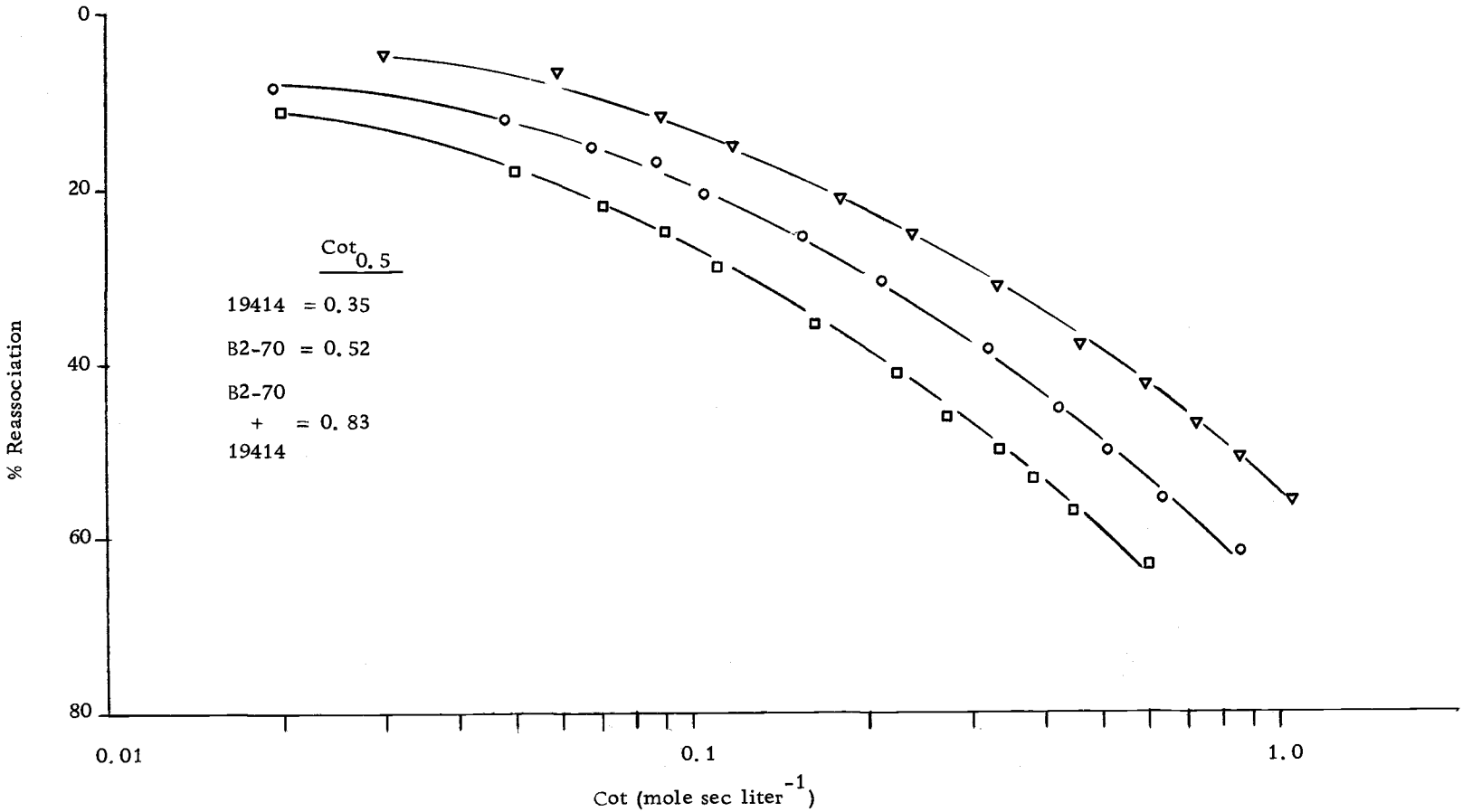

Figure 16. Cot (mole sec liter^{-1} vs. % reassociation for (O) Lactobacillus B2-70, (□) E. rhusiopathiae 19414 and (△) their mixture.

again that it has been misnamed. The results are plotted in Figure 17. The data for the relationship between L. casei var. casei and Lactobacillus MSH are identical as determined by both procedures. The remainder of the data in Table 21 show that the procedures support each other.

Figure 18 shows the optical density profile of Lactobacillus MSH DNA from the cesium chloride ethidium bromide density gradient. As can be seen, there is a definite satellite DNA peak along with the chromosomal DNA peak. This particular sample had added cold chromosomal DNA (DNA extracted as done for the homology experiments). The chromosomal peak extends between the 22nd and the 28th fractions, whereas the satellite peak extends between 11th and 14th fractions, suggesting the existence of extrachromosomal or plasmid DNA. Radioactivity of the fractions was assayed as described in Materials and Methods and also are plotted in the figure. This confirmed the presence of satellite DNA which was radioactive. To check for satellite DNA in the normally-extracted sample, a similar cesium chloride-ethidium bromide density gradient was run with cold DNA extracted as in the hybridization experiments. From Figure 19, it may be seen that only chromosomal DNA is present.

Once the satellite DNA peak fractions were established, they were collected, dialyzed and treated with RNase to remove any contaminating RNA. They then were deproteinized with Sevag's solution

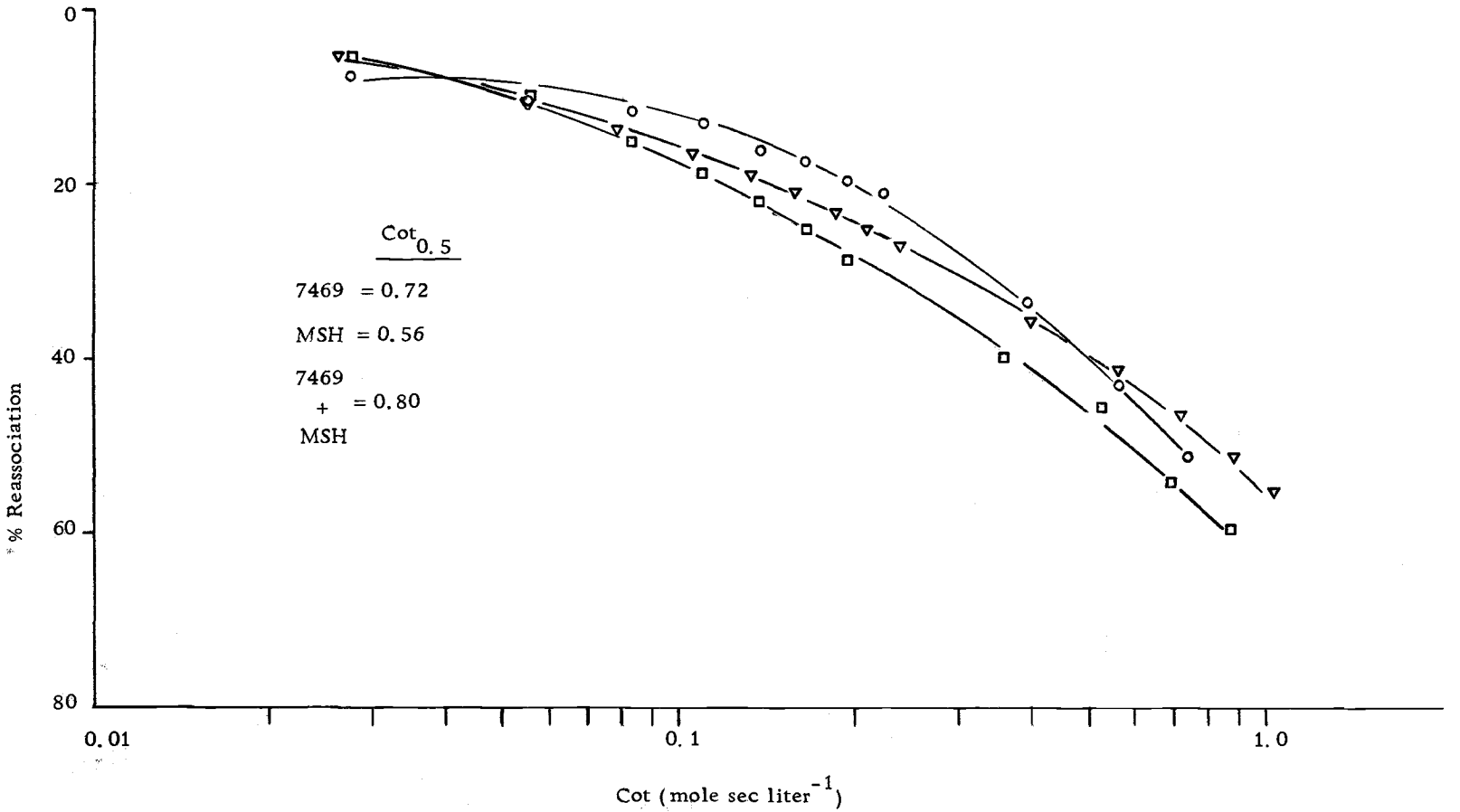

Figure 17. Cot (mole sec liter^{-1}) vs. % reassociation for (O) L. casei 7469, (□) Lactobacillus MSH and (△) the mixture of the two.

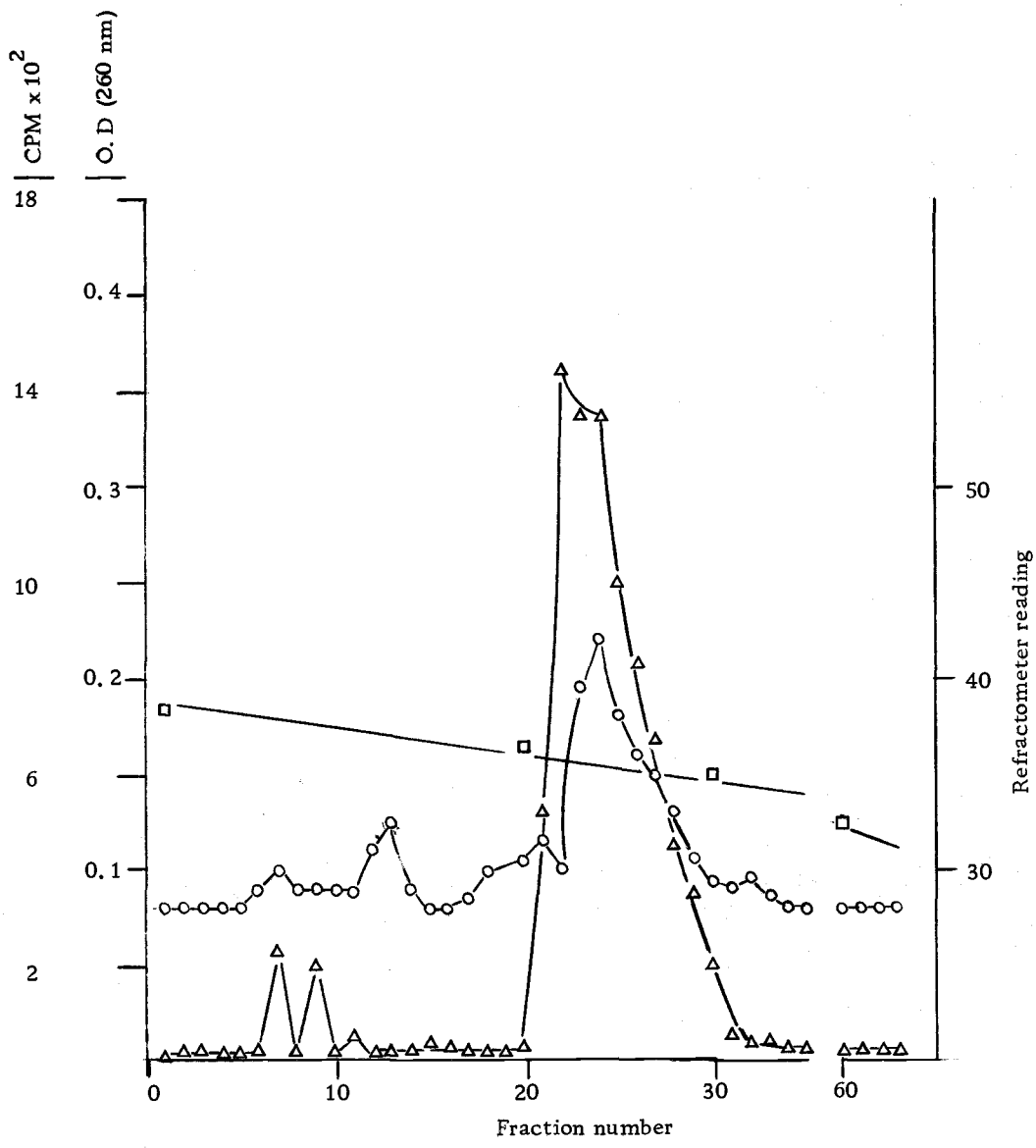

Figure 18. Optical density and Trichloroacetic acid precipitable radioactivity profiles of the fractions from cesium chloride ethidium bromide density gradient.

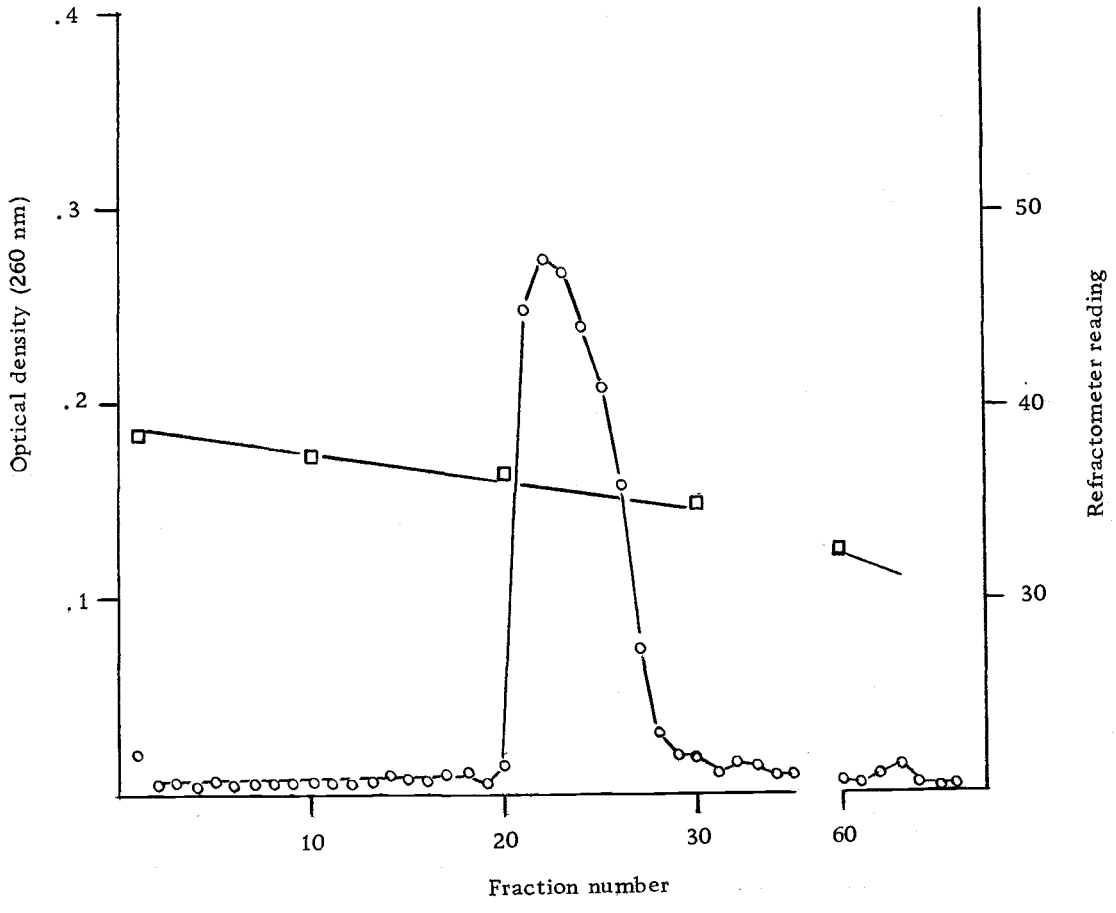

Figure 19. Optical density (260 nm) profile of the cesium chloride-ethidium bromide density gradient centrifugation fractions (cold DNA extracted as done for the hybridization experiments) from cesium chloride ethidium bromide density gradient.

and analyzed by sucrose gradients and agarose gel electrophoresis.

Neutral and alkaline sucrose gradients of the supernatant and pooled fractions from the CsCl-EtBr density gradient centrifugation exhibited an unusually broad and low peak for a plasmid. Also, it usually stayed on the top of the gradient even after three hours in the centrifuge. This led us to speculate the following:

a) the supernatant was contaminated with chromosomal DNA; this appeared evident from the CsCl-EtBr gradient in Figure 18.

b) the organism had more than two plasmids of various sizes.

c) the plasmid was too small for it to travel in the sucrose gradients used.

Since it was evident from the radioactivity assay plot of the density gradient centrifugation fractions that the supernatant was contaminated with chromosomal DNA, the supernatant fluids were subjected to density gradient purification before using for further analysis. Supernatants from normal (uncured) and cured L. plantarum MSH were then subjected to agarose gel electrophoresis. The results of the radioactivity assay of fractions are plotted in Figures 20 and 21.

In Figure 20 the sample consisted of supernatant fluid from uncured L. plantarum MSH. As a marker, ^{14}C-labelled bacteriophage lambda DNA was included. The samples were counted as described in the Materials and Methods section. It appears from Figure 20 that the lambda DNA migrated a little faster than the

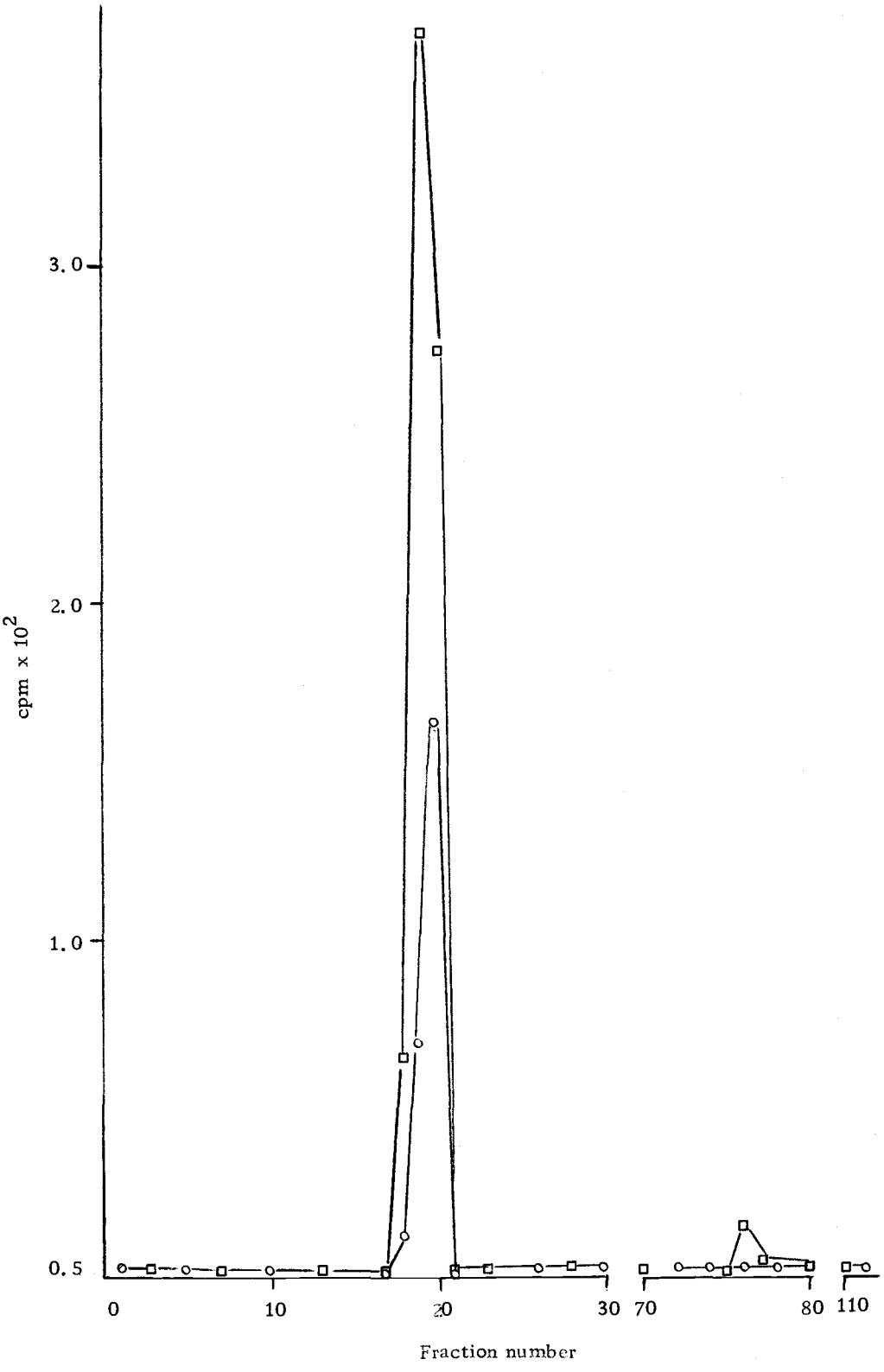

Figure 20. Radioactivity of fractions resulting from Gel electrophoresis: (□) ^{3}H-plasmid DNA, (O) ^{14}C-Lambda DNA.

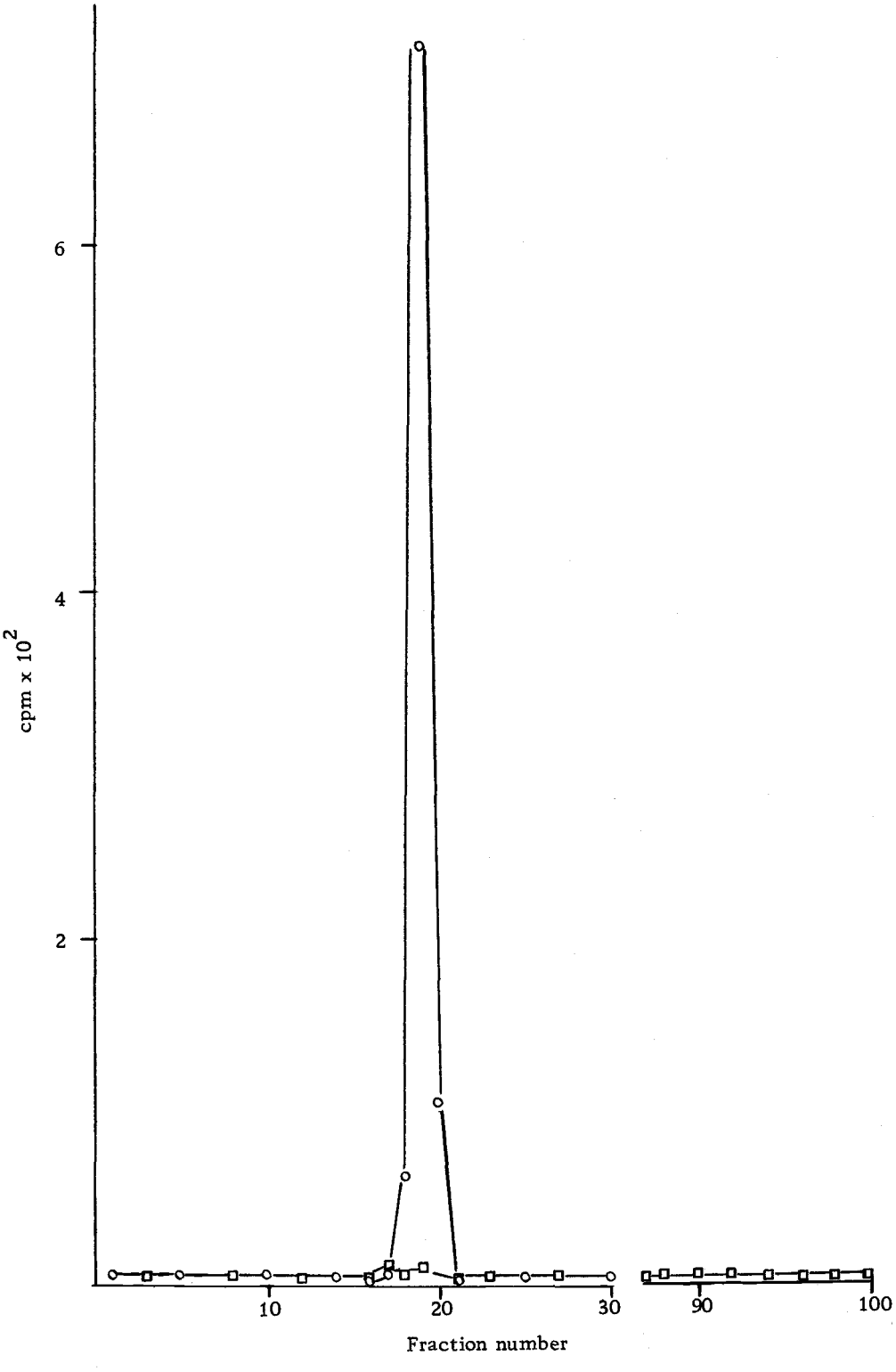

Figure 21. Radioactivity of fractions resulting from Gel electrophoresis: (O) ^{3}H-plasmid DNA, (□) ^{3}H-plasmid supernatant from cured L. plantarum MSH.

plasmid DNA, suggesting that the plasmid DNA is larger than lambda or that super-coiled plasmid DNA travels slower.

In Figure 21, radioactivity of two samples are plotted; one is the supernatant from uncured L. plantarum MSH and the second is the supernatant from cured MSH. Curing was accomplished by growing in different concentrations of ethidium bromide (Clowes, 1972). It may be seen that no peak resulted from the sample that had been cured, indicating that the plasmid has been eliminated by the ethidium bromide.

This effect appears more dramatic in pictures (Fig. 11) taken under ultra-violet light after incubation of the gels in a buffer containing ethidium bromide. While a faint band also appeared in the cured sample, it was not visible to the naked eye and may have been due to the closeness of the plasmid-containing gel, forming a reflection on the other gel. Fractions from the same sample did not yield any radioactivity but it is possible that the organism was not totally cured of all the plasmids.

In effort to determine characters the plasmid DNA may code for, cured as well as uncured L. plantarum MSH were subjected to a series of biochemical tests. The results indicated that none of the characters tested for were plasmid-mediated since both the cured and the uncured organisms behaved identical except for the partial fermentation of glycerol by the cured organism. The result of the

antibiotic sensitivity tests conducted on all the organisms used in the present study are tabulated in Table 22. The results of this experiment showed that the plasmid DNA did not code for resistance against any of nine drugs tested, suggesting that the plasmid was not an R factor.

Animal pathogenicity tests conducted on cured and uncured L. plantarum MSH indicated that the plasmid DNA might carry pathogenicity determinants; the LD_{50} increased from $5x10^8$ cells for the uncured sample to above $1x10^9$ cells for the cured sample. This is surely not a strong indication for the pathogenicity, since $5x10^8$ cells of most non-pathogens will cause disease symptoms when injected into mice.

An electron photomicrograph of the supernatant fluid from uncured strain MSH is shown in Figure 12. The plasmid DNA molecules are approximately 5000 A in length, which would allow for about 1500 base pairs and a molecular weight of about 1,000,000 daltons. This is somewhat small for a plasmid. Such a molecular weight would allow coding for only about 500 amino acids and provide genetic information for one or two average-sized proteins.

Table 22. Results of antibiotic sensitivity tests.

Organism ATCC or NCDO no.	Antibiotics used														
	PEN	S	S	AM	AM	CB	GM	C	C	TE	TE	FD	FD	K	K
11742	+	-	-	+	+	+	±	+	+	+	+	+	+	-	-
25258	+	±	+	+	+	+	+	+	+	+	+	+	+	±	-
4356	+	-	±	+	+	+	-	+	+	+	+	-	-	-	-
B2-70	±	-	-	-	+	+	-	+	+	+	+	+	+	-	-
LRPKI-70	+	-	-	+	+	+	-	+	+	+	+	+	+	-	-
8018	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
15009	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-
521	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
14917	+	-	-	+	+	+	-	+	+	+	+	-	-	-	-
8014	+	-	-	+	+	+	-	+	+	-	+	+	+	-	-
MSH	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
7469	+	-	-	+	+	+	-	+	+	+	+	+	+	-	-
473	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+
12315	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11842	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9649	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
4797	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
215	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+
927	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-
S. inulinus	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-

+ = sensitive

- = resistant

±= doubtful

Pen = Penicillin

CB - Carbenicillin 50 mcg

GM - gentamicin 10 mcg

AM = ampicillin 2 mcg

AM = ampicillin 10 mcg

C = chloromycitin 5 mcg

S = streptomycin 2 mcg

S = streptomycin 10 mcg

TE = tetracycline 5 mcg

TE = tetracycline 30 mcg

FD = furadantin 50 mcg

FD = furadantin 300 mcg

K = kanamycin 5 mcg

K = kanamycin 30 mcg

C = chloromycitin 30 mcg

DISCUSSION

The membrane filter technique of Gillespie and Spiegelman (1965) has been used recently for the determination of DNA homology by several workers (Denhardt, 1966; Legault et al., 1967; De Ley and Tijtgat, 1970; Moore and Hirsch, 1972; Dellaglio et al., 1973; Johnson, 1973). Pointing out the significance of the incubation temperature, Marmur and Doty (1961) concluded that a temperature 25 C below the T_m was optimal for DNA renaturation. Recently McConaughy et al. (1969) and Rogul et al. (1970) showed that leaching of the DNA from the incubating membrane filters used in competition experiments was markedly increased by the high temperature employed for stringent renaturation. To effect a reduction in the T_m, so that hybridization could be carried out at a lower temperature, Bonner et al. (1967) included formamide in the renaturation mixture. Later McConaughy et al. (1969) showed that this chemical would lower the T_m by 0.72 C and that increased specificity and renaturation rate could be achieved by the appropriate choice of temperature, formamide and salt concentrations.

In view of this work, experiments were carried out during the first phases of the present study to determine optimum hybridization conditions. These experiments indicated that 6xSSC containing 40% formamide was a suitable solvent for the hybridization at incubation

temperatures of 40 to 60 C. Additional experiments were conducted to determine the effect of this buffer and the incubation temperature on leaching, pH and the optical density. The results (Table 12) clearly showed that there was little or no leaching. While there was a slight fall in pH during the incubation, it remainted between 7 and 8 even after 48 h. The only significant change observed was an increase in optical density between 6 and 18 h of incubation, but even this stabilized after the initial increase; this did not interfere with the results since optical density was not used in measurements of competition.

Okanishi and Gregory (1970) attempted to find a method to prevent leaching of DNA from membrane filters during hybridization. However, leaching has not been a problem for all workers. For example, Gillespie and Spiegelman (1965), Moore and McCarthy (1969) and Moore and Hirsch (1972) did not experience leaching. Nevertheless, in the present study all precautions were taken to minimize it.

T_m determinations allow calculation of the average moles % CG content of DNA, and only bacteria having similar values may be related to each other. If there is a difference of 5 to 7 moles % GC content, the probability of two organisms having high homology is low, indicating that the organism in question belongs to a different species (Jones and Sneath, 1970). It was with these facts in mind that the species selection of lactobacilli was made for all the

hybridization experiments conducted. Since none of the heterofermentative lactobacilli have a GC content comparable to the heterofermentative sourdough bacteria, the low GC homofermentative organisms were used because of their similarity in GC content to the sourdough strains (Table 6). The average GC content of sourdough bacteria DNA was between 38 and 39 moles %. Therefore the choice for homology studies with these heterofermentative lactobacilli was L. viridescens and L. brevis with GC contents of 42.7 and 45% respectively. The other two species of the subgenus Betabacterium, L. cellobiosus and L. fermenti, are about 12 moles % GC higher and could not have any polynucleotide sequences in common with L. sanfrancisco; therefore no known heterofermentative lactobacilli are related to these bacteria (De Ley, 1969).

The high homology obtained among the four sourdough bacteria was not surprising. They have almost identical morphological, cultural and biochemical characteristics. The 23 to 37% homology shown between the four group I homofermentative lactobacilli and the sourdough bacteria indicate the capacity of the hybridization method to differentiate between genetically unrelated organisms with similar GC content. This becomes more evident from the still lower percent homology (6 to 32%) obtained between these organisms under stringent hybridization conditions. The slightly higher percent homology of 39% was obtained with heterofermentative L. brevis, suggesting the

possibility that their genetic homology, though not great is related to fermentation type.

There were no known streptobacteria with a GC content sufficiently similar to conduct hybridization experiments with the fish isolates B2-70 and LRPKI-70. They belonged to the low GC group of organisms with 36 moles % GC. According to the biochemical classification, they were members of the subgenus Streptobacterium, except for their ability to hydrolyze arginine. Therefore organisms of the low GC group were utilized in the competition experiments.

During the biochemical study of these organisms, it was realized that characters observed were very similar to those shown by bacteria of genus Erysipelothrix. In addition, the fish isolates were resistant to potassium tellurite, which is one of the salient characters of the family Corynebacteriacae in which Ersipelothrix resides. The only organism from this family which was catalase negative and had a similar GC content was E. insidiosa. Therefore it was also included in these experments.

High homology observed between the two fish isolates (B2-70 and LRPKI-70) were in agreement with the biochemical, cultural and morphological characters (Table 3). The apparent partial homologies of 20 to 45% with the low GC group of lactobacilli and the 14% homology observed with E. insidiosa, suggests that the fish isolates do belong to the genus Lactobacillus rather than to the genus

Erysipelothrix. Generally the homology values at the more stringent temperature of T_m - 15 C were lower compared to those at T_m - 25 C. This is in agreement with other workers' observations for unrelated organisms (Brenner et al., 1969, 1972a, b). The lack of high homology values by these organisms with similar GC content, suggests that the fish isolates B2-70 and LRPKI-70 are new species. The complete homology observed between these two organisms show that they both represent a single new species of the genus Lactobacillus. It is suggested that this organism be called L. piscium.

Lactobacilli have been traditionally classified according to their ability to ferment carbohydrate into only lactic acid (homofermenters) or lactic acid plus other end products (heterofermenters); this appears to be an unrealistic approach. Only a few more enzymes are required to produce acetate, ethanol and CO_2 from the carbohydrates. In addition, these enzymes could be coded for by a few genes constituting a small portion of the genome of the organism. Therefore only a fraction of the genome is considered when these organisms are classified on the basis of such selected phenotypic characters.

Genome size determinations (Tables 6, 7 and 8) indicated clearly that the lactobacilli have the capacity to code for approximately 1,000 to 1,200 enzymes of average size made up of about 300 amino acids. This is about half the genetic capability of E. coli. When we

realize that lactobacilli with such a strict nutritional dependency can still code for approximately 1,000 enzymes, it seems unwise to depend on only 50 or 60 odd characters in their classification. This is even more unwise when it is realized that some of the characters used actually may not be coded for by the organism.

Recently Wittenberger et al. (1971) and Brown and Wittenberger (1971) have shown that Streptococcus faecalis, a known homofermentative organism, has the enzymatic capability to carry out the heterofermentative degradation of sugar by the way of hexose monophosphate pathway. They have also shown that the heterofermentative pathway is repressed in whole cells by the glycolytic pathway intermediate fructose-1-6-diphosphate. It is conceivable, therefore that a homofermentative organism through mutation and selection in an unsuitable environment may lose the fructose-1-6-diphosphate control. This potential becomes evident since it has been shown that all homofermentative lactobacilli possess glucose-6-phosphate dehydrogenase (Williams, 1971), a key enzyme in the heterofermentative hexose monophosphate pathway.

Recently Sakaguchi et al. (1974) have shown some of the difficulties which are encountered using biochemical classification of organisms. They showed that a respiratory deficient mutant of E. coli (H7) physiologically resembled hemofermentative lactobacilli. The organism was negative for catalase and cytochromes, it grew

best in the absence of air (microaerophilic), it produced lactic acid even under aerobic conditions and it required amino acids for growth. It could only be identified as E. coli by DNA hybridization with the parent strain. The organism remained gram negative but it is well known that lactobacilli become gram negative during post-logarithmic growth. The authors also drew evolutionary implications from their findings, suggesting that lactobacilli may have evolved from E. coli.

These observations show how important it is to know the genetic capability of bacteria in determining their interrelationship. At present we do not have the ability to read directly all the information in the genetic material. The closest approach to this is what has been carried out in the present study.

L. salivarius 11742, revealing no or very slight polynucleotide sequence similarity with all the low GC group of organisms, showed that it is a legitimate species. Homology values for L. helveticus 15009 with L. jugurt 521 revealed the problem in using a single sugar fermentation reaction for differentiating species. L. jugurt is probably a name given to lactobacilli originally found in yogurt; later the differentiating sugar fermentation reaction (maltose) was identified. Even recently organisms have been classified on the basis of a few sugar fermentation reaction differences (Rogosa et al., 1953). Sharpe et al. (1973a) separated species of L. ruminis and L. vitulinus on the basis of cell wall peptidoglycan, motility, type

of lactic acid formed and DNA base composition. Base composition differences would be sufficient when one is differentiating only two organisms. But it has been shown that organisms with similar GC contents may or may not show any homology. Sharpe (1971) has warned against carrying subdivisions of species too far by separation on the basis of action on a single sugar. She has been able to produce a non-maltose fermenting variety of L. helveticus by UV irradiation. Such mutants would be indistinguishable from L. jugurt. It seems appropriate therefore to designate L. jugurt as a non-maltose fermenting variety of L. helveticus. This view is also supported by the results of Dellaglio et al. (1973) and others (Rogosa, 1970; Sharpe, 1971; Williams, 1971).

L. helveticus did not show any significant homology with all the type species of the low GC group of organisms tested, indicating that it is a justifiable species.

In the medium GC group of organisms, L. plantarum and L. casei var. casei (probably L. casei var. alactosus) have the most ideal disposition to be grouped as a subgenus. The hybridization data from this group clearly indicate the necessity for the creation of a new species for L. casei var. rhamnosus. Having shown only 20 to 40% homology with L. casei and L. plantarum it still belongs to the same subgenus. This is at least partially supported by the sugar fermentation pattern and the cell wall antigens. Here the value

of the hybridization results becomes obvious from the fact that they separate species of organisms with very similar GC contents which share some of the same biochemical characters.

Johnson (1973) has shown that L. casei var. casei and L. casei var. alactosus are genetically very similar with an average of 80% homology, suggesting that these two organisms are the same species. He has also shown that L. casei var. casei has about 35% homology with L. casei var. rhamnosus. Present homology results of 15 to 20% competition between these organisms under stringent conditions of hybridization show that L. casei var. rhamnosus indeed is a new species. Williams (1971) stated that L. fermenti and L. cellobiosus are alike in many biochemical properties. In taxonomic tables commonly used to identify species of Betabacterium (Sharpe et al., 1966), these species differ in five of 16 features. Two of these five are temperature relationships, which Leifson (1966) regards as of little taxonomic value compared with tests such as carbohydrate fermentation. By contrast, strains of Streptobacterium that differ by five of 15 features are regarded as varieties of L. casei rather than as a separate speceis. The present observations and the results of Johnson (1973) suggest that the above is true and that L. casei var. rhamnosus needs to be a new species; it should be called L. rhamnosus.

The results of the hybridization studies involving L. lactis,

L. bulgaricus, L. leichmanii and L. delbrueckii show that they are highly related to each other genetically and probably belong to a single species rather than to the four species as at present. They are very similar in their biochemical properties except for their sugar fermentation reactions and vitamin requirements. It would seem appropriate to designate L. leichmanii as the type species because of its ability to ferment more sugars than the other three. Rogosa (1970) expressed the same view, suspecting the validity of the four species based on biochemical patterns. Miller et al. (1971) showed that L. leichmanii and L. lactis were highly related to each other by the DNA-RNA hybridization method. Though the lower values revealed between L. leichmanii and L. delbrueckii are not in close agreement with the present observations, it should be noted that the present method of homology determinations was by DNA-DNA hybridization and not DNA-RNA. Furthermore, results of the present study are supported, at least partially, by the findings of Simonds et al. (1971) and Dellaglio et al. (1973). These workers showed that L. lactis and L. bulgaricus were highly related to each other genetically.

In the heterofermentative group, L. brevis appears to be a distinct species revealing little homology with all the organisms of similar GC content. On the other hand, L. fermenti and L. cellobiosus appear to be the same species with high genetic homology.

The biochemical patterns also are very similar, except for sugar fermentations. Miller (1971) showed that these organisms were related by DNA-RNA hybridization. Williams (1971), while studying cell wall composition and enzymology of lactobacilli, suggested that they may belong to a single species.

All these observations point out one thing: that the genus Lactobacillus need extensive taxonomic study using procedures to determine genetic homology between as well as among species to derive a workable classification for the future. In this respect it seems appropriate to mention the observation made long ago by Orla-Jensen: "Old species names are just labels."

It should be noted that each of the three GC groups of lactobacilli contained homofermentative as well as heterofermentative types. This emphasizes that in the future, all new Lactobacillus isolates will need to be classified on the basis of their genetic relatedness rather than on the basis of only their biochemical characteristics.

The homology values obtained in this study for lactobacilli by renaturation kinetics confirmed values obtained by the membrane method. Both types of data indicated that L. plantarum MSH is closely related to L. casei var. rhamnosus; also biochemical properties for this organism were quite similar to those of L. casei var. rhamnosus. Since L. casei var. rhamnosus itself is a new species, it is suggested that it be called L. rhamnosus.

The DNA analysis of L. plantarum MSH suggested the existence of at least one plasmid at $1x10^6$ daltons in molecular weight. Though small in size, it would code for a couple of proteins. While the animal studies suggested that the plasmid may carry pathogenicity determinants, further work is needed to confirm this. It is interesting to consider the potential pathogenicity of lactobacilli. Their association with dental caries (Canby and Bernier, 1936; Steinle et al., 1967) and avitaminosis is well known, but their role as invasive pathogenic organisms is open to question. It also seems desirable from an aesthetic point of view to question their pathogenic nature, since they are widely used in food fermentations.

Axelrod et al. (1973) document the first known case of endocarditis caused in U.S.A., by an organism they called L. plantarum, but which was shown in the present studies to be a strain of L. rhamnosus. Other cases of endocarditis caused by lactobacilli have been reported in other countries (Marschall, 1938; Biocca and Sepilli, 1947; Sharpe et al., 1973b).

Recently Sharpe et al. (1973b) documented several cases in which lactobacilli were shown to be pathogenic. It should be noted that almost all the cases reported so far were in individuals with a history of congenital heart disease or previously damaged hearts or valves associated with dental infections. Some of the patients may have been on cortisone and antibiotics for a fairly long time. From this, it

becomes clear that lactobacilli, in rare conditions, become pathogenic and set up infections in debilitated patients. Therefore, healthy individuals should not be concerned about consuming the many fermented foods which depend on growth of lactobacilli for their production.

It has been the practice of microbiologists to use Bergey's Manual of Determinative Bacteriology (1957) as the authority in determining the classification of a particular organism. Presently a new eight edition is being prepared, which reportedly will contain many revisions, especially those based on genetic considerations such as presented in this thesis. The present edition of Bergey s Manual (1957) gives the following information on classification for the genus Lactobacillus:

Gram positive, non-motile, non-sporeforming rods. They are microaerophilic and do not reduce nitrate. They are catalase negative and ferment sugars to lactic acid or lactic acid, acetic acid, acetaldehyde, ethanol and carbon dioxide. They are fastidious and are found in the mouth and intestinal tract of man and other animals. They are also found in food and dairy products and in fermenting vegetables and plant products.

Key to the species of genus Lactobacillus

I. Homofermentative, producing only traces of end-products other than lactic acid from glucose. Sub-genus Lactobacillus Beijerinck (Arch. neetl. d. sci. exact. et nat., Haarlem, Ser. 2, 7, 1901, 212).

A. Optimum temperature, between 37° and 60° C. or higher.

1. Produce acid from lactose.

a. Optimum temperature, between 37° and 45° C.

1. Produce acid from lactose.

a. Optimum temperature, between 37° and 45° C.

b. Produce levo rotatory lactic acid.

1. Lactobacillus caucasicus.

2. Lactobacillus lactis.

bb. Produce optically inactive or dextro rotatory lactic acid.

c. Microaerophilic.

3. Lactobacillus helveticus.

4. Lactobacillus acidophilus.

cc. Anaerobic in freshly isolated cultures.

5. Lactobacillus bifidus.

aa. Optimum temperature, between 45° and 62° C.; usually no acid from maltose.

6. Lactobacillus bulgaricus.

7. Lactobacillus thermophilus.

2. Does not produce acid from lactose.

8. Lactobacillus delbrueckii.

B. Optimum temperature, between 28° and 32° C.

1. Produce optically active lactic acid.

a. Produces dextro rotatory lactic acid. Often prefers lactose to sucrose and maltose.

9. Lactobacillus casei.

aa. Produces levo rotatory lactic acid.

10. Lactobacillus leichmanii

2. Produces optically inactive lactic acid.

11. Lactobacillus plantarum

II. Heterofermentative, producing considerable amounts of end-products other than lactic acid from glucose (carbon dioxide, alcohol and acetic acid; mannitol from fructose). Sub-genus Saccharobacillus van Laer (Contribution a l'Histoire des Ferments des Hydrates de Carbone. Mem. Acad. Royale de Belgique, 47, 1892, 5).

A. Optimum temperature, between 28° and 32° C. Usually ferment arabinose.

1. Ferment raffinose, sucrose and lactose.

12. Lactobacillus pastorianus.

13. Lactobacillus buchneri.

2. Does not ferment raffinose and often does not ferment sucrose or lactose.

14. Lactobacillus brevis.

B. Optimum temperature, between 35° and 40° C. or higher. Usually does not ferment arabinose.

15. Lactobacillus fermenti.

The present thesis suggests a classification system significantly different from the traditional scheme based on biochemical and cultural properties. The new system is based on DNA GC content, genome size, homology data and some of the important biochemical characteristics as follows:

Genus Lactobacillus:

Gram positive, non-motile, non-sporeforming rods with a GC content between 33 and 50 moles percent. They are microaerophilic organisms with a genome size of approximately 800 to 1, 200 million daltons. They are catalase negative and do not reduce nitrate. They are fastidious. Speciation is determined by DNA homology, type of lactate isomer produced and resistance to 0.05% potassium tellurite. The genus is divided into three groups on the basis of GC content. Each

group is further divided into homofermentative and hetero-fermentative organisms.

Group I. Organisms with 33 to 39 moles % GC content.

a. Homofermentative.

1. Resistant to 0.05% potassium tellurite; produces L(+) lactic acid.

Lactobacillus piscium.

2. Not resistant to 0.05% potassium tellurite; produces D(-) lactic acid.

Lactobacillus jensenii

3. Not resistant to 0.05% potassium tellurite; produces DL lactic acid; DNA homology data required.

Lactobacillus helveticus

Lactobacillus salivarius

Lactobacillus acidophilus

b. Heterofermentative.

Lactobacillus sanfrancisco

Group II. Organisms with 42 to 48 moles % GC content.

a. Homofermentative.

1. produces DL lactic acid.

Lactobacillus plantarum.

2. Produces L(+) lactic acid:

DNA homology data required.

Lactobacillus casei

Lactobacillus rhamnosus

b. Heterofermentative:

DNA homology data required.

Lactobacillus brevis

Lactobacillus viridescens

Group III. Organisms with 48.4 to 50 moles % GC content.

a. Homofermentative; DNA homology data required.

Lactobacillus leichmanii

b. Heterofermentative; DNA homology data required.

Lactobacillus fermenti

Varieties of species are not listed in the new classification scheme but would be defined in narrative accompanying the genus description. The taxonomy of some lactobacilli such as *L. postorianus*, a common beer contaminant, would require further study by nucleic acid hybridization to determine their true identity.

SUMMARY

Results of the deoxyribonucleic acid composition studies, genome size determinations and DNA-DNA hybridization experiments with type, neotype and stock strains of species of the genus Lactobacillus showed the following:

1) L. salivarius, L. sanfrancisco and L. piscium are distinct species in the low GC group of organisms.

2) L. helveticus and L. jugurt are the same species and L. jugurt is a maltose negative variant of L. helveticus.

3) L. casei var. casei and L. casei var. rhamnosus are two different species rather than varieties of a single species.

4) L. plantarum and L. casei var. casei have 20 to 40% homology and belong to a single group as described at present. L. casei var. rhamnosus should be called L. rhamnosus; it showed 20% homology within the group.

5) L. lactis, L. bulgaricus, L. delbrueckii and L. leichmanii are genetically as variable as different isolates of E. coli. L. leichmanii should be named the type species as it ferments the most sugars; the other three should be given varietal status.

6) L. fermenti and L. cellobiosus are the same species. L. cellobiosus appears to be a cellobiose fermenting variety of L. fermenti.

These studies support the division of the genus, on the basis of the DNA GC content, into low GC (33.1 to 39.7), intermediate GC (44.0 to 48.0) and high GC (48.4 to 50.0) groups. In addition the data indicate that species consolidation is justified and that workers in the field should include GC values, genome sizes and DNA homology data along with biochemical and other characteristics in describing a new species.

Finally, plasmid isolation procedures revealed the presence of super coiled DNA, one million daltons in size, in L. rhamnosus MSH, an organism which caused endocarditis in man.

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