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Abstract approved: _____
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The base composition of deoxyribonucleates from recognized species and strains of lactobacilli was determined by thermal denaturation. Compositional heterogeneity within the genus Lactobacillus was evident, since deoxyribonucleic acid (DNA) base compositions ranged from 32.4 to 51.9 moles % guanine plus cytosine (GC). The data also substantiated and complemented results previously obtained by chemical and buoyant density methods.

Lactobacilli were placed in three groups on the basis of moles % GC content. Group I included species with a GC content between 32.4 and 38.3 moles %; Lactobacillus jugurti, L. helveticus, L. salivarius, L. (jugurti) bulgaricus and L. bulgaricus. Group II included species with a GC content between 42.7 and 48.0 moles %; L. buchneri, L. brevis, L. casei, L. viridescens and L. plantarum. Group III included species with a GC content between 49.0 and 51.9 moles %; L. lactis, L. leichmannii, L. delbrueckii, L. fermenti

and L. cellobiosus.

Further distinctions within each of the above groups were apparent when the results of DNA-ribonucleic acid (RNA) hybridizations were assessed. The following classification, consisting of six homofermentative and two heterofermentative subgroups, was suggested. Group I contained (a) L. helveticus, L. jugurti, L. (jugurti) bulgaricus and L. bulgaricus ATCC 12278, which was indistinguishable from L. helveticus, and (b) L. salivarius. Group II contained (a) L. casei, (b) L. plantarum and (c) L. buchneri, which was further subdivided into (c_1) L. brevis and (c_2) L. viridescens. Group III contained (a) L. fermenti and L. cellobiosus and (b) L. lactis, L. leichmannii and L. delbrueckii. L. acidophilus was tentatively placed in Group III as an additional homofermentative subgroup.

The division of lactobacilli indicated above agreed in many respects with previously reported classifications based on serological reactions and physiological characteristics. Serologically distinct species were easily distinguished by the homology technique employed. Although a majority of known species of lactobacilli was examined, further investigations with additional strains are needed before a definite classification scheme can be proposed. Nevertheless, since authentic strains of a well established species will invariably show the same degree of genetic homology, the data constitute a firm foundation for species identification and classification.

The possibility of a phylogenetic relationship between selected lactobacilli and Bacillus coagulans was discussed also. Tritiated RNA, synthesized using B. coagulans ATCC 8038 DNA as primer, was reacted with 8038 DNA and DNA from L. casei, L. plantarum and Sporolactobacillus inulinus. No appreciable cross reactions were observed for the latter three strains. This obvious lack of DNA homology appeared to preclude any possibility of a phylogenetic relationship. However, evolutionary development may have followed a number of independent paths, each resulting in extensive changes in polynucleotide sequence from a primary DNA of a particular composition.

Several species and strains of lactobacilli, B. coagulans and S. inulinus produced detectable amounts of extracellular nuclease. All species capable of depolymerizing DNA also hydrolyzed RNA. Further investigation with purified enzyme preparations was suggested to determine whether the enzymatic activity observed was the result of two distinct enzymes or a single enzyme, such as a non-specific phosphodiesterase.

Deoxyribonucleic Acid Homology in the Genus *Lactobacillus*

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DEOXYRIBONUCLEIC ACID HOMOLOGY IN THE GENUS LACTOBACILLUS

INTRODUCTION

Orla-Jensen (51) divided the genus Lactobacillus into three subgenera: Thermobacterium, Streptobacterium and Betabacterium on the basis of results obtained from nutritional and physiological studies. In general, this classification has been substantiated by other methods such as computer-aided numerical analysis (57) and infra-red spectroscopy (36). Additional physiological (7) and serological studies (58, 60), as well as chromatographic analyses (16, 17, 18), have also aided in species separation and identification.

Methods of deoxyribonucleic acid (DNA) base composition analyses are also recognized as valuable taxonomic tools and the literature is replete with information on bacterial DNA base compositions. The DNA-base composition of the genus Lactobacillus has been studied extensively by several investigators (9-13, 20, 33, 34), however, substantial differences were observed when data obtained by chemical analyses, buoyant density measurements and by thermal denaturation were compared. Therefore, preliminary thermal denaturation studies on the base composition of recognized species of lactobacilli were conducted to determine the extent of homology in their DNA structure and to clarify, if possible, the variations previously

obtained using different methodologies.

Nucleic acid homology methods, based on DNA-DNA or DNA-ribonucleic acid (RNA) interactions, have proven useful in bacterial systematics by enabling comparisons of various bacteria at a molecular level. Since these methods involve the bacterial genome, they are of unlimited value for the confirmation of other methods commonly used in classification. The literature contains a wealth of information on bacterial polynucleotide homology, however, no such data are, at present, available for species of the genus Lactobacillus. Therefore, DNA-RNA hybridization techniques were employed in this study to examine possible genetic interrelationships among recognized species of lactobacilli. It was anticipated that the results obtained from thermal denaturation and DNA-RNA hybridization studies would further complement the literature available with respect to species identification and classification.

HISTORICAL REVIEW

Taxonomy of the Lactobacilli

Orla-Jensen (51) divided the genus Lactobacillus into three subgenera: Thermobacterium, Streptobacterium and Betabacterium on the basis of optical type of lactic acid produced, sugar fermentations, the temperature range of growth and nitrogen requirements. The thermobacteria and streptobacteria are homofermentative and display differences in the temperature range of growth while the betabacteria are heterofermentative. For species contained in each of the three subgenera and differentiating characteristics, the reader is referred to Sharpe (59) and Rogosa and Sharpe (54). The current recognition of species in the genus Lactobacillus is based essentially on the classical monograph of Orla-Jensen (51) with recent improvements and modifications by Rogosa and Sharpe (54). Other aspects of classification are cited by Davis (23) and a comprehensive survey of lactobacilli, with special reference to taxonomy, was compiled by Briggs and Briggs (8).

Methods of Classification

Cultural and Biochemical Characteristics

Davis (22) proposed a classification scheme for lactobacillus

strains based on selected morphological and physiological taxonomic criteria. The strains examined were classified into five species: Lactobacillus acidophilus, L. casei, L. plantarum, L. fermenti and L. brevis. The data presented by Davis (22) were in close agreement with those of Rogosa et al. (55).

Briggs (7) classified representative species of lactobacilli into eight groups (six homofermentative and two heterofermentative) on the basis of the results of six physiological tests. Group I contained named cultures of L. acidophilus, L. bulgaricus and L. delbrueckii. Group II contained strains of L. acidophilus and L. bulgaricus. Group III contained L. bulgaricus, L. helveticus, L. lactis and L. delbrueckii. Group IV contained L. casei and L. helveticus. Group V contained L. lactis, L. leichmannii and L. thermophilus. Group VI contained L. casei and L. plantarum. Group VII contained L. brevis, L. buchneri and additional heterofermentative species. Group VIII contained L. fermenti. Homofermentative groups I, II and III, though separated from the remaining groups, were not clearly distinct from each other.

Wheater (66) reported the differentiating characteristics of L. plantarum, L. helveticus and L. casei as well as those of L. acidophilus and L. bulgaricus (67) and showed that the corresponding physiological groups of Briggs (7) were generally consistent with species differentiation in the thermobacteria and streptobacteria.

Serological Reactions

Williams (68) identified four major antigenic components (A, B, C, and D) in oral lactobacilli by use of monospecific sera in agglutination tests. Orland (52), also using agglutinin-adsorption, extended the antigenic analyses and observed additional antigens which he designated G, H and I. The presence of a common antigen F was also recognized. Miller and Hansen (44) observed an antigen K in several strains of L. lactis (Orla-Jensen) Holland which so far has not been reported in any other species of lactobacilli. It was different from the antigens A, B, C, D, E, F, G, H, and I found by Williams (68) and Orland (52). A new terminology for designating antigenic components of homofermentative and heterofermentative lactobacilli was proposed by Williams, Norris and György (69).

The application of agglutination reactions to the classification of lactobacilli has several limitations: auto-agglutination of antigen suspensions, a low degree of immunogenicity of many strains and irregular cross reactions (71). The latter was explained by the presence of a masking substance or envelope which inhibited agglutination but did not affect the immunogenic properties of the cell. The utilization of "unmasked" cells as tube antigens, to increase the sensitivity of agglutination tests, was suggested.

Representative species of lactobacilli were also studied

serologically by Sharpe (58). The majority of species were classified by precipitin tests into six groups and one subgroup: (1) L. bulgaricus, (2) L. casei-helveticus, (3) L. casei, (4) L. fermenti, (5) L. plantarum and (6) L. lactis, which also contained subgroup L. brevis. This classification was in close agreement with that of Briggs (7). Each serological group was almost entirely represented in one physiological group, except that L. bulgaricus serological group organisms were distributed over Briggs' physiological groups I, II and III and strains in the L. casei-helveticus serological group occurred in physiological groups IV and VI. Several physiologically defined species: L. delbrueckii, L. acidophilus, L. leichmannii and L. jugurti, still remain unclassified serologically because of difficulties encountered in the preparation of group sera. Efthymiou and Hansen (29) observed the absence of a common group-specific component for several strains of L. acidophilus, however, a definite pattern of cross reactivity exhibited by certain strains revealed the presence of common antigens. Apparently, L. acidophilus does not represent a species which can be regarded as serologically homogeneous.

Little agreement between the precipitin data of Sharpe (58) and the agglutination data of Orland (52) was observed. Agglutination tests suggested a large number of heterologous organisms and were considered to indicate type relationships; precipitin tests, however, indicated broader groups, usually compatible with physiological

characteristics.

Sharpe and Wheater (60) proposed the following nomenclature for serological groups of lactobacilli:

New Designation	Former Designation[a]	Species Included in Group
Group A	L. bulgaricus	L. bulgaricus, L. helveticus
Group B	L. casei	L. casei
Group C	L. casei-helveticus	L. casei, L. casei var. rhamnosus
Group D	L. plantarum	L. plantarum
Group E	L. lactis-brevis	L. lactis, subgroup L. brevis
Group F	L. fermenti	L. fermenti

[a]As given in Sharpe (58).

Results obtained by Coetzee, de Klerk and Sacks (19), utilizing phage-typing techniques, appeared to confirm some of the serological relationships encountered by Sharpe (58) and Sharpe and Wheater (60). L. casei phages 300 and 316 and L. casei var. rhamnosus phage 356 acted on different strains of L. casei, L. casei var. rhamnosus and L. plantarum. The former two phages also acted on various L. fermenti strains, thereby complementing the weak serological relationship among L. fermenti, L. casei and L. casei var. rhamnosus strains, previously reported by Sharpe (58).

Infra-Red Spectroscopy

Goulden and Sharpe (36) examined the infra-red absorption spectra of recognized species of lactobacilli and found that the absorption patterns divided them into three groups corresponding to the three subgenera of Orla-Jensen (51). Strains, possessing the X-type of spectrum, were divided into the XL-type; containing L. acidophilus, L. lactis and L. delbrueckii and the XM-type; containing L. bulgaricus and L. helveticus. The Y-type of spectrum was also divided into species; L. plantarum, YLa and L. casei, YLb. Type YM contained L. brevis and L. fermenti. Infra-red absorption patterns also revealed that organisms with the X-type spectrum contained relatively larger amounts of nucleic acid than those possessing the Y-type.

Numerical Analysis

Seyfried (57), using computer-aided numerical analysis, divided several species of lactobacilli into three major taxonomic groups which corresponded to the subgenera described by Orla-Jensen (51). The first group contained six of the Thermobacterium species: L. bulgaricus, L. helveticus, L. lactis, L. acidophilus, L. delbrueckii and L. leichmannii. The second group corresponded to the subgenus Betabacterium and included L. viridescens, L. brevis and L. fermenti. The third group contained the following streptobacteria:

L. plantarum, L. casei and L. pentosus.

Barre (4) established taxonomic relationships between 65 strains of lactobacilli isolated from wines and four named cultures by adonsonian analysis. Seven 80 phenons and two 72 phenons were described; the latter included the subgenera Streptobacterium and Betabacterium. Two 80 phenons corresponded to the species L. casei and L. plantarum, however, the other 80 phenons of more than one strain did not correspond to any known species.

Chromatography

Cummins and Harris (21) examined hydrolysates of cell wall preparations from several strains of lactobacilli by paper chromatography. The amino acids present in the cell wall were characteristic of the genus and the relative proportions of the sugars and hexosamines appeared to differentiate the individual species within the genus.

Baddiley and Davison (3) investigated the occurrence of teichoic acids in the cell walls and cell contents of lactobacilli. The nature of the teichoic acid present was determined by extracting with dilute trichloroacetic acid, precipitating with ethyl alcohol and identifying the precipitated polymer by hydrolysis to ribitol or glycerol phosphates and other recognizable degradation products. The serological groups of organisms studied were compared with the teichoic acid of their

cell walls. The serological designations used were those of Sharpe (58) and Sharpe and Wheater (60). L. arabinosus 17-5 and L. plantarum, serological group D, contained ribitol teichoic acid in their walls. Group E organisms; L. brevis, L. buchneri and L. lactis, as well as group A organisms; L. bulgaricus and L. helveticus, contained a glycerol teichoic acid. Groups B, C and F contained no cell wall teichoic acid. Therefore, the type of teichoic acid in the cell walls appeared to correlate with available serological data and provided a preliminary means of classification.

Other chromatographic techniques have also been used in the differentiation and identification of species and strains of lactobacilli (16-18). Amino acids and peptides are extracted from cells with dilute acetic acid and the extracts subjected to two-dimensional paper partition chromatography. The resultant chromatographic patterns are used for species differentiation. Species in the subgenus Thermobacterium were distinguished by different distributions of amino acids. Bottazzi (6) indicated a possible separation of L. lactis from L. acidophilus, L. bulgaricus and L. helveticus. Alanine was always stronger than threonine for the latter three strains and was equal to or less than threonine for L. lactis. It was not possible to differentiate between L. bulgaricus and L. helveticus. Several strains of L. leichmannii gave chromatograms which differed slightly from the L. lactis patterns (61) and L. salivarius was considered closer to L. casei

than to L. lactis.

With reference to the streptobacteria, L. casei was differentiated from L. plantarum by the absence of valine, leucine and tyrosine in the latter organism (17). In addition, the serological division of L. casei into groups B and C could generally be recognized chromatographically.

Distinctions among the heterofermentative species, betabacteria, were not clear (18). No distinction could be made between L. brevis and L. buchneri nor was any pattern relationship shown with any of the other fermentation groups. Nevertheless, distinct species patterns were often observed.

Deoxyribonucleic Acid (DNA) Base Composition

Methods of DNA base composition analyses have been recognized as valuable taxonomic tools and the literature contains considerable information on bacterial DNA base composition.

The DNA base composition of the genus Lactobacillus has been studied by several investigators (9-13, 20, 33, 34), however, substantial differences were observed when data obtained by chemical analyses, buoyant density measurements and by thermal denaturation were compared.

Gasser and Mandel (33) compared the results of buoyant density determinations of DNA from known species of lactobacilli with results

previously obtained by chemical analysis (34). They established a definite linearity between the buoyant density of DNA in CsCl and the guanine plus cytosine (GC) content determined by chemical methods. In addition, when GC content values derived from buoyant density measurements were compared with those previously reported and determined by thermal denaturation (9-13), several significant variations were observed. These variations were attributed to the use of different strains, improper species designation or inaccuracies in the physical determinations.

Recently, Mandel et al. (40) demonstrated a linear relationship between the denaturation temperature (T_m) and the buoyant density in CsCl for samples of double-stranded bacterial DNA, having GC contents in the range of 27 to 72 moles %.

Gasser and Sebald (34) placed recognized species of lactobacilli in three groups on the basis of chemical determinations of DNA base composition:

Group I included species with a GC content between 33 and 37.9 moles %; L. jugurti, L. helveticus, L. salivarius and L. acidophilus.

Group II included species with a GC content between 42 and 47 moles %; L. buchneri, L. brevis, L. casei and L. plantarum.

Group III included species with a GC content between 47 and 53 moles %; L. bulgaricus, L. lactis, L. leichmannii, L. delbrueckii,

L. fermenti and L. cellobiosus.

The chemical data indicated certain subgroups within each of the above major groups, but the ranges for the strains of different species appeared to overlap, so it was difficult to make further subdivisions with certainty. However, the buoyant density results (33) permitted further distinctions within each group.

Cantoni, Hill and Silvestri (12) examined the DNA base composition of strains of lactobacilli belonging to the subgenera Betabacterium and Streptobacterium by thermal denaturation. Base composition of the streptobacteria ranged from 43 to 47.5 moles % GC. The L. casei strains had very similar values, 46 to 47.5 moles %; whereas L. plantarum, 43 moles %, was considered appreciably different. Additional data on L. plantarum have been reported by Cantoni (11). Values for strains of betabacteria ranged from 44 to 51.5 moles % GC. Since a variability in DNA base composition within both subgenera was apparent, the authors indicated that a distinction on the basis of DNA composition between the two subgenera recognized in traditional classifications could not be made. Similar studies by Cantoni (9, 10) indicated DNA base composition heterogeneity within the genus Lactobacillus. Heterogeneity was also observed by Gasser and Sebald (34) and Gasser and Mandel (33).

The relationship between DNA base composition and temperature range of growth was investigated by Craveri, Manachini and

Cantoni (20). A correlation between moles % GC values and minimum temperatures of growth was noted. Strains of the subgenus Thermobacterium, which had minimum growth temperatures of approximately 20 C, possessed DNA of lower GC content than those of the Betabacterium and Streptobacterium, which had minimum growth temperatures of approximately 10 C.

Nucleic acid homology methods have proven useful in bacterial systematics by enabling comparisons of various bacteria at a molecular level. Since these methods involve the bacterial genome, they are of unlimited value for the confirmation of other methods commonly used in classification. Therefore, DNA-RNA hybridization techniques were employed in this study to examine possible genetic interrelationships among recognized species of lactobacilli.

MATERIALS AND METHODS

Organisms

Representative strains of lactobacilli and Bacillus coagulans were obtained from the stock culture collection of the Department of Microbiology, Oregon State University. Additional lactic strains were kindly contributed by M. Elizabeth Sharpe, National Institute for Research in Dairying, University of Reading, England. Sporolactobacillus inulinus was a gift from K. Kitahara and J. Suzuki, Tokyo University of Agriculture, Setagaya-ku, Tokyo, Japan. Lactobacillus acidophilus IFO 3532 was received from the culture collection of the Institute for Fermentation; Osaka, Japan, during the latter phases of this research. All cultures were plated for purity and selected isolates reevaluated as to species designation by the methods of Rogosa et al. (55), Rogosa and Sharpe (54) and by standard bacteriological procedures (62).

Culture Conditions and Media

All lactobacilli were grown in MRS-medium (25) at their respective optimum growth temperatures, 32 or 37 C. B. coagulans and S. inulinus were grown in lactic broth (30) at 37 C. All cells were collected during the early stationary phase of growth by

centrifugation at 12,000 x g, washed several times with distilled water and stored in pellet form at -25 C until needed.

Isolation of Deoxyribonucleic Acid (DNA)

The method of DNA extraction and purification was essentially the same as that described by Marmur (41) with minor modifications. L. helveticus, B. coagulans and S. inulinus were susceptible to lysis by lysozyme (2-4 mg/ml) followed by the addition of 2 ml of a 25% solution of sodium lauryl sulfate (w/v). The remaining lactobacilli could not be lysed by the above procedure, but were lysed substantially by a dual enzyme system containing lysozyme and lytase (Baltimore Biological Laboratory). When necessary, 3-5 g of cells (wet weight) were heated at 70 C for 30-60 minutes in 20 ml of saline-ethylenediaminetetraacetate (0.15 M NaCl plus 0.1 M EDTA, pH 8.0) and cooled to room temperature. Lysozyme (2 mg/ml) and lytase, reconstituted to 5 ml with NaCl-EDTA, were added and incubation continued at 37 C for 24-48 hours. If no increase in viscosity was evident, the heat-treatment and addition of enzymes were repeated. Viscous suspensions were then treated with sodium lauryl sulfate as indicated above.

The lysate was then heated in a water bath at 60 C for 10 minutes to inactivate any deoxyribonuclease present and allowed to cool to room temperature. The sodium ion concentration was adjusted to

1 M by the addition of 5 M sodium perchlorate to facilitate the dissociation of protein from nucleic acid. The resultant mixture was transferred to a 250 ml Erlenmeyer flask to which an equal volume of chloroform-isoamyl alcohol (24:1 v/v) was added and shaken on a reciprocal shaker (200 strokes/min) for 30 minutes at room temperature. The resultant emulsion was separated into three layers by centrifugation for five minutes at 12,000x g. The top layer, crude nucleic acid fraction, was collected using a wide-bore pipette without disturbing the underlying protein layer. Approximately two volumes of 95% ethyl alcohol were gently layered over the crude extract and the nucleic acids collected by winding on a glass stirring rod. The collected material was drained of excess alcohol and dispersed in 10-15 ml of dilute saline-citrate (DSC); 0.015 M NaCl plus 0.0015 M trisodium citrate, pH 7.0 by gently aspirating into and discharging from a Pasteur pipette. The solution was adjusted to standard saline-citrate (SSC) concentration (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0 $\pm$ 0.2) by the addition of concentrated saline-citrate (CSC), 1.5 M NaCl plus 0.15 M trisodium citrate and subjected to a series of 15 minute deproteinizations until little, if any, denatured protein appeared at the interface. After the final centrifugation, the nucleic acids were again precipitated with ethyl alcohol (95%), collected and dispersed in DSC as previously described. The solution was again adjusted to SSC concentration. Ribonuclease (0.2 % in

0.15 M NaCl, pH 5.0), preheated at 80 C for ten minutes to inactivate any contaminating deoxyribonuclease, was added to a final concentration of 50 μg/ml and the mixture incubated at 37 C for 30 minutes. Twenty μg/ml of subtilisin (Nutritional Biochemicals Corp., Cleveland, Ohio) were added to remove ribonuclease. After a 30 minute incubation period at 37 C, the resultant digest was again subjected to a series of deproteinizations. When protein removal was considered complete, the nucleic acids were precipitated, collected and dissolved in 9 ml of DSC to which 1 ml of acetate-EDTA (3.0 M sodium acetate plus 0.001 M EDTA, pH 7.0) was added. The DNA was selectively precipitated upon addition of approximately 0.54 volumes of isopropyl alcohol and collected on a glass rod attached to a stirring motor. The final fibrous precipitate was washed free of acetate and salt in increasing concentrations of ethyl alcohol; 70, 80 and 95%. The DNA was generally dissolved in 5 ml of DSC and adjusted to SSC concentration by the addition of 0.45 ml of CSC. All extracts were stored at 2 C over several drops of chloroform until needed.

The determination of DNA concentrations was performed as described by Reich et al. (53). An optical density of 0.025 was assumed to be equivalent to a DNA concentration of approximately 1 μg/ml.

All DNA extracts, used in subsequent hybridization experiments, were precipitated at least once more from SSC by addition of two

volumes of ethyl alcohol (95%). The DNA fibers, collected by vigorous mixing on a vortex mixer, were washed with ethyl alcohol (80 and 95%), air dried and stored at 2-5 C. Stock solutions of DNA (1 mg/ml) were prepared as needed.

Thermal Denaturation of DNA

Thermal denaturation temperature (T_m) values of purified DNA preparations were determined as described by Marmur and Doty (42) employing a Gilford (Model 2000) automatic spectrophotometer equipped with dual thermospacers, auxiliary input channel and automatic cuvette positioner. The DNA was at a concentration of approximately 20 μg/ml in SSC in glass-stoppered silica cuvettes having a 1 cm light path. A Haake (Model F) circulating, thermostatted water bath was used to raise the temperature of the cuvette chamber by circulating ethylene glycol through thermospacers located on either side of it. Tap water at room temperature was slowly circulated through an adjacent set of thermospacers to protect the photocell from excessive heat.

The DNA samples were equilibrated at 25 C for 15 minutes and the absorbance at 260 nm was recorded. The temperature of the chamber was raised to 70 C, equilibrated and a linear temperature program was initiated. When DNA is heated in solution, a sharp increase in its extinction coefficient occurs at the temperature where

the transition takes place from the double-stranded helical configuration to a disordered or random coil. No further increase in absorbance indicates complete denaturation or strand separation. Optical densities calculated from the sigmoidal recordings were corrected for thermal expansion of solution by multiplying the observed reading by the ratio V_t/V_{25C} (Table 1). Relative absorbance (corrected absorbance divided by the value at 25 C) was plotted against temperature of solution. Table 2 lists absorbance readings and their corrected values for a typical T_m determination. The T_m values recorded were calculated as described by Marmur and Doty (42) (Figure 1) and by normal probability plots of melting data (39) (Table 3) and (Figure 2). Moles % guanine plus cytosine (GC) was calculated according to the linear relation of Marmur and Doty (42): $T_m = 69.3 + 0.41$ (GC). Normal distribution curves were constructed from absorbance-temperature denaturation profiles by plotting the change in slope per degree increase in temperature ($\Delta \text{Tan}\phi/C$) against temperature (39) (Figure 3). The compositional distribution of DNA molecules was also examined by treating the absorbance data at 260 nm as a differential plot in terms of the increment of absorbance per unit of temperature (Δ optical density at 260 nm per degree C) as a function of temperature (31) (Figure 4).

Escherichia coli DNA (Nutritional Biochemicals Corp., Cleveland, Ohio) was used as a reference and control in all T_m determinations.

Table 1. Conversion factors (V_t/V_{25C}) for thermal expansion of solution.

Temperature (C)	V_t/V_{25C} [a]	Temperature (C)	V_t/V_{25C} [a]
25	1.0000	61	1.0146
26	1.0003	62	1.0152
27	1.0005	63	1.0157
28	1.0008	64	1.0162
29	1.0011	65	1.0168
30	1.0014	66	1.0174
31	1.0017	67	1.0180
32	1.0020	68	1.0185
33	1.0024	69	1.0191
34	1.0027	70	1.0197
35	1.0030	71	1.0203
36	1.0034	72	1.0209
37	1.0037	73	1.0215
38	1.0041	74	1.0221
39	1.0045	75	1.0228
40	1.0049	76	1.0234
41	1.0053	77	1.0240
42	1.0057	78	1.0247
43	1.0061	79	1.0253
44	1.0065	80	1.0260
45	1.0069	81	1.0266
46	1.0073	82	1.0273
47	1.0078	83	1.0280
48	1.0082	84	1.0287
49	1.0087	85	1.0293
50	1.0091	86	1.0300
51	1.0096	87	1.0308
52	1.0100	88	1.0314
53	1.0105	89	1.0321
54	1.0110	90	1.0329
55	1.0115	91	1.0336
56	1.0120	92	1.0343
57	1.0125	93	1.0351
58	1.0131	94	1.0358
59	1.0135	95	1.0365
60	1.0141	96	1.0373

Table 1. (continued)

Temperature (C)	V_t/V_{25C} [a]	Temperature (C)	V_t/V_{25C} [a]
97	1.0379		
98	1.0387		
99	1.0395		
100	1.0404		

[a] V_t/V_{25C} = volume of water at temperature t divided by volume at 25 C. Values for V obtained from Smithsonian Tables in handbook of Chemistry and Physics (Chemical Rubber Company, CRC) 46th ed. (1965-1966) PF-4.

Table 2. Absorbance, corrected absorbance and relative absorbance from a melting determination of DNA isolated from L. plantarum 17-5.

Temperature (C)	Absorbance 260 nm	Corrected Absorbance[a]	Relative Absorbance[b]
25	0.282	0.282	1.00
80	0.282	0.289	1.02
82	0.282	0.290	1.03
83	0.285	0.293	1.04
84	0.289	0.297	1.05
85	0.297	0.306	1.09
86	0.309	0.318	1.13
87	0.327	0.337	1.20
88	0.348	0.359	1.27
89	0.366	0.378	1.34
90	0.376	0.388	1.38
91	0.379	0.392	1.39
92	0.381	0.394	1.40
93	0.381	0.394	1.40

[a] Corrected absorbance $= A_t \cdot \frac{V_t}{V_{25C}}$; A_t = observed absorbance at temperature t, $\frac{V_t}{V_{25C}}$ = volume of water at temperature t divided by volume at 25C.

[b] Relative absorbance = corrected absorbance divided by absorbance at 25C.

Table 3. Absorbance, corrected absorbance and percent increase in absorbance obtained from a melting determination of DNA isolated from L. helveticus OSU.

Temperature (C)	Absorbance 260 nm	Corrected Absorbance[a]	Absorbance Increase	Percent Increase in Absorbance[b]
78	0.375	0.375	0.000	0.0
80	0.377	0.387	0.012	6.8
84	0.419	0.431	0.056	32.1
85	0.443	0.456	0.081	46.5
86	0.471	0.485	0.110	63.2
88	0.511	0.527	0.152	87.3
90	0.525	0.542	0.167	95.9
92	0.531	0.549	0.174	100.0

[a] Corrected absorbance = $A_t \cdot \frac{V_t}{V_{25C}}$; A_t = observed absorbance at temperature t and $\frac{V_t}{V_{25C}}$ = volume of water at temperature t divided by volume at 25 C.

[b] Absorbance increment between corrected absorbance at 78 C and 92 C was set equal to 100%. Results were expressed as a percentage of the total increase.

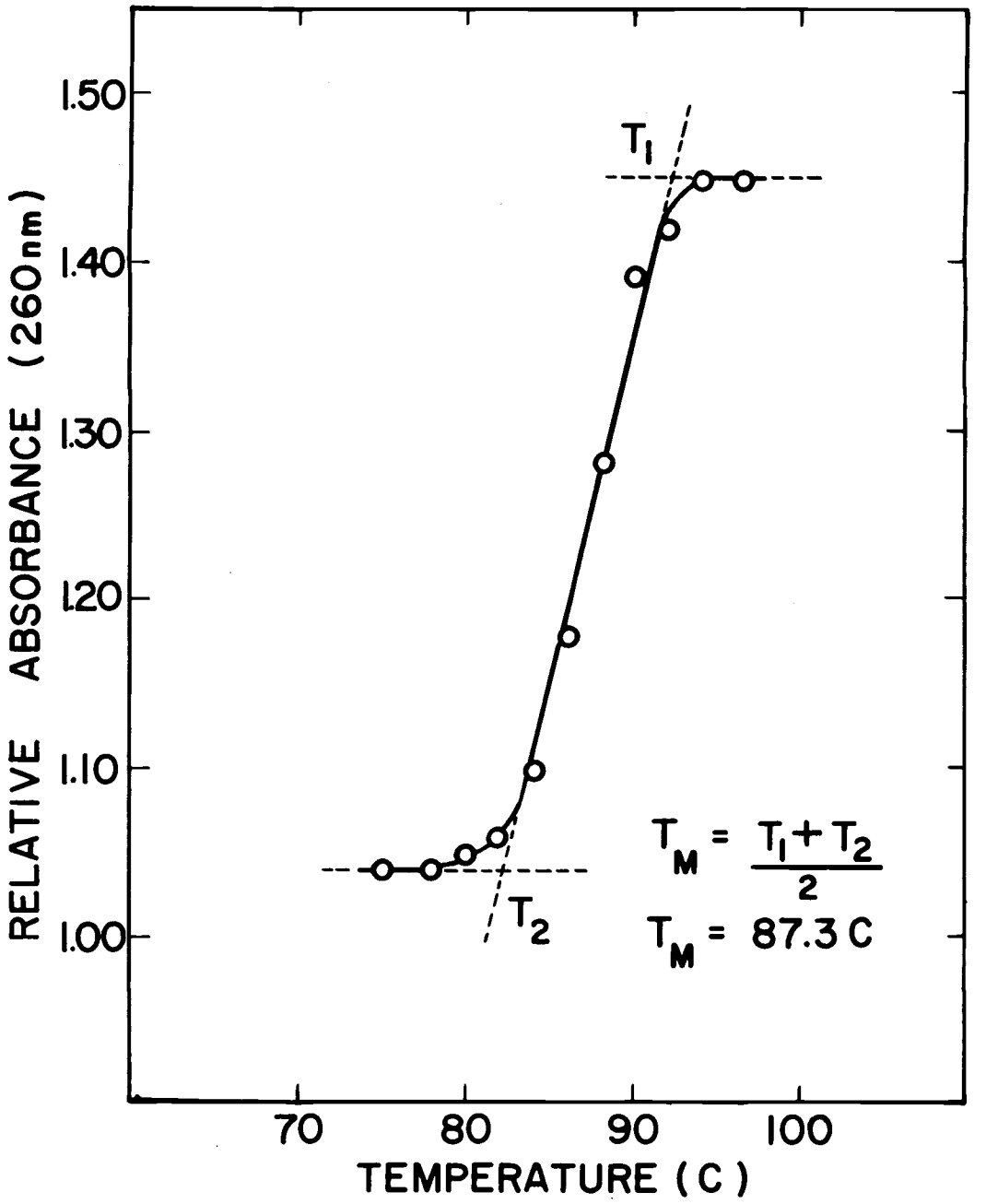


Figure 1. Absorbance-temperature denaturation profile of salmon sperm DNA.

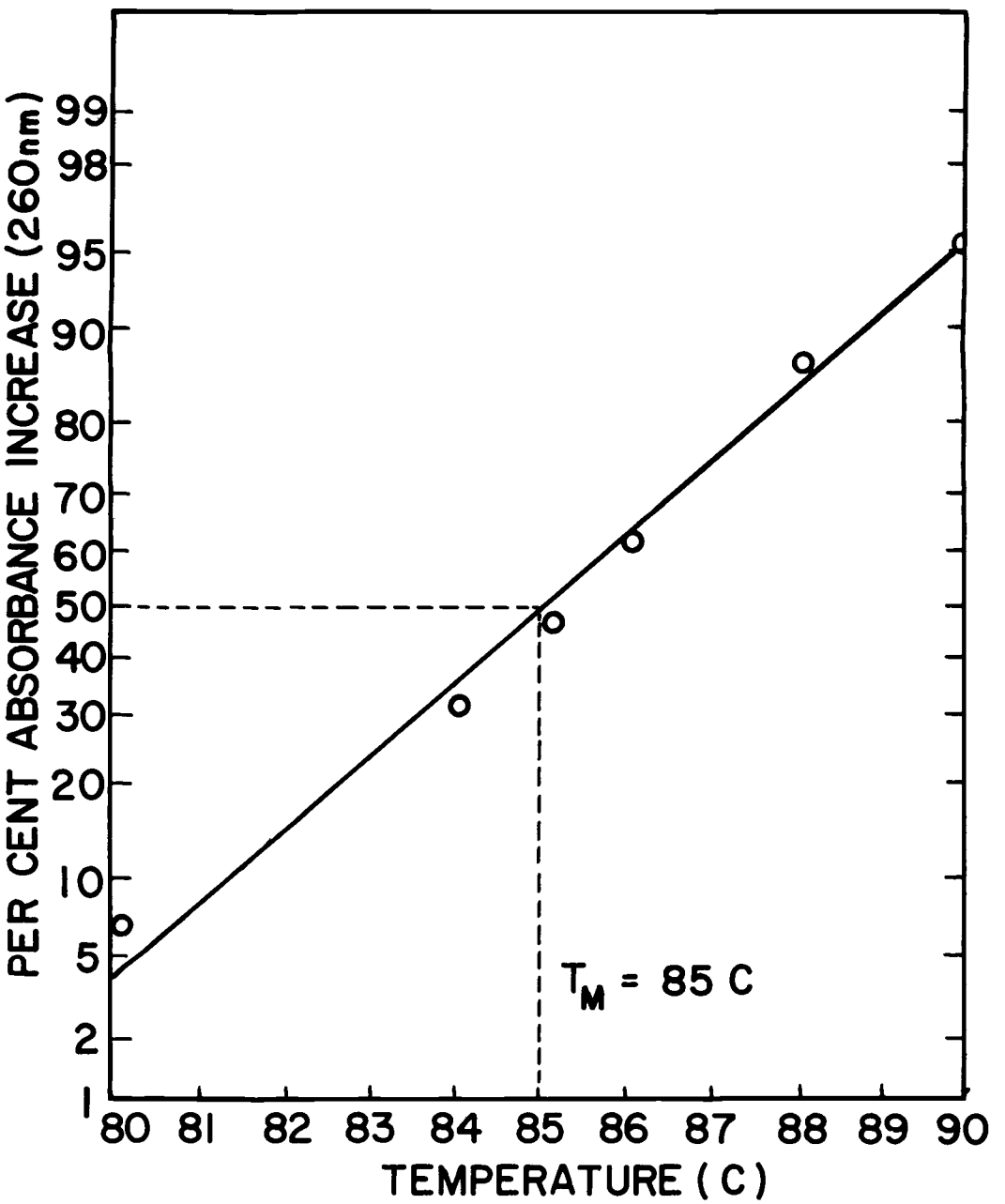


Figure 2. Normal probability plot of thermal denaturation data for L. helveticus OSU DNA.

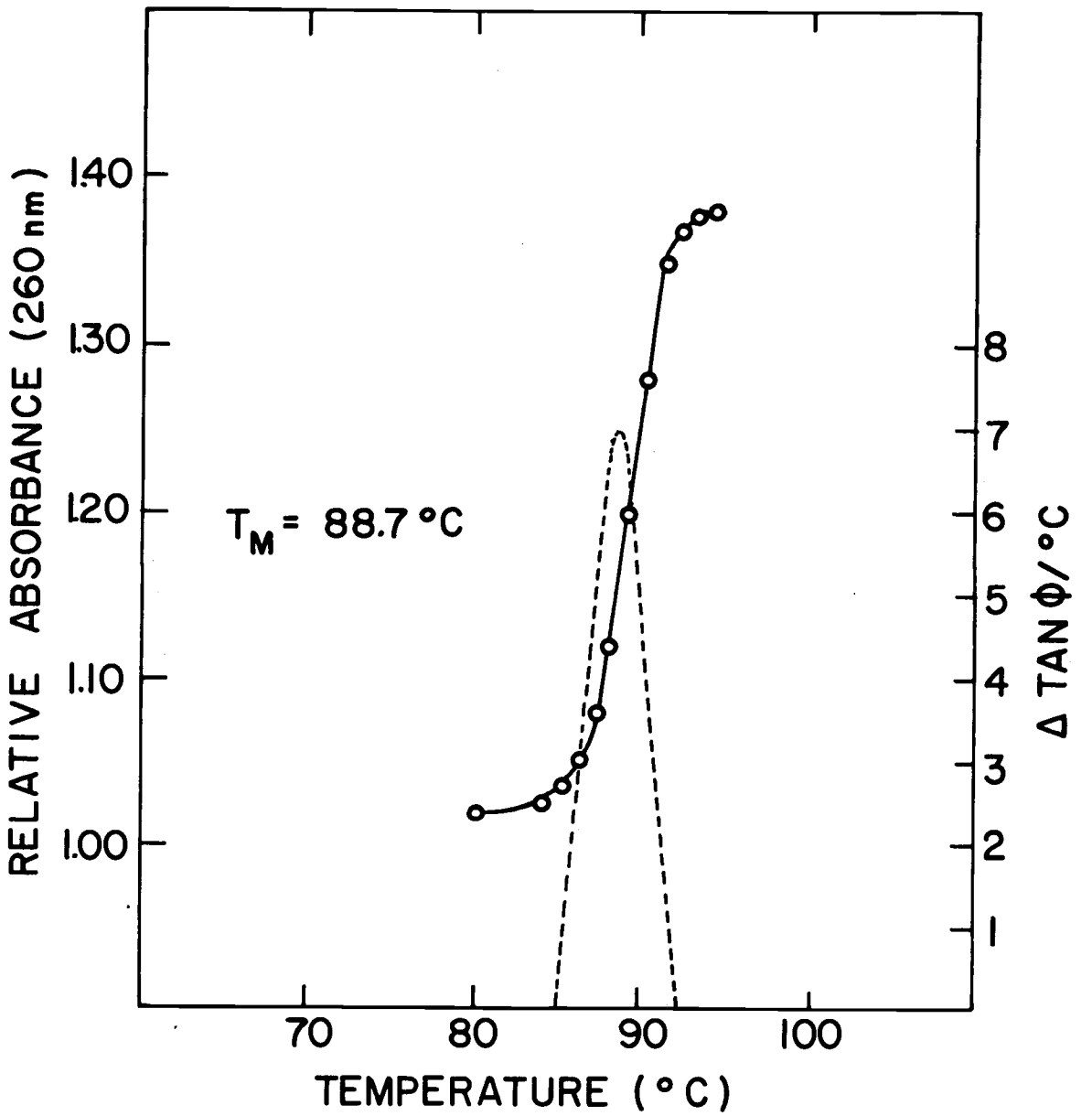


Figure 3. Absorbance-temperature denaturation profile for L. casei C5 (solid line); normal distribution curve (dotted line).

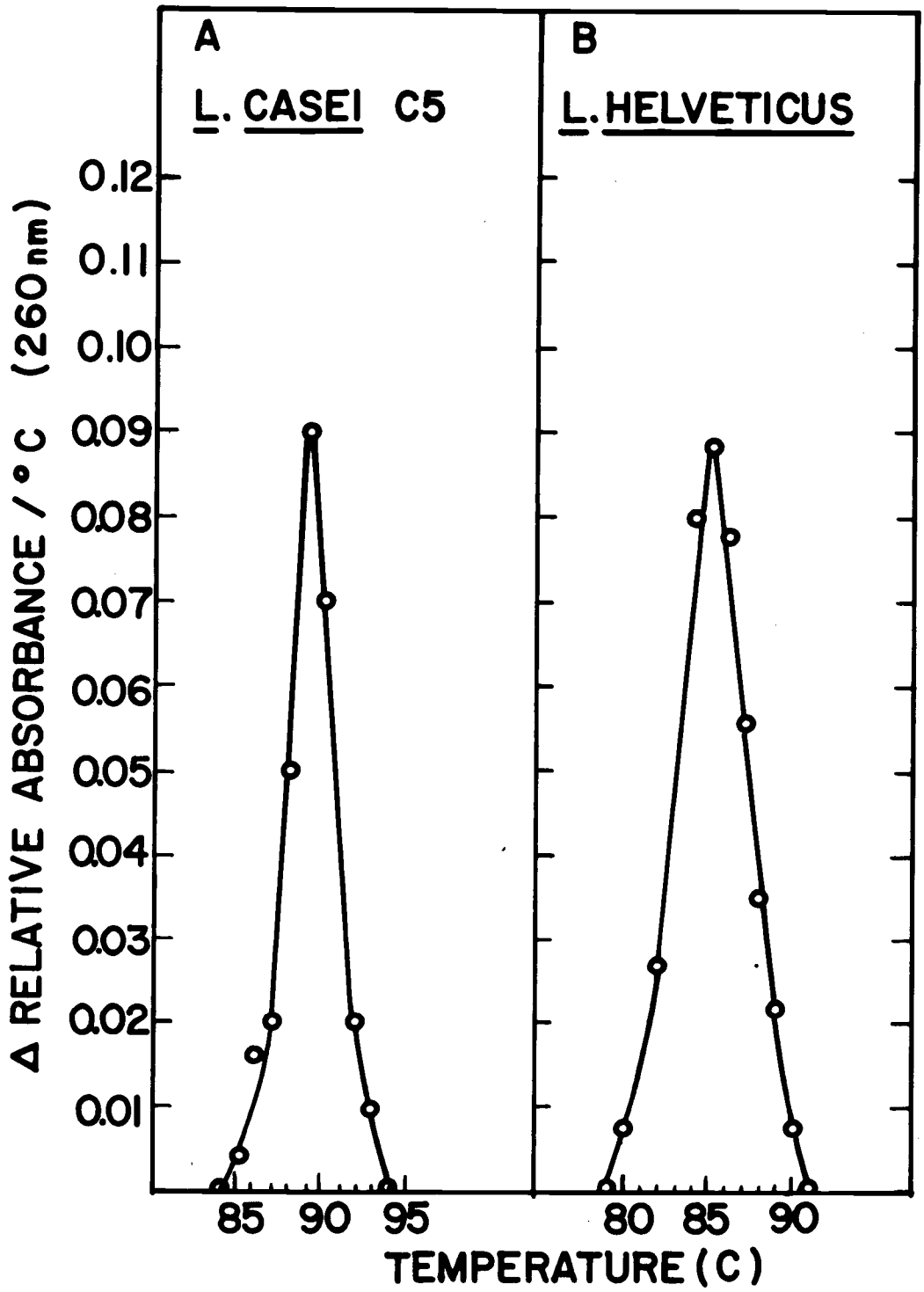


Figure 4. Derivatives of DNA thermal denaturation plots for (A) L. casei C5 and (B) L. helveticus OSU.

RNA Polymerase Reaction

Tritiated adenosine 5'-triphosphate (ATP-^{3}H), uridine 5'-triphosphate (UTP-^{3}H), cytidine 5'-triphosphate (CTP-^{3}H) and guanosine 5'-triphosphate (GTP-^{3}H) were obtained from Schwarz Bio Research; Van Nuys, California. The specific activities were adjusted to: ATP-^{3}H, 2.45 c/mmole; UTP-^{3}H, 1.8 c/mmole; CTP-^{3}H, 1.35 c/mmole and GTP-^{3}H, 1.16 c/mmole (Appendix 1). Ribonucleic acid polymerase (E. coli, specific activity 2,000 units/mg) was purchased from Biopolymers Inc.; Dover, New Jersey. All dilutions of RNA polymerase were made in buffer containing 0.01 M tris-(hydroxymethyl) aminomethane, pH 7.9; 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol; 5×10^{-5} M EDTA and 1 mg/ml of crystalline bovine serum albumin (15). Yeast RNA and deoxyribonuclease I were obtained from Schwarz Bio Research, Orangeburg, New York. Other chemicals used were of analytical reagent grade.

Radioactive complementary RNA was synthesized under essentially the same conditions as described by Chamberlin and Berg (15) and Reich et al. (53). Briefly, 20 μg of the desired template DNA, 20 mμmoles of each tritiated nucleoside triphosphate, 20 units of RNA polymerase, 10 μmoles of tris-(hydroxymethyl) aminomethane; pH 7.9, 0.5 μmole of $MnCl_2 \cdot 4H_2O$, 2.0 μmoles of $MgCl_2 \cdot 6H_2O$ and 0.5 μmole of β-mercaptoethanol were contained in a total volume of 0.25 ml. After incubation at 37 C for 90 minutes, yeast RNA (1.5 mg) and

deoxyribonuclease I (1.5 μg) were added. The mixture was incubated at 37 C for an additional 15 minutes. Protein was removed by extraction with 90% hot phenol (60 C) and the tritiated RNA, precipitated upon addition of 20% potassium acetate and 99% ethyl alcohol, was collected by centrifugation and dissolved in a minimal volume (0.14-0.18 ml) of 0.015 M NaCl plus 0.0015 M sodium citrate, pH 6.7. When necessary, the extraction procedure was repeated. Acid-precipitable radioactivity was measured at 0, 30, 60 and 90 minutes as described by Reich et al. (53). A 0.005 ml portion was added to 3 ml of cold 5% trichloroacetic acid and filtered through a B-6 nitrocellulose membrane filter (Schleicher and Schuell Company, Keene, New Hampshire). The filter was washed with 10 ml of 5% trichloroacetic acid and assayed for radioactivity as described below.

DNA-RNA Hybridization

The method of Nygaard and Hall (50) and Reich et al. (53) were used to detect and assess the extent of DNA-RNA complex formation. The reaction mixture, total volume 0.1 ml, contained the following: subtilisin (50 μg), 0.01 ml; NaCl-citrate (0.6 M plus 0.06 M), 0.05 ml to give a final concentration of 0.3 M NaCl plus 0.03 M citrate, pH 6.7; tritiated complementary RNA, 0.02 ml and, unless stated otherwise, 20 μg of DNA (0.02 ml). The mixtures, previously incubated at 37 C for 15 minutes, were heated at 100 C for 10 minutes

in sealed 1 ml ampoules to denature the DNA and incubation was continued at 66-67 C for 18 hours. The contents of each ampoule were diluted 1:150 by addition of 15 ml of 0.5 M NaCl plus 0.01 M tris-(hydroxymethyl) aminomethane, pH 7.3 and filtered through membrane filters previously washed with 10 ml of the above diluent at 60 C. The filters were assayed for radioactivity as described below.

Radioactivity Assay

All nitrocellulose membrane filters were washed with 25 ml of 80% ethyl alcohol, thoroughly air dried and placed in scintillation counting vials. Scintillation solution was prepared with toluene and liquifluor (New England Nuclear Corp., Boston, Massachusetts) as directed by the manufacturer. Fifteen milliliters of scintillation solution were added to each vial and the filters were assayed for radioactivity in a Packard Tri-Carb (Model 524) liquid scintillation spectrometer.

Non-specific retention of radioactivity was corrected for by subtraction of values obtained when tritiated RNA was incubated without DNA and filtered. The amount of radioactivity retained by the filters under these conditions varied from 0.1 - 0.5 %. Percent incorporation, RNA bound relative to total radioactivity, was calculated by dividing the radioactivity retained by the filters after correction for non-specific retention by the total activity. For each hybridization

experiment, the quantity of radioactivity retained on the filters was expressed as a percentage of the counts per minute retained with primer or homologous DNA.

Extracellular Nuclease Assay

Extracellular enzyme activity of all cultures was tested using deoxyribonuclease test agar (Difco) plates and MRS-broth or agar (25) supplemented with either herring sperm DNA (2 g/L) or Torula RNA (2 g/L); Calbiochem, Los Angeles, California. The latter medium was modified by omitting Tween 80 and reducing the beef extract and glucose to 0.3 and 0.5 %; respectively. In addition, tryptone was substituted for proteose peptone. All plates were heavily inoculated with the desired organisms, previously grown in MRS (25) or lactic broth (30), and incubated at 32 or 37 C for three days. Extracellular hydrolytic activity was assayed by lightly flooding the plates with 1 N HCl (37). Clear zones, due to nucleic acid fragmentation, are formed around colonies possessing enzymatic activity. Serratia marcescens was used to illustrate a positive reaction; E. coli, devoid of extracellular nucleases, was used as a negative control (Figure 5).

Extracellular deoxyribonuclease activity was also determined using the quantitative procedures of Anfinsen et al. (2) and Eaves and Jeffries (28). The reaction mixture contained the following: 1 ml of

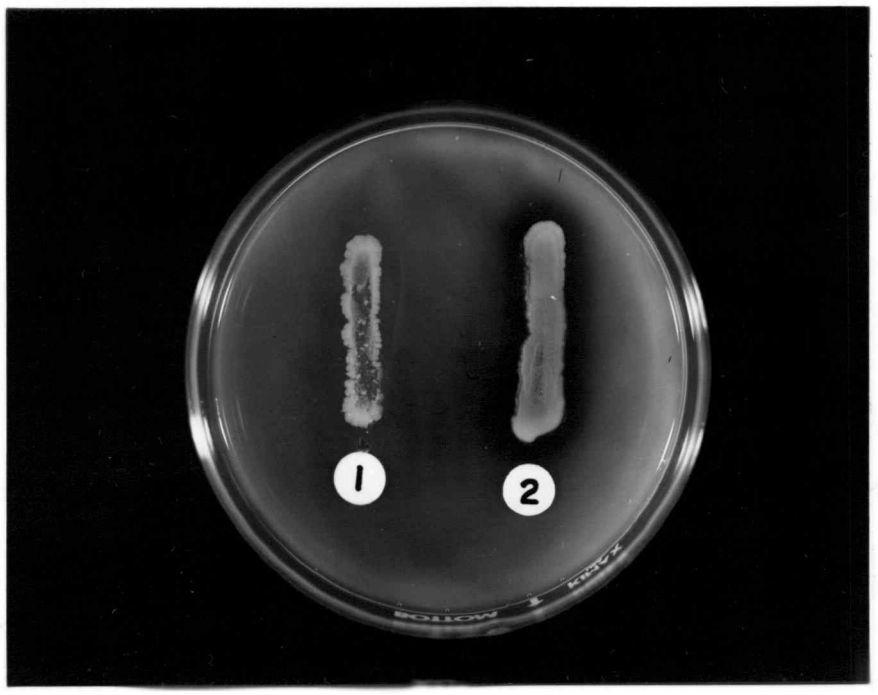


Figure 5. Extracellular nuclease production: (1) E. coli, negative reaction and (2) S. marcescens, positive reaction.

the substrate solution (2 mg DNA/ml), 0.5 ml of 0.4 M tris-(hydroxymethyl) aminomethane-HCl buffer (pH 8.5) containing 0.04 M $MgCl_2 \cdot 6H_2O$ and 0.5 ml of the culture supernatant. The reaction mixture was incubated in a water bath at 37 C for 20 minutes after which the reaction was terminated by addition of 0.5 ml of 0.75% uranyl acetate in 25% perchloric acid. The solutions were held on ice for 10 minutes, centrifuged at 8000 x g for 5 minutes and the supernatant fluids removed and properly diluted with water. The absorbance of these solutions at 260 nm was read against a blank reaction in which buffer had been substituted for the culture filtrate or enzyme solution.

Ribonuclease activity was detected by the same method described above for deoxyribonuclease, however, the substrate solution contained Torula RNA (2 mg/ml). A unit of deoxyribonuclease or ribonuclease activity was defined as that amount of enzyme causing an increase of 1.0 in absorbance at 260 nm in 20 minutes at 37 C.

RESULTS AND DISCUSSION

Thermal Denaturation Studies

Preliminary studies on the base composition of deoxyribonucleates from a number of recognized species and strains of lactobacilli were conducted to determine the extent of structural homology. All T_m values reported are an average of at least two determinations and were reproducible for a given sample within 0.3 C; the T_m for different DNA preparations of the same culture was reproducible also within 0.3 C. The T_m values determined for salmon sperm DNA, calf thymus DNA and E. coli; 87.3, 86.8 and 89.9 C, respectively (Figures 1, 6, 7) were similar to reported literature values (42). All DNA preparations gave monophasic absorbance-temperature denaturation profiles and the compositional distribution of DNA molecules was relatively narrow and unimodal (Figures 3, 4).

T_m values, determined by two methods, and moles % GC values of several lactobacilli are given in Table 4. The T_m values calculated as described by Marmur and Doty (42) are in close agreement with those determined by normal probability plots of melting data (39). In addition, T_m values ranged from 82.6 C for L. salivarius ATCC 11742 to 90.6 C for L. fermenti F1 in a manner directly proportional to the GC content (Figure 8).

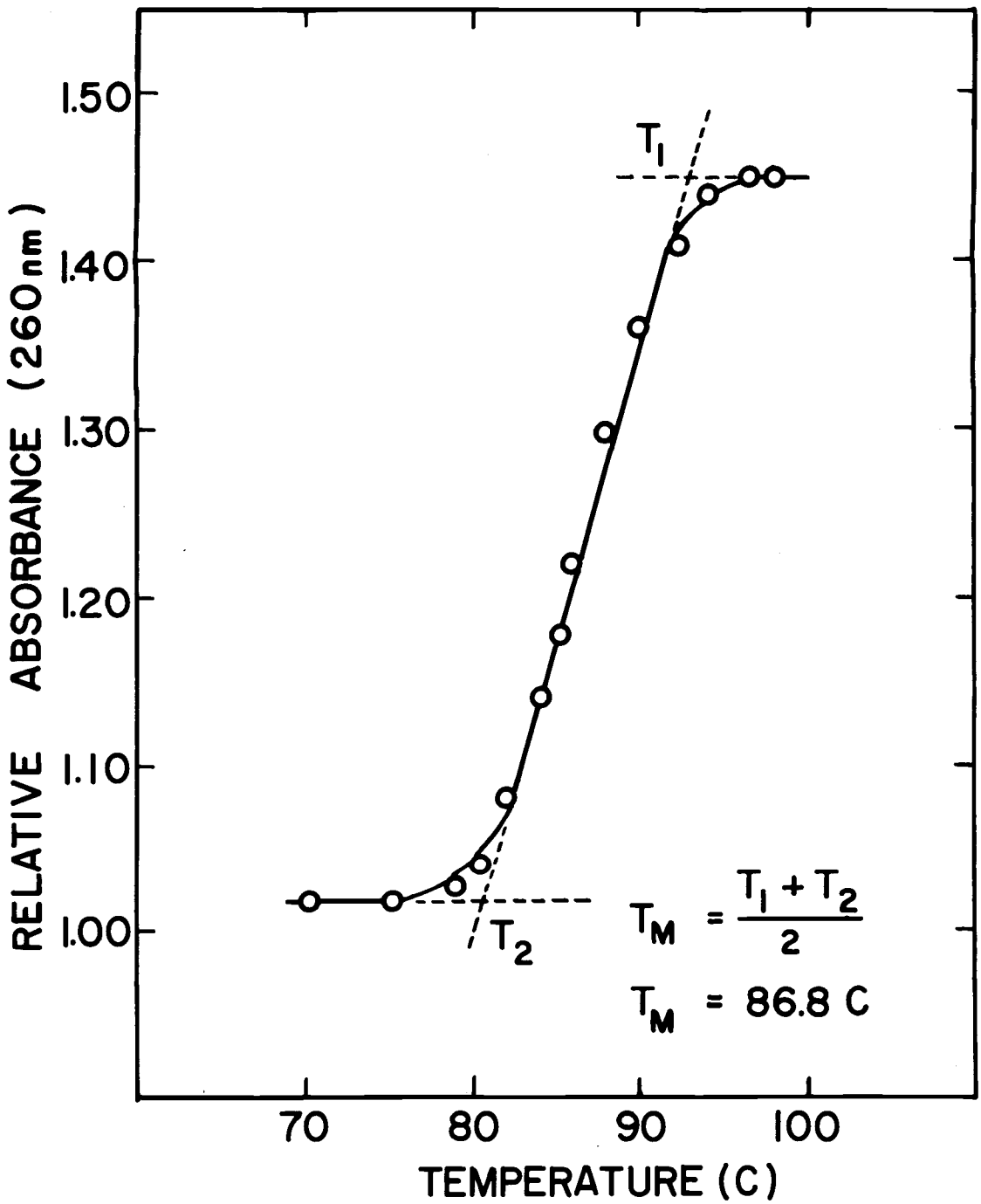


Figure 6. Absorbance-temperature denaturation profile of calf thymus DNA.

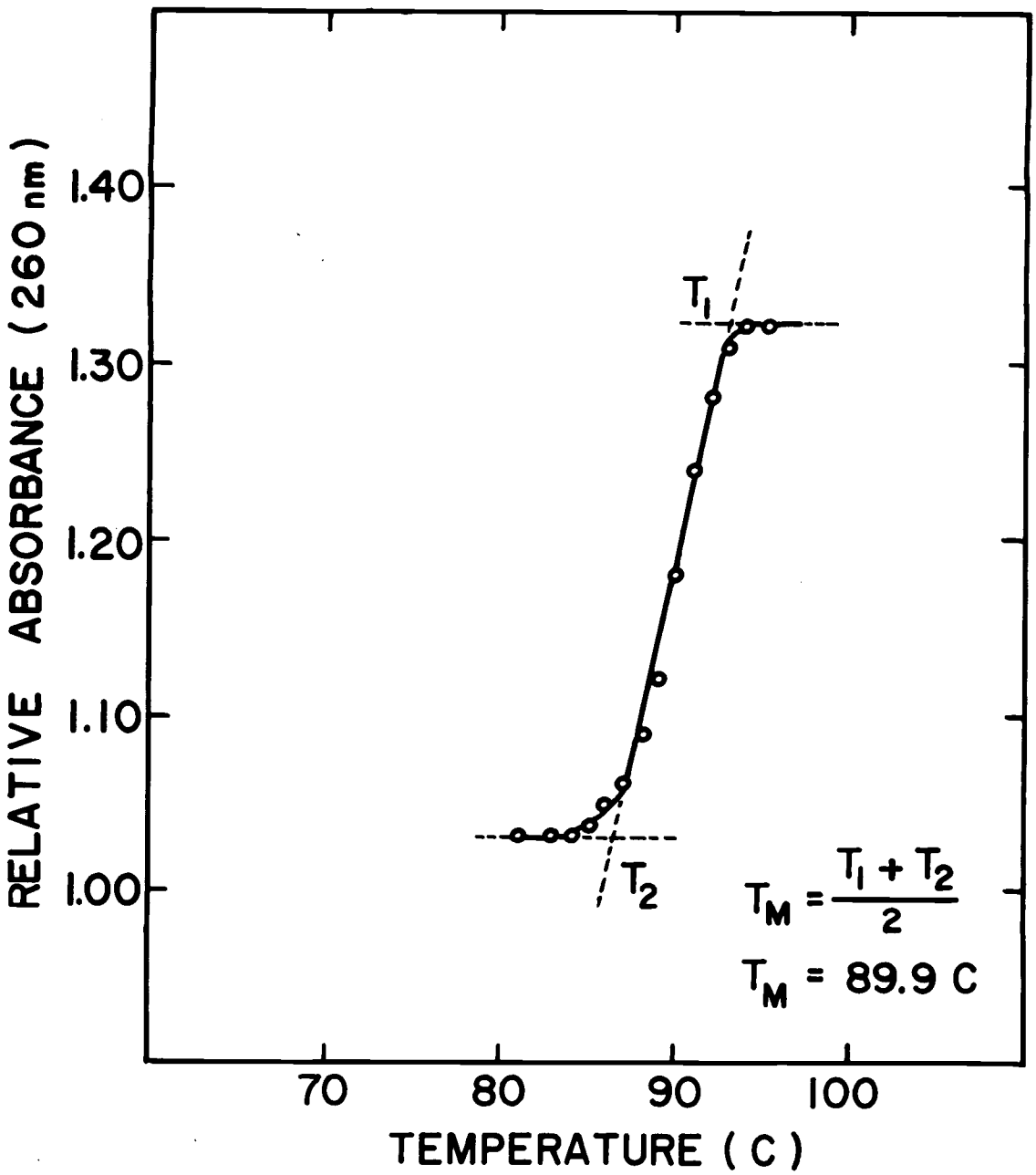


Figure 7. Absorbance-temperature denaturation profile of E. coli DNA.

Table 4. GC content (moles %) and thermal denaturation (T_m) values of lactobacilli.

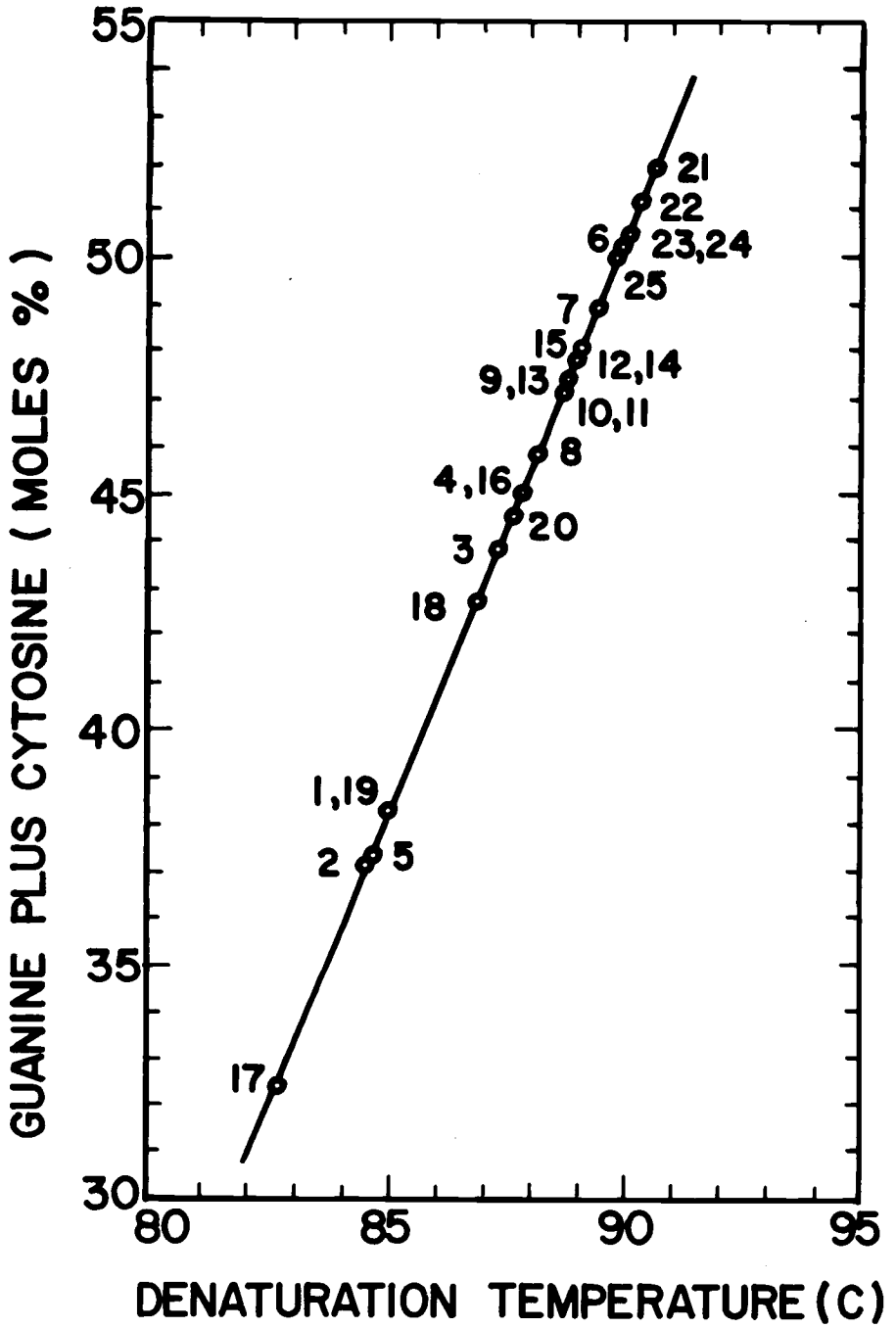
Organisms and Origin[a]	T_m(C)	T_m(NPP)[b]	Moles % GC[c]
L. helveticus OSU	85.0	85.0	38.3
L. jugurti ATCC 521	84.5	84.5	37.1
L. plantarum 17-5 (ATCC 8014)	87.8	87.8	45.1
L. plantarum P-5 (NCDO 343)	87.3	87.4	43.9
L. (jugurti) bulgaricus ATCC 7993	84.6	84.5	37.3
L. acidophilus Farr	89.9	89.7	50.2
L. lactis 39-A	89.4	89.3	49.0
L. casei ATCC 9595	88.1	88.2	45.8
L. casei OSU	88.8	88.6	47.5
L. casei ATCC 7469 (NCDO 243)	88.7	88.5	47.3
L. casei C5 (NCDO 151)	88.7	88.8	47.3
L. casei 780	88.9	89.0	47.8
L. casei 356	88.8	88.8	47.5
L. casei 300	88.9	88.8	47.8
L. casei 316	89.0	88.9	48.0
L. brevis X1 (NCDO 473)	87.8	87.9	45.1
L. salivarius ATCC 11742	82.6	82.3	32.4
L. viridescens ATCC 12706	86.8	86.8	42.7
L. bulgaricus ATCC 12278 (GA)	85.0	84.8	38.3
L. buchneri BC1 (NCDO 110)	87.6	87.8	44.6
L. fermenti F1 (NCDO 215)	90.6	90.4	51.9
L. cellobiosus G1 (NCDO 927)	90.3	90.1	51.2
L. leichmannii ATCC 7830	90.0	90.0	50.5
L. leichmannii ATCC 4797	90.0	90.1	50.5
L. delbrueckii ATCC 9649	89.8	89.5	50.0

[a] ATCC: American Type Culture Collection. NCDO: National Collection of Dairy Organisms, Reading, England.

[b] T_m values obtained from normal probability plots of melting data.

[c] Moles % GC content calculated from T_m values in Column 2.

Figure 8. Dependence of the denaturation temperature, T_m, on the guanine plus cytosine (GC) content of DNA. The numbers refer to the following lactobacilli from which DNA was extracted. (1) L. helveticus OSU, (2) L. jugurti ATCC 521, (3) L. plantarum P5, (4) L. plantarum 17-5, (5) L. (jugurti) bulgaricus ATCC 7993, (6) L. acidophilus Farr, (7) L. lactis 39-A, (8) L. casei ATCC 9595, (9) L. casei OSU, (10) L. casei ATCC 7469, (11) L. casei C5, (12) L. casei 780, (13) L. casei 356, (14) L. casei 300, (15) L. casei 316, (16) L. brevis X1, (17) L. salivarius ATCC 11742, (18) L. viridescens ATCC 12706, (19) L. bulgaricus ATCC 12278, (20) L. buchneri BC1, (21) L. fermenti F1, (22) L. cellobiosus G1, (23) L. leichmannii ATCC 7830, (24) L. leichmannii ATCC 4797 and (25) L. delbrueckii ATCC 9649.


55
50
45
40
35
30
80
85
90
95
GUANINE PLUS CYTOSINE (MOLES %)
DENATURATION TEMPERATURE (C)
21
22
6
23,24
25
7
15
12,14
9,13
10,11
8
4,16
20
3
18
1,19
2
5
17

Since DNA base compositions ranged from 32.4 to 51.9 moles % GC, heterogeneity within the genus Lactobacillus was quite evident. This observation is not unique for the lactobacilli because compositional heterogeneity has been reported also within other genera: Proteus, 39 to 50 moles % GC (32); Mycoplasma, 24 to 41 moles % GC (49, 70); Neisseria, 40 to 50 moles % GC (14) and Bacillus, 33 to 50 moles % GC (42). For bacteria having substantially different mean DNA base compositions, it can be said that they will have few DNA molecules in common (63).

Comparison of data from this study with compiled GC contents for lactobacilli from previous reports (9-13, 33) is shown in Table 5. Moles % GC values given in Column 2 did not concur entirely with those previously reported by Cantoni and co-workers (Column 3). Significant differences were noted for L. leichmanni, L. delbrueckii and L. lactis. Additional variations were observed for L. helveticus, L. jugurti, L. cellobiosus and L. salivarius. With the exception of L. acidophilus and L. bulgaricus, the data obtained in this investigation substantiated results obtained by buoyant density measurements (Table 5) as well as by chemical analyses (34).

The correlation of GC contents determined by buoyant density measurements with those obtained by thermal denaturation for selected species, indicated in Figure 9, was examined further. The correlation coefficient calculated from buoyant density-T_m data was

Table 5. Comparison of reported GC contents (moles %) of various lactobacilli.

Organism	Moles % GC		
	Thermal Denaturation[a]	Thermal Denaturation[b]	Buoyant Density[c]
L. helveticus	38.3	31.8	39.3
L. jugurti	37.1	33.5	39.0±0.9
L. plantarum	43.9 45.1	41.9-43.0	45.0±1.0
L. acidophilus	50.2	31.9-39.0	36.7±0.7
L. lactis	49.0	35.7 36.8	50.3±1.4
L. casei	47.4[d]	46.8-47.3	46.4±0.8
L. brevis	45.1	41.3-45.5	42.7±1.5 46.4±1.0
L. salivarius	32.4	28.2 29.8	34.7±1.4
L. viridescens	42.7	----	35.7 42.3
L. bulgaricus	38.3	41.4	50.3±1.0
L. buchneri	44.6	43.9-44.5	44.8±1.1
L. fermenti	51.9	51.5 51.8	53.4±0.5
L. cellobiosus	51.2	45.5 45.7	53.1±0.8
L. leichmannii	50.5	30.4 31.7	50.8±0.5
L. delbrueckii	50.0	28.9	50.0

[a] Data from this study.
[b] Data previously reported in the literature (9-13) and compiled by Gasser and Mandel (33).
[c] From Gasser and Mandel (33).
[d] Mean GC content for 8 strains.

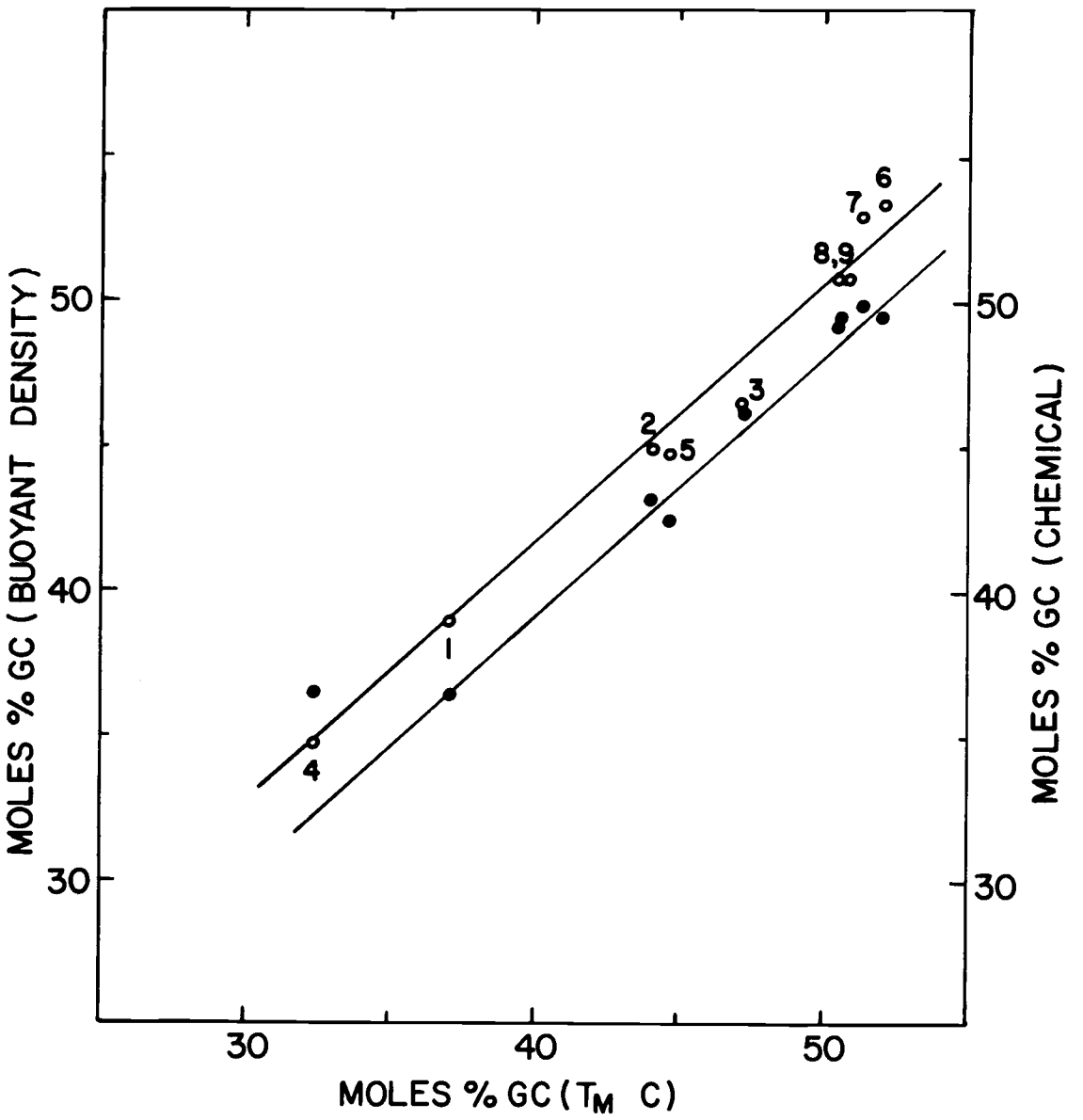


Figure 9. Comparison of GC content (moles %) of selected lactobacilli determined by thermal denaturation, buoyant density and chemical methods. (o), T_m - buoyant density; (•), T_m -chemical. Numbers refer to organisms: (1) L. jugurti ATCC 521, (2) L. plantarum P5, (3) L. casei NCDO 151, (4) L. salivarius ATCC 11742, (5) L. buchneri NCDO 110, (6) L. fermenti NCDO 215, (7) L. cellobiosus NCDO 927, (8) L. leichmannii ATCC 7830 (9) L. leichmannii ATCC 4797.

0.99. The relationship between chemical and T_m data for the same species was assessed also and a correlation coefficient of 0.98 was obtained. The linear relationship among the data obtained by the above three methods is shown in Figure 9. In addition, when GC contents from this study were compared to those reported by Cantoni (9-13), a correlation coefficient of 0.48 was obtained. The variations observed between the data reported herein and that previously reported by Cantoni and associates may be due to the use of different strains, improper species designation or inaccuracies in the physical determinations.

The GC content and T_m values of species of lactobacilli contained in the three subgenera of Orla-Jensen (51) are given in Table 6. The base composition of the thermobacteria ranged from 32.4 to 50.5 moles % GC, however, L. lactis, L. leichmannii and L. delbrueckii had similar values, 49.0 to 50.5 moles %. Values for species included in the subgenus Streptobacterium ranged from 43.9 to 47.8 moles %. The three strains of L. casei had similar values, 47.3 to 47.8 moles %, whereas L. plantarum, 43.9 moles %, was slightly different. Values for the betabacteria ranged from 42.7 to 51.9 moles %. The results indicate that a distinction on the basis of GC content between the subgenera recognized by traditional classifications (51, 54) cannot be made.

The relationship between DNA base composition and temperature

Table 6. GC content (moles %) and thermal denaturation (T_m) values of recognized species of lactobacilli.

Organism and Origin	T_m (C)	Moles % GC
Subgenus Thermobacterium		
L. helveticus OSU	85.0	38.3
L. jugurti ATCC 521	84.5	37.1
L. lactis 39-A	89.4	49.0
L. leichmannii ATCC 7830	90.0	50.5
L. delbrueckii ATCC 9649	89.8	50.0
L. salivarius ATCC 11742	82.6	32.4
Subgenus Streptobacterium		
L. plantarum P5	87.3	43.9
L. casei var. casei C5	88.7	47.3
L. casei var. rhamnosus ATCC 7469	88.7	47.3
L. casei 300	88.9	47.8
Subgenus Betabacterium		
L. fermenti F1	90.6	51.9
L. buchneri BC1	87.6	44.6
L. brevis XI	87.8	45.1
L. cellobiosus G1	90.3	51.2
L. viridescens ATCC 12706	86.8	42.7

range of growth was examined by Craveri, Manachini and Cantoni (20). A correlation was observed between moles % GC values and minimum temperatures of growth. Strains of the subgenus Thermobacterium, which had minimum growth temperatures of approximately 20 C, possessed DNA of lower GC content than those of the Betabacterium or Streptobacterium, which had minimum growth temperatures of approximately 10 C. Results of this study (Table 7) did not agree with the above observations. The thermobacteria, L. leichmannii, L. lactis and L. delbrueckii, had GC contents of 50.5, 49.0 and 50.0 moles %, respectively. Therefore, no correlation between temperature of growth and GC content among the three subgenera was apparent.

On the basis of GC content, the lactobacilli were placed in three groups similar to those proposed by Gasser and Sebald (34) and Gasser and Mandel (33). This is illustrated by the frequency curve of DNA base compositions for 24 species and strains, omitting L. acidophilus Farr (Figure 10).

Group I included species with a GC content between 32.4 and 38.3 moles %; L. jugurti, L. helveticus, L. salivarius, L. (jugurti) bulgaricus ATCC 7993 and L. bulgaricus ATCC 12278. Gasser and Sebald (34) also included L. acidophilus, however, our strain, L. acidophilus Farr, had a GC content of 50.2 moles %. Identical fermentation reactions were observed for L. acidophilus Farr and L.

Table 7. Comparison of growth temperatures, T_m and moles % GC values of recognized species of lactobacilli.

Organism and Origin	T_m(C)	Moles % GC	Temperature (C) of Growth
			10 15 20 30 40 45 50
Subgenus Thermobacterium			
L. helveticus OSU	85.0	38.3	
L. jugurti ATCC 521	84.5	37.1	
L. lactis 39-A	89.4	49.0	
L. leichmannii ATCC 7830	90.0	50.5	
L. delbrueckii ATCC 9649	89.8	50.0	
L. salivarius ATCC 11742	82.6	32.4	
Subgenus Streptobacterium			
L. plantarum P5	87.3	43.9	
L. casei var. casei C5	88.7	47.3	
L. casei var. rhamnosus ATCC 7469	88.7	47.3	
L. casei 300	88.9	47.8	
Subgenus Betabacterium			
L. fermenti F1	90.6	51.9	
L. buchneri BC1	87.6	44.6	
L. brevis X1	87.8	45.1	
L. cellobiosus G1	90.3	51.2	
L. viridescens ATCC 12706	86.8	42.7	

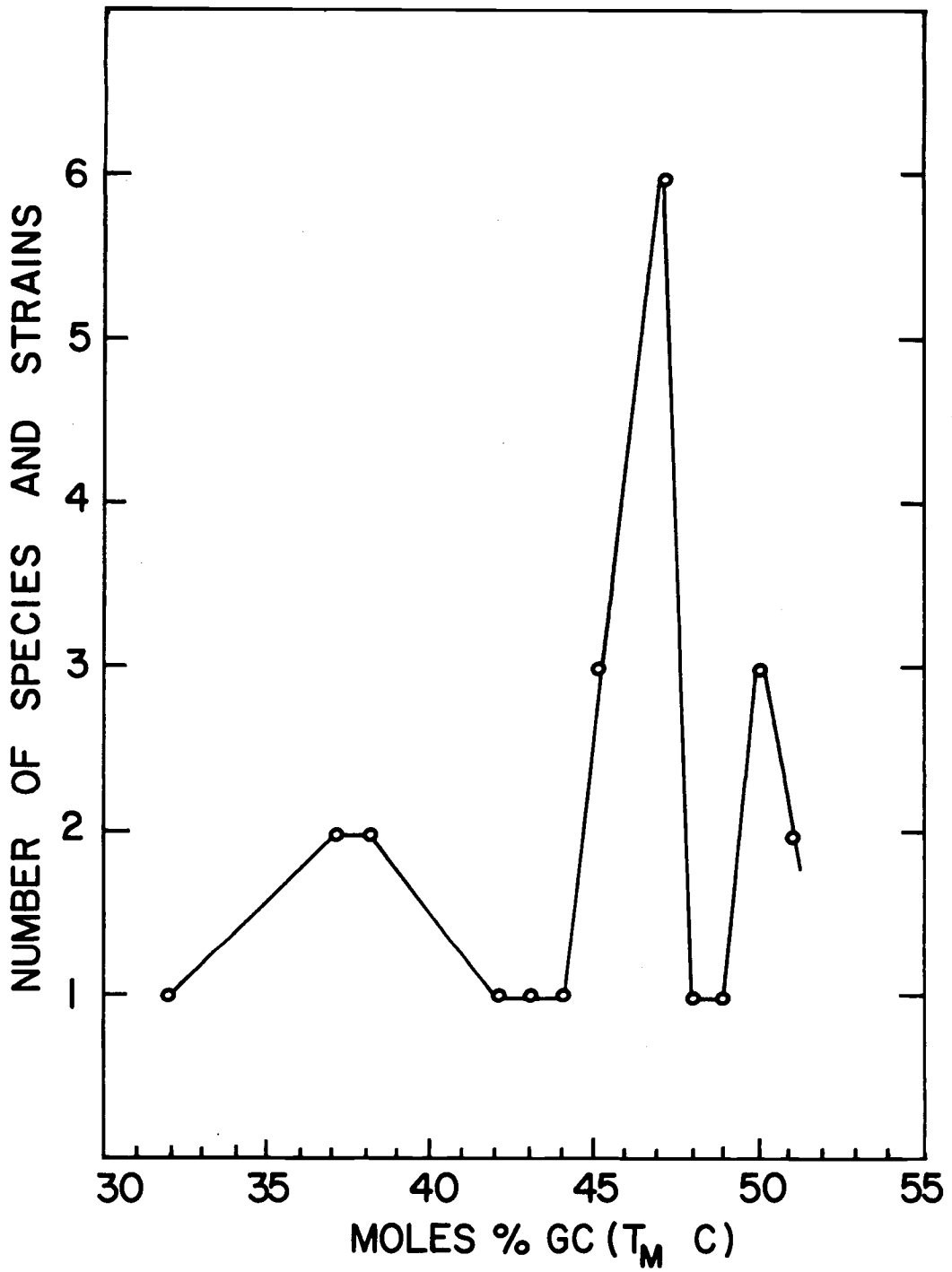


Figure 10. Frequency curve of DNA base compositions for 24 species and strains of lactobacilli.

lactis 39-A. Both organisms fermented fructose, galactose, maltose, mannose, lactose, salicin, sucrose, trehalose, glucose, α-methyl-D-glucoside and dextrin; raffinose was fermented with delay. Arabinose, rhamnose, sorbose, amygdalin, cellobiose, mannitol, melibiose, sorbitol, adonitol, dulcitol, erythritol, inositol and melezitose were not fermented. It is generally agreed that an accurate classification cannot be made solely on the basis of fermentation reactions. However, since L. acidophilus Farr and L. lactis 39-A also have similar GC contents, 50.2 and 49.0 moles %, respectively, strain Farr may have been classified erroneously and should be redesignated L. lactis.

Group II included species with a GC content between 42.7 and 48.0 moles %: L. buchneri, L. brevis, L. casei, L. viridescens and L. plantarum. L. viridescens was placed in this group even though species heterogeneity may exist (33).

Group III included species with a GC content between 49.0 and 51.9 moles %: L. lactis, L. leichmannii, L. delbrueckii, L. fermenti and L. cellobiosus. Gasser and Sebald (34) included L. bulgaricus, however, L. bulgaricus ATCC 12278 had a GC content of 38.3 moles % and was placed in Group I above. Typical fermentation reactions were observed for strain 12278; fructose, galactose, mannose, lactose and glucose were fermented while a wide variety of other carbohydrates were not utilized.

A number of the above species have similar to identical GC contents: L. helveticus, L. jugurti and L. (jugurti) bulgaricus, 37.1 to 38.3 moles %; L. buchneri and L. brevis, 44.6 and 45.1 moles %, respectively. Additional similarities are also evident in Table 4. However, organisms having similar base compositions can also be completely unrelated: Pseudomonas aeruginosa and Mycobacterium phlei, 66.0 moles % GC; B. circulans and Mycoplasma gallisepticum, 35.0 moles % GC and Salmonella typhimurium and Neisseria meningitidis, 51.5 moles % GC (42). It should be emphasized that it is the sequence of bases in DNA which is significant in comparisons for similarity. The magnitude of relatedness can be assessed only by appropriate DNA-DNA or DNA-RNA hybridization experiments which will be discussed below.

RNA Polymerase Reaction

With few exceptions, the rate of DNA-directed synthesis of RNA catalyzed by RNA polymerase from E. coli remained constant for approximately 30 minutes and then decreased with time (Figure 11). Dilution of the enzyme into solutions not containing a sulfhydryl compound (β-mercaptoethanol) resulted in over 95% inactivation. Other parameters of the enzyme reaction have been thoroughly investigated by Chamberlin and Berg (15) and will not be discussed here.

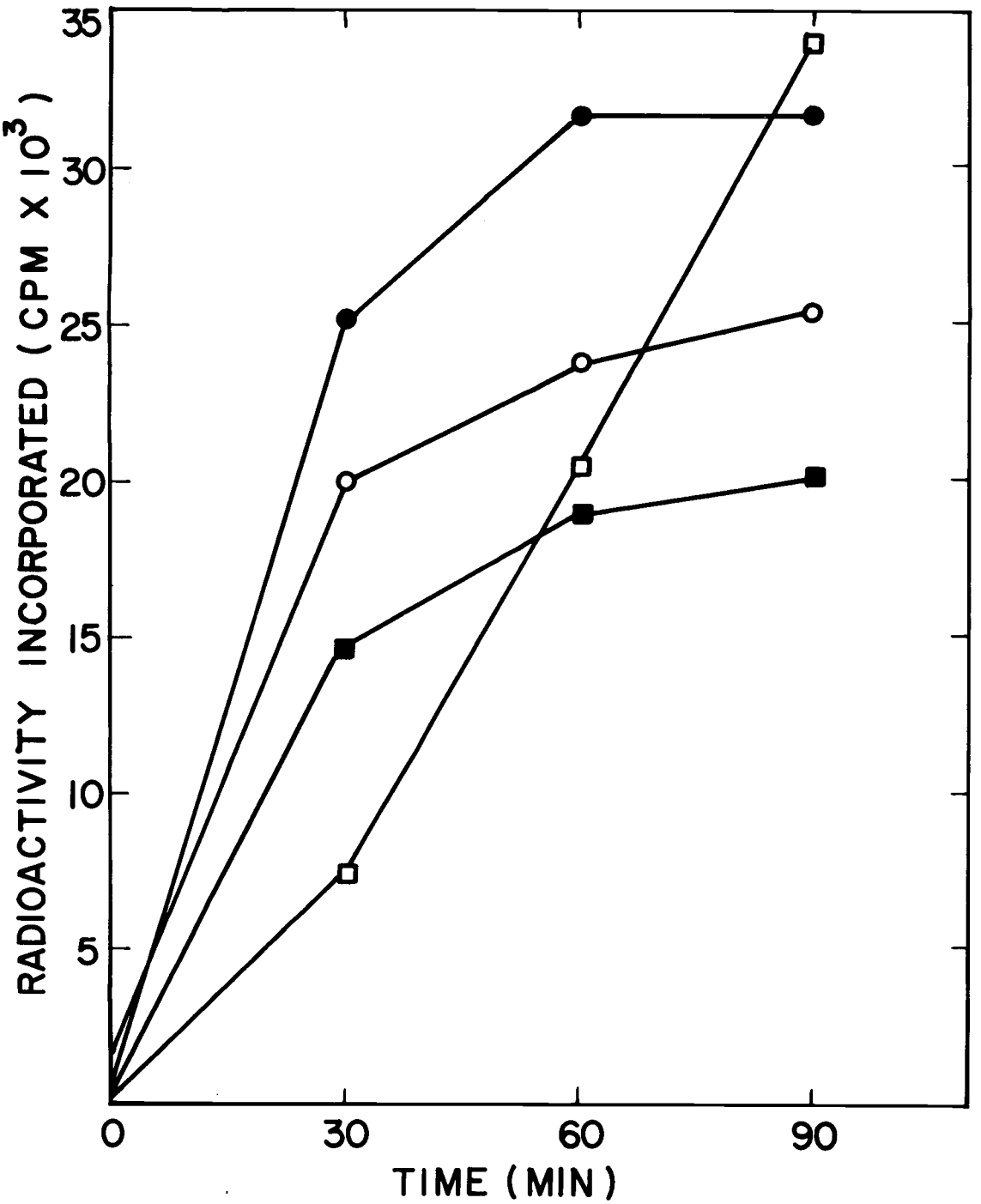


Figure 11. The effect of different DNA preparations on the rate of RNA synthesis by RNA polymerase. L. helveticus DNA, (□); L. casei 780 DNA, (●); B. coagulans ATCC 8038 DNA, (o); and L. leichmannii ATCC 7830 DNA (■).

Relationship of L. helveticus OSU DNA to DNA from Other Group I Organisms

Tritiated complementary RNA synthesized using L. helveticus OSU DNA as primer, was reacted with OSU DNA and DNA from selected thermobacteria (Table 8). L. bulgaricus ATCC 12278 gave a 94.8% reaction, indicating an extensive homology in base sequence with L. helveticus DNA. Identical DNA base compositions, 38.3 moles % GC (Table 4), support this observation. Davis (22) also suggested a close relationship between L. helveticus and L. bulgaricus and Sharpe and Wheater (60) have shown that L. helveticus (Orla-Jensen) Holland is a member of the serological group L. bulgaricus, group A. However, Rogosa and Sharpe (54) also indicated that L. bulgaricus is a member of serological group E. Since strains of L. bulgaricus are often confused with L. helveticus or L. jugurti, L. bulgaricus ATCC 12278 may have been classified erroneously and should be redesignated L. helveticus. The only consistent difference observed between L. bulgaricus ATCC 12278 and L. helveticus OSU was the fermentation of maltose by the latter strain. However, a difference in the fermentation of one carbohydrate, involving the mutation of one gene, is insufficient evidence for species separation and identification.

L. (jugurti) bulgaricus ATCC 7993 and L. jugurti ATCC 521

Table 8. Hybridization of DNA from several thermobacteria with L. helveticus OSU tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to OSU DNA[b]
	%	counts/minute	%
L. helveticus OSU	(1) 28.5 (2) 32.4	18,096 20,539	100.0
L. jugurti ATCC 521	16.9	10,680	55.3
L. (jugurti) bulgaricus ATCC 7993	12.8	8,109	42.0
L. bulgaricus ATCC 12278 (GA)	28.9	18,317	94.8
E. coli	0.0	0	0.0

[a] RNA bound relative to total radioactivity, acid-precipitable activity of OSU tritiated RNA was 63,376 counts/minute.

[b] Quantity of radioactive RNA (counts/minute) retained by the filter with OSU DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with OSU tritiated RNA.

gave cross reactions of 42.0 and 55.3%, respectively (Table 8). These strains appeared to be more closely related to each other than to either L. helveticus or L. bulgaricus ATCC 12278. The American Type Culture Collection (personal communication) suggested that strain 7993 may have mutated and can no longer be distinguished from L. helveticus. However, species separation was possible on the basis of polynucleotide homology. In addition, strain 7993 fermented glucose, galactose, mannose and lactose; fructose and maltose, as well as a variety of other carbohydrates, were not utilized. The inability to ferment fructose and maltose is characteristic of Orla-Jensen's Thermobacterium jugurti. Therefore, strain ATCC 7993 appeared to be a possible neotype for L. jugurti Rogosa and Sharpe (1).

The data for L. helveticus and L. jugurti ATCC 521 (Table 8) indicated that 45% of the DNA base sequences of the latter strain are different from those of L. helveticus. Although these two species appeared to be separate and distinct, they may also be varieties of a single species as suggested by Rogosa and Sharpe (54).

The demonstration of DNA-RNA complex formation between DNA isolated from strains ATCC 7993, ATCC 521 and ATCC 12278 and RNA synthesized with strain OSU DNA as primer was expected from examination of available physiological and serological data. In addition, Mills (46) has shown a close immunological relationship

between L. jugurti ATCC 521 and several strains of L. helveticus and L. bulgaricus.

Relationship of L. casei 316 DNA to DNA from Other Group II Organisms

Tritiated RNA, synthesized using L. casei 316 DNA as primer, was reacted with 316 DNA and DNA from L. casei C5, L. plantarum P5, L. buchneri BC1 and L. brevis X1. (L. viridescens DNA was not hybridized.) L. casei C5 gave a 96.5% reaction, indicating extensive DNA homology with L. casei 316 (Table 9). No cross reactions were observed for L. plantarum, L. buchneri, L. brevis and E. coli; the unrelated control. Since the heterofermentative species, L. buchneri and L. brevis, differ so markedly from L. casei, the lack of an appreciable reaction was not unexpected. The phenotypic similarities often observed for L. casei and L. plantarum were not reflected in their DNA homology, and these two species were easily differentiated. Species separation of L. casei and L. plantarum has also been accomplished by chromatographic techniques (17) and by infra-red spectroscopy (36). Serologically, strains of L. plantarum belong in group D, while strains of L. casei possess either the group B or C antigen (59). In addition, L. plantarum is the only Lactobacillus species which contains diaminopimelic acid as a cell wall constituent.

Tritiated RNA was also synthesized using L. casei 780 DNA as

Table 9. Hybridization of DNA from selected lactobacilli with L. casei 316 tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to 316 DNA[b]
	%	counts/minute	%
L. casei 316	16.0	6,810	100.0
L. casei C5	15.5	6,576	96.5
L. plantarum P5	0.0	0.0	0.0
L. buchneri BC1	0.0	0.0	0.0
L. brevis X1	0.0	0.0	0.0
E. coli	0.0	0.0	0.0

[a]RNA bound relative to total radioactivity, acid-precipitable activity of 316 tritiated RNA was 42,400 counts/minute.

[b]Quantity of radioactive RNA (counts/minute) retained by the filter with 316 DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with 316 tritiated RNA.

primer and was reacted with 780 DNA and DNA from other L. casei strains. The relationship of 780 DNA to DNA from strains C5, 300, 316, 356, C17, OSU and ATCC 9595 is shown in Table 10. A 90% or greater reaction was observed for the two non-lactose fermenting strains, 300 and 316, and the L. casei var. casei strains, 356, C5 and ATCC 9595. L. casei var. rhamnosus, C17 and OSU, gave cross reactions of 50.1 and 58.4%, respectively. Strains of L. casei var. casei contain either group B or C antigen, while L. casei var. rhamnosus strains possess only the group C antigen. Generally, the serological division of L. casei into groups B and C can be recognized by chromatographic patterns of cellular amino acids and peptides (17) and by infra-red absorption spectra (36). The homology methods employed also appear to be useful for strain as well as species separation, however, a more extensive study, involving additional strains and reciprocal reactions is required for confirmation.

Relationship of L. cellobiosus G1 DNA to DNA from Other Group III Organisms

Tritiated RNA, synthesized using L. cellobiosus G1 DNA as primer, was reacted with G1 DNA and DNA from L. fermenti F1, L. leichmannii ATCC 7830, L. delbrueckii ATCC 9649, L. lactis 39-A and E. coli; unrelated control (Table 11). L. fermenti F1 gave a 76.4% reaction, indicating substantial homology with L. cellobiosus

Table 10. Hybridization of DNA from several strains of L. casei with L. casei 780 tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to 780 DNA[b]
	%	counts/minute	%
L. casei 780	15.2	6,278	100.0
L. casei C5	15.7	6,474	100.0
L. casei 300	13.7	5,675	90.4
L. casei 316	15.1	6,225	99.2
L. casei 356	14.0	5,796	92.3
L. casei C17	7.6	3,143	50.1
L. casei OSU	8.9	3,665	58.4
L. casei ATCC 9595	19.3	7,956	100.0
E. coli	0.0	0.0	0.0

[a] RNA bound relative to total radioactivity, acid-precipitable activity of 780 tritiated RNA was 41,316 counts/minute.

[b] Quantity of radioactive RNA (counts/minute) retained by the filter with L. casei 780 DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with 780 tritiated RNA.

Table 11. Hybridization of DNA from selected lactobacilli with L. cellobiosus G1 tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to G1 DNA[b]
	%	counts/minute	%
L. cellobiosus G1	(1) 18.0 (2) 16.8	8,493 7,959	100.0
L. fermenti F1	(1) 13.0 (2) 13.6	6,159 6,422	76.4
L. leichmanni ATCC 7830	0.2	117	1.4
L. delbrueckii ATCC 9649[c]	0.0	0.0	0.0
L. delbrueckii ATCC 9649[d]	0.7	333	4.0
L. lactis 39-A	0.4	212	2.5
E. coli	0.0	0.0	0.0

[a] RNA bound relative to total radioactivity, acid-precipitable activity of tritiated RNA was 47,168 counts/minute.

[b] Quantity of radioactive RNA (counts/minute) retained by the filter with G1 DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with G1 tritiated RNA.

[c] L. delbrueckii DNA, 10-12μg.

[d] L. delbrueckii DNA, 21 μg.

G1 DNA. Similar DNA base compositions (Table 4) and several common physiological characteristics (54) confirm this species similarity. However, heterofermentative L. fermenti and L. cellobiosus do differ, mainly in the fermentation of cellobiose and hydrolysis of esculin by the latter organism. Insignificant cross reactions (4% or less) were observed for L. leichmanni, L. delbrueckii, L. lactis and E. coli (Table 11). Therefore, L. fermenti and L. cellobiosus comprised a distinct subdivision within Group III.

Since L. leichmannii, L. lactis and L. delbrueckii may be related (33), tritiated RNA, synthesized using L. leichmannii ATCC 7830 primer DNA, was reacted with 7830 DNA and DNA from L. leichmannii ATCC 4797, L. delbrueckii ATCC 9649 and L. lactis 39-A (Table 12). Additional results entered in Table 12 will be discussed individually below. Strains 4797 and 39-A gave reactions of 100 and 98.4%, respectively. L. delbrueckii gave a cross reaction of 34.4%, however, when correction was made for the amount of DNA hybridized, a value of 57% was obtained. Similar results were obtained for L. delbrueckii in a subsequent experiment in which the concentration of DNA was increased to 18 μg. Therefore, it appears that L. lactis is more closely related to strains of L. leichmannii than to L. delbrueckii. Several strains of L. leichmannii were examined by Silva and Cheeseman (61) and all gave chromatographic patterns which differed little from those of L. lactis.

Table 12. Hybridization of DNA from selected lactobacilli and S. inulinus with L. leichmannii ATCC 7830 tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to 7830 DNA[b]
	%	counts/minute	%
L. leichmannii ATCC 7830	9.4	2,136	100.0
L. leichmannii ATCC 4797	10.0	2,269	100.0
L. delbrueckii ATCC 9649[c]	3.2	736	34.4
L. lactis 39-A	9.2	2,103	98.4
L. acidophilus Farr	12.3	2,802	100.0
L. acidophilus IFO 3532	0.7	160	7.4
S. inulinus	0.0	0.0	0.0
E. coli	0.7	166	7.7

[a] RNA bound relative to total radioactivity, acid-precipitable activity of 7830 tritiated RNA was 22,624 counts/minute.

[b] Quantity of radioactive RNA (counts/minute) retained by the filter with 7830 DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with 7830 tritiated RNA.

[c] L. delbrueckii DNA, 10-12 μg.

L. acidophilus Farr and L. acidophilus IFO 3532 gave reactions of 100 and 7.4%, respectively (Table 12). The high count rate observed for strain Farr may be due either to excess DNA or to variations in the ability to hybridize. The above reasons may also account for the high value previously noted for L. casei ATCC 9595 (Table 10). The 7.7% value noted for E. coli may represent short segments of homology with L. leichmannii DNA, however, it is more likely due to contamination or technical error. It was previously suggested that L. acidophilus Farr may have been classified erroneously and should be re-designated L. lactis. The above hybridization data (Table 12) substantiate such a species change, however, it should be emphasized that over years of continued transfer and associative growth, L. lactis may have been isolated and carried in our culture collection under the misnomer L. acidophilus.

Suzuki and Kitahara (65) reported a value of approximately 39 moles % GC for S. inulinus DNA. When purified DNA from the same strain was examined by thermal denaturation, a T_m value of 88.7 C (Figure 12), which corresponded to a GC content of 47.3 moles %, was obtained. Since a possible relationship between S. inulinus and L. leichmannii was suggested (65), denatured S. inulinus DNA was hybridized with tritiated 7830 DNA. The lack of a demonstrable reaction (Table 12) indicated the absence of homologous DNA regions.

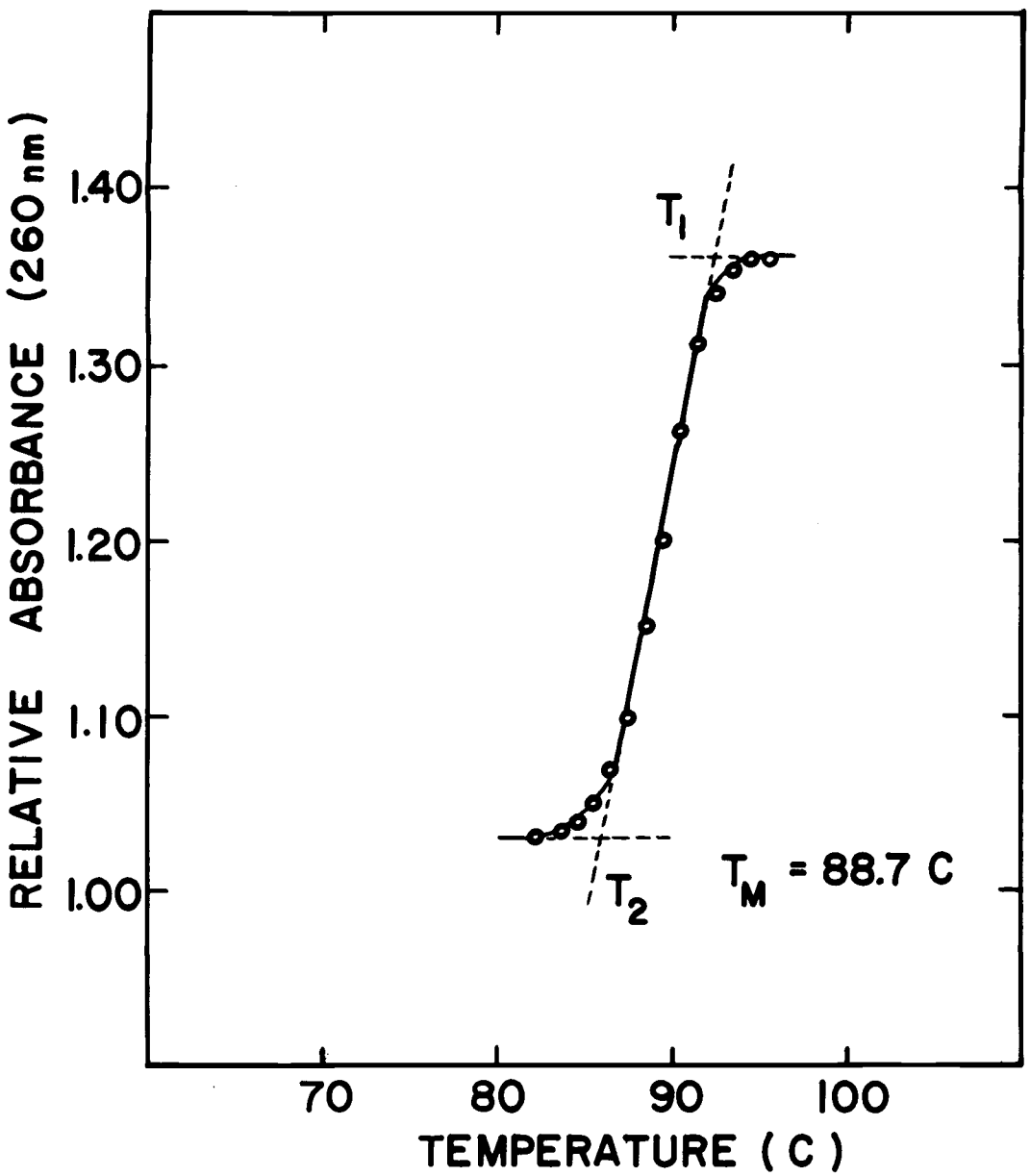


Figure 12. Absorbance-temperature denaturation profile of S. inulinus DNA.

Therefore, it remains to be determined whether S. inulinus is related to other recognized species of lactobacilli.

Relationship of DNA from Recognized Species of Lactobacilli to DNA from Selected Group I, II and III Organisms

The relationship of DNA from several species of lactobacilli to DNA from L. helveticus OSU (Group I), L. buchneri BC1 (Group II) and L. lactis 39-A (Group III) was examined. Tritiated RNA, synthesized using primer DNA from strains OSU, BC1 and 39-A, was reacted with DNA from other recognized Lactobacillus species. The quantity of radioactivity retained on the filters was expressed as a percentage of the counts/minute/microgram retained with homologous DNA. No appreciable cross reactions between Groups I, II and III were observed (Table 13). The reactions recorded for L. helveticus tritiated RNA and DNA from L. jugurti and L. bulgaricus were similar to those presented in Table 8. No significant cross reaction occurred with L. helveticus tritiated RNA and DNA from L. salivarius. Tritiated L. buchneri RNA reacted with L. brevis DNA to an extent of only 6.7% (Table 13). This was surprising, since these two heterofermentative species have very similar GC contents (Table 4) and are often considered closely related. Nevertheless, L. buchneri and L. brevis do have many variable characteristics (54). No appreciable reactions between L. buchneri and any of the other heterofermentative

Table 13. Hybridization of DNA from recognized species of lactobacilli with tritiated RNA from L. helveticus OSU, L. buchneri BC1 and L. lactis 39-A.

Source of DNA	Source of Tritiated RNA[a]: L. helveticus OSU — Radioactivity Retained per μg of DNA	L. helveticus OSU — Homology[b]	L. buchneri BC1 — Radioactivity Retained per μg of DNA	L. buchneri BC1 — Homology[b]	L. lactis 39-A — Radioactivity Retained per μg of DNA	L. lactis 39-A — Homology[b]
Group I	counts/minute	%	counts/minute	%	counts/minute	%
L. helveticus OSU	4,085	100.0	43	4.2	48	6.2
L. jugurti ATCC 521	2,368	57.9	27	2.6	74	9.6
L. bulgaricus ATCC 12278 (GA)	3,650	89.3	23	2.2	45	5.8
L. salivarius ATCC 11742	23	0.5	2	0.2	16	2.0
Group II						
L. casei 780	0	0.0	34	3.3	13	1.7
L. plantarum P5	14	0.3	20	2.0	6	0.7
L. viridescens ATCC 12706	6	0.1	6	0.6	7	0.9
L. buchneri BC1	28	0.6	1,005	100.0	14	1.8
L. brevis X1	174	4.2	68	6.7	9	1.1
Group III						
L. lactis 39-A	79	1.9	41	4.0	769	100.0
L. leichmannii ATCC 7830	16	0.4	5	0.5	---	---
L. delbrueckii ATCC 9649	15	0.4	0	0.0	---	---
L. fermenti F1	44	1.0	48	4.7	27	3.5
L. cellobiosus G1	25	0.6	8	0.8	19	2.4
L. acidophilus IFO 3532[c]	13	0.3	10	1.0	10	1.3
E. coli (unrelated control)	0	0.0	0	0.0	0	0.0

[a] Acid-precipitable activity of tritiated RNA; 23,000 - 107,000 counts/minute.

[b] The counts/minute of radioactive RNA retained by the membrane filter per microgram of homologous DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with homologous tritiated RNA.

[c] L. acidophilus IFO 3532 was placed in Group III solely on the basis of GC content.

species, L. viridescens, L. fermenti and L. cellobiosus, were evident. Cheeseman and Silva (18) could not distinguish L. brevis from L. buchneri on the basis of chromatographic behavior, nor could they show any pattern relationship with any of the other fermentation groups. A 9.6% or less reaction was observed when L. lactis tritiated RNA was reacted with DNA from L. helveticus, L. jugurti and L. bulgaricus. These values may represent only short segments of homology with L. lactis DNA.

The hybridization results presented, indicate the need for further subdivisions within each of the three major groups previously proposed. The following classification, consisting of six homofermentative and two heterofermentative subgroups, is suggested.

Group I contains (a) L. helveticus, L. jugurti, L. (jugurti) bulgaricus and L. bulgaricus ATCC 12278, which was indistinguishable from L. helveticus, and (b) L. salivarius.

Group II contains (a) L. casei, (b) L. plantarum, and (c) L. buchneri, which was further subdivided into (c_1) L. brevis and (c_2) L. viridescens.

Group III contains (a) L. fermenti and L. cellobiosus and (b) L. lactis, L. leichmannii and L. delbrueckii.

Since intermediate strains of L. acidophilus, as well as other species of lactobacilli exist, difficulty was encountered in grouping L. acidophilus IFO 3532. In addition, some strains of L. acidophilus

are often confused with Actinomyces bifidus (56). Gasser and Mandel (33) reported a mean GC value of 36.7 moles % for several strains of L. acidophilus. Suzuki and Kitahara (65) observed a value of 47.2 moles % GC for L. acidophilus 506. When DNA from strain IFO 3532 was examined by thermal denaturation, a value of 48.5 moles % GC was obtained. Therefore, solely on the basis of GC content, strain IFO 3532 was tentatively placed in Group III as an additional homofermentative subgroup.

The division of lactobacilli indicated above agreed in many respects with previously reported classifications based on serological reactions (58) and physiological characteristics (7). Serologically distinct species were easily distinguished by the homology technique employed and further subdivisions within several of the physiological groups of Briggs (7) were apparent. Although a majority of the recognized species of lactobacilli was examined, further investigations with additional strains are needed before a definite classification scheme can be proposed. Nevertheless, since authentic strains of a well established species will invariably show the same degree of genetic homology, the data constitute a firm foundation for species identification and classification. The only difficulties that one might encounter would be those related to the use of intermediate strains or improperly designated species.

The hybridization data also further magnified the extent of

compositional heterogeneity within the genus Lactobacillus. Several species can be considered significantly different as to question their classification as lactobacilli. For example, L. casei and L. plantarum have virtually no DNA base sequences in common, yet they are both considered lactobacilli. Davis (24) suggested that L. casei might represent the further evolution of L. plantarum and that heterofermenters evolved from homofermenters. Homofermentative species produce primarily lactic acid from fermentable carbohydrates, while heterofermenters produce acetic acid, ethyl alcohol, glycerol and carbon dioxide, in addition to lactic acid. Undoubtedly, several of the enzymes involved in carbohydrate metabolism have common functions which might be attributed to similar active sites (64). The lack of DNA homology between species would indicate that the rest of the protein or enzyme molecule was structurally quite different. Therefore, if one assumes a common phylogenetic origin for Lactobacillus species, compositional heterogeneity must be the result of a divergent evolutionary process. Since ribosomal RNA (r-RNA) sequences in bacteria (26, 27, 47), yeasts (5), plants (43) and protozoa (35) are more conserved than the base sequences of the rest of the genome, evolutionary divergence may be estimated by determining the extent of r-RNA homology. Since a frequent lack of DNA homology was observed among many of the species examined, a further investigation of species relatedness, as measured by 23s r-RNA hybrid formation,

should be considered. Even though DNA species are highly divergent, it may be possible to relate the divergent species to type species by comparing their r-RNA sequences.

Kitahara (38) suggested that certain "wild" lactobacilli were intermediate forms between homofermentative lactobacilli and the genus Bacillus. Nakayama (48) subsequently classified these intermediates as B. coagulans and called them spore-forming lactic acid bacteria. In addition, Davis (24) postulated that the genus Bacillus may be a convenient source from which lactobacilli might have originated. L. plantarum was considered to be the primitive lactobacillus which might link the genera Lactobacillus and Bacillus.

In a preliminary assessment of relatedness among lactobacilli and bacillus species, the DNA base compositions of three strains of B. coagulans were examined. The T_m values obtained for B. coagulans ATCC 8038, 8083 and 8043 were 87.7, 88.0 and 88.1 C, respectively. The mean GC content, 45.4 moles %, was similar to the mean value for L. plantarum and the GC content of L. casei ATCC 9595, 44.5 and 45.8 moles %, respectively (Table 4). Similar values for other strains of L. casei and L. plantarum have been reported (33, 34). Since a possible relationship between B. coagulans and the above streptobacteria may well exist, tritiated RNA, synthesized using B. coagulans ATCC 8038 DNA as primer, was reacted with 8038 DNA and DNA from L. casei, L. plantarum and additional

strains of B. coagulans. S. inulinus DNA, 47.3 moles %, was also hybridized. All strains of B. coagulans gave a 100% or greater reaction (Table 14). The high count rates observed for strains 8043 and 8083 were attributed to excess DNA. This was confirmed by determining the concentrations of the DNA stock solutions spectrophotometrically, as previously described, and re-evaluating the hybridization data. For example, strain 8043 retained an excess of 113 counts/minute/microgram of DNA when compared to the homologous reaction, 290 counts/minute/microgram. This difference in count rate was equivalent to 38%, thereby explaining the 138% reaction observed for strain 8043. No cross reactions were observed for L. casei, L. plantarum, S. inulinus or E. coli (Table 14). This obvious lack of DNA homology, indicative of the absence of similar genes, appeared to preclude any possibility of a phylogenetic relationship among the organisms examined. However, evolutionary development may have followed a number of independent routes, each resulting in extensive changes in polynucleotide sequence from a primary DNA of a particular structure or composition. Further investigation with additional species and strains is required before definite conclusions can be made as to phylogenetic relationships between the genera Bacillus and Lactobacillus. As previously stated, hybridizations involving r-RNA could possibly lead to a more complete understanding of the phylogenetic origin of lactobacilli.

Table 14. Hybridization of DNA from selected lactobacilli and S. inulinus with B. coagulans ATCC 8038 tritiated RNA.

Source of DNA	Incorporation[a]	RNA Retained on Filter	RNA Bound Relative to 8038 DNA[b]
	%	counts/minute	%
B. coagulans ATCC 8038	6.8	2,554	100.0
B. coagulans 8043	10.7	4,030	100.0
B. coagulans 8083	13.5	5,099	100.0
L. plantarum P5	0.0	0.0	0.0
L. casei ATCC 9595	0.0	0.0	0.0
S. inulinus	0.0	0.0	0.0
E. coli	0.0	0.0	0.0

[a] RNA bound relative to total radioactivity, acid-precipitable activity of 8038 tritiated RNA was 37,720 counts/minute.

[b] Quantity of radioactive RNA (counts/minute) retained by the filter with 8038 DNA was set equal to 100%. Results for each DNA were expressed as a percentage of the counts/minute of DNA retained with 8038 tritiated RNA.

Although the stabilities of homologous and heterologous hybrid duplexes were not examined, the base sequence complementarities observed in this study were considered specific and were generally reproducible (±5%) within and between experiments. In addition, several reciprocal reactions gave similar to identical results with the same degree of reproducibility.

The percentages of homology reported herein should not be considered exact values, since some degree of species specificity among RNA polymerases may exist.

Extracellular Nuclease Studies

Since some group N lactic streptococci have been reported to produce extracellular deoxyribonuclease (45), a preliminary examination for this enzyme in lactobacilli was conducted using deoxyribonuclease test agar (Difco) plates. Several strains, both homofermentative and heterofermentative, were capable of producing detectable amounts of nuclease (Table 15). The extracellular enzyme produced by *L. casei* strains OSU, C5 and 300 (Figure 13) and 780 and 316 (Figure 14) was easily discernible. Enzyme activity of *L. delbrueckii*, *L. leichmannii* and *L. fermenti* is shown in Figure 15. Under the experimental conditions employed, *L. lactis* 39-A, *L. plantarum* 17-5 and P5, *L. casei* ATCC 9595 (Table 15) and *S. inulinus* were devoid of enzyme activity. Equivocal or negative

Table 15. Extracellular nuclease production by strains of lactobacilli.

Strain	Extracellular Nuclease[a]	
	DNA-Agar	DNA-Agar Plus Glucose (2%)
L. leichmannii ATCC 7830	±	+
L. leichmannii ATCC 4797	±	+
L. viridescens ATCC 12706	+	+
L. lactis 39-A	-	-
L. salivarius ATCC 11742	+	+
L. brevis X1	-	±
L. (jugurti) bulgaricus ATCC 7993	s	s
L. delbrueckii ATCC 9649	+	+
L. cellobiosus G1	±	±
L. fermenti F1	+	+
L. buchneri BC1	s	+
L. bulgaricus ATCC 12278	s	+
L. jugurti ATCC 521	s	+
L. helveticus OSU	s	±
L. plantarum P5	-	-
L. plantarum 17-5	-	-
L. acidophilus Farr	±	±
L. casei ATCC 7469	±	-
L. casei OSU	+	-
L. casei C5	+	±
L. casei 300	+	-
L. casei 356	-	±
L. casei 780	+	-
L. casei ATCC 9595	-	-
L. casei 316	+	-

[a] ± = weak positive reaction.

+ = positive reaction.

- = negative reaction.

s = slight growth.

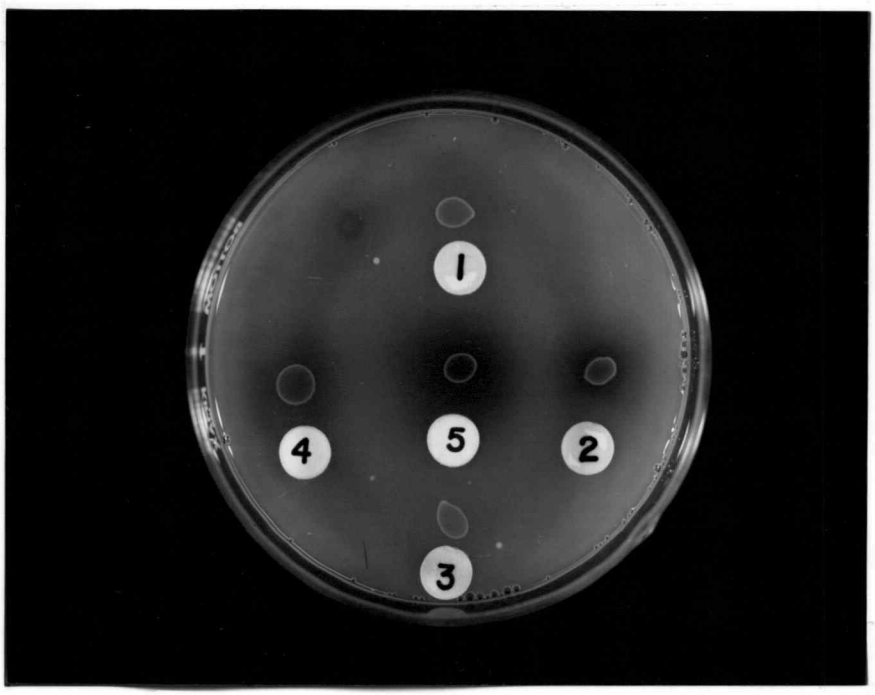


Figure 13. Extracellular nuclease production by several strains of L. casei. Strains: (1) L. casei ATCC 7469, (2) L. casei OSU, (3) L. casei C17 (same as ATCC 7469), (4) L. casei C5 and (5) L. casei 300.

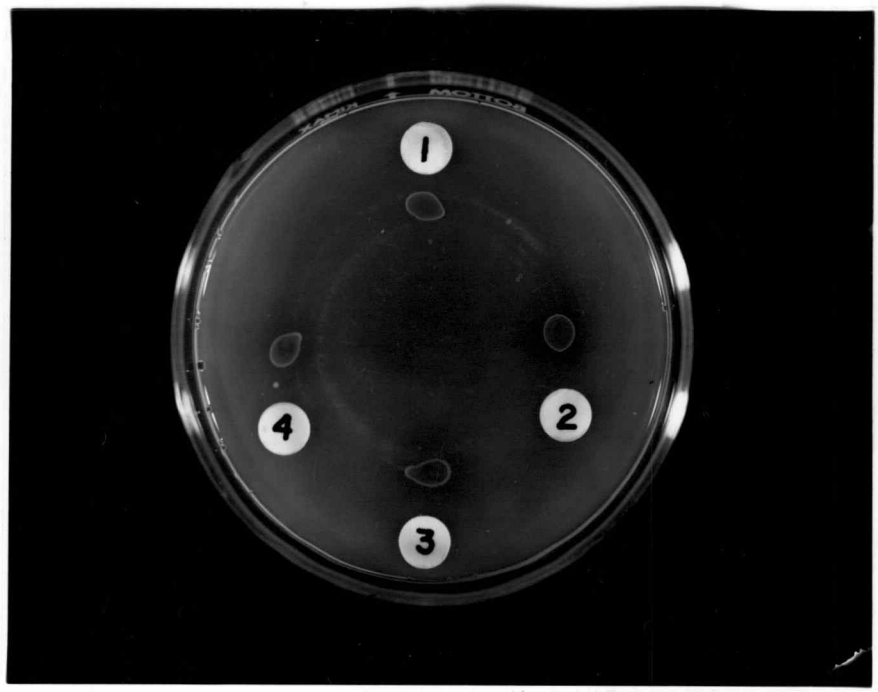


Figure 14. Extracellular nuclease production by strains of L. casei. Strains: (1) L. casei 356, (2) L. casei 780, (3) L. casei 316 and (4) L. casei ATCC 9595.

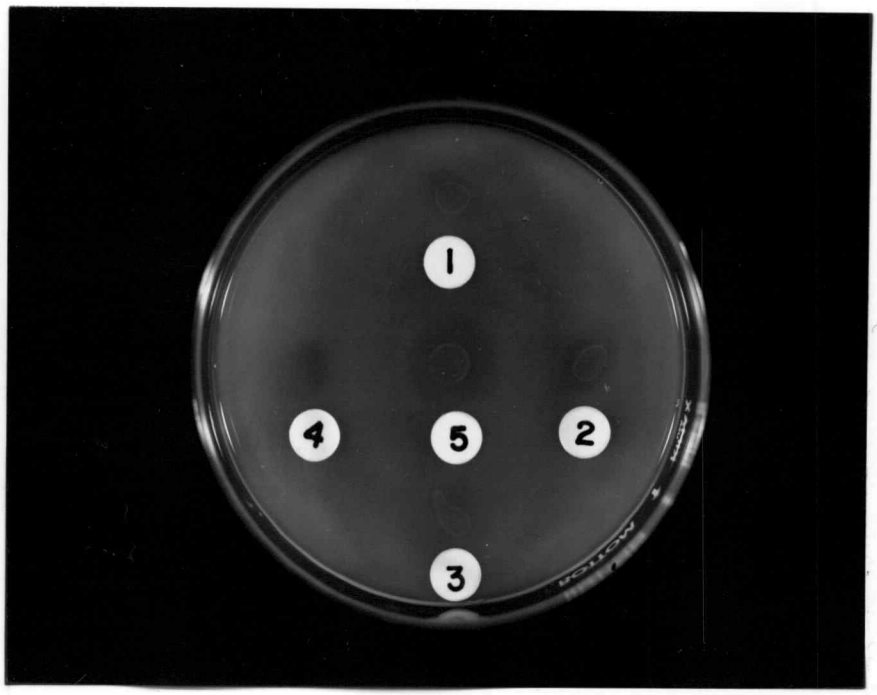


Figure 15. Extracellular nuclease production by several strains of lactobacilli. Strains: (1) L. leichmannii ATCC 4797, (2) L. delbrueckii ATCC 9649, (3) L. cellobiosus G1, (4) L. leichmannii ATCC 7830 and (5) L. fermenti F1.

results, however, do not necessarily imply that the culture completely lacked extracellular nuclease activity, since variations in the enzyme or rate of enzyme synthesis may exist. In addition, the medium and cultural conditions employed did not allow optimal growth of all species (Table 15).

Hydrolytic activity was observed also for all strains of B. coagulans (Figure 16). When the assay medium was supplemented with glucose (0.5, 1.0 and 2.0%), growth of B. coagulans was more luxuriant but enzyme production was decreased markedly. The inhibitory effect of glucose was also noted for L. casei strains ATCC 7469, OSU, 300, 780 and 316 (Table 15); however, enzyme production by several other species of lactobacilli appeared to be stimulated by the presence of 2% glucose (Table 15).

Nuclease activity was also examined using modified MRS-medium supplemented with either DNA or RNA as described previously. All species capable of depolymerizing DNA also hydrolyzed RNA (Table 16). Depending on the species examined, zones of hydrolysis ranged from 1 to 4 mm in diameter; L. fermenti and B. coagulans exhibited the most activity. L. casei 356 and ATCC 9595, L. plantarum P5 and 17-5 and L. brevis X1, incapable of producing detectable amounts of nuclease on Difco deoxyribonuclease plates, gave positive reactions on MRS-medium supplemented with either DNA or RNA. S. inulinus also hydrolyzed both substrates when

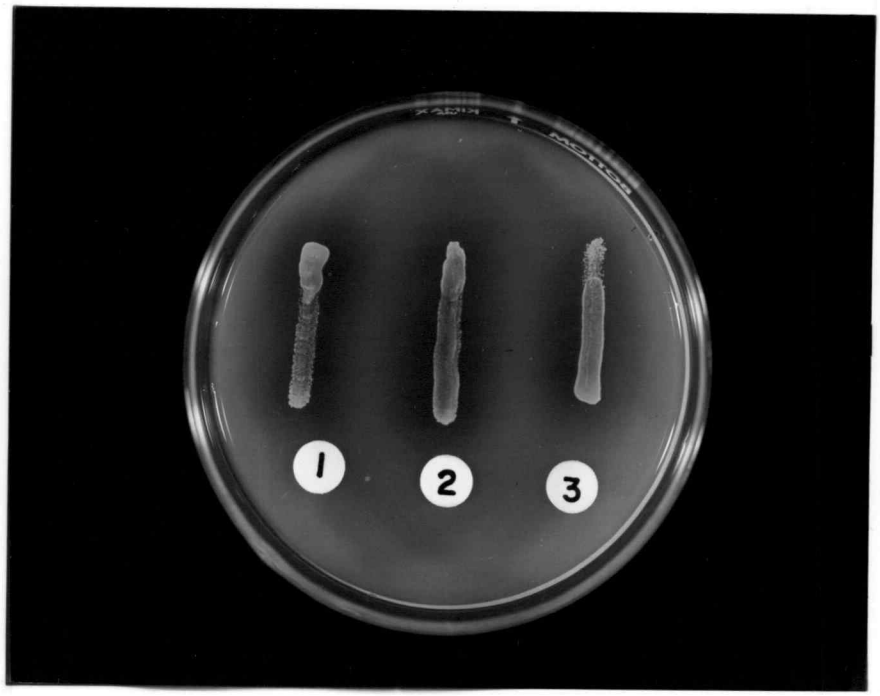


Figure 16. Extracellular nuclease production by B. coagulans. Strains: (1) B. coagulans 8083, (2) B. coagulans 8043 and (3) B. coagulans ATCC 8038.

Table 16. Extracellular nuclease production by lactobacilli and B. coagulans.

Species and Strains	Extracellular Nuclease	
	DNA-MRS Agar	RNA-MRS Agar
L. helveticus OSU	+[a]	+
L. jugurti ATCC 521	+	+
L. plantarum P5	+	+
L. plantarum 17-5	+	+
L. (jugurti) bulgaricus ATCC 7993	s[b]	s
L. acidophilus Farr	s	s
L. lactis 39-A	s	s
L. casei ATCC 7469	+	+
L. casei C5	+	+
L. casei 300	+	+
L. casei ATCC 9595	+	+
L. casei OSU	+	+
L. casei 780	+	+
L. casei 356	+	+
L. casei 316	+	+
L. brevis X1	+	+
L. salivarius ATCC 11742	s	s
L. viridescens ATCC 12706	+	+
L. bulgaricus ATCC 12278 (GA)	s	s
L. buchneri BC1	+	+
L. fermenti F1	++[c]	++
L. cellobiosus G1	+	+
L. leichmannii ATCC 7830	s	s
L. leichmannii ATCC 4797	s	s
L. delbrueckii ATCC 9649	s	s
B. coagulans ATCC 8038	++	++
B. coagulans 8083	++	++
B. coagulans 8043	++	++

[a] + = positive reaction, zones of hydrolysis extended 1 to 2 mm from colony edge.

[b] s = slight growth.

[c] ++ = positive reaction, zones of hydrolysis extended 3 to 4 mm from colony edge.

incubated at 37 C for three days in a candle jar.

Enzymatic degradation of DNA and RNA was observed also in cell-free, MRS-broth supernatants from strains of L. casei, B. coagulans and L. fermenti grown at 37 C for 48 hours. Since cellular lysis was not evident, the nucleolytic activity (6-8 units/ml) exhibited by the above species further substantiated the extracellular nature of the enzyme or enzymes involved.

Further investigation with purified enzyme preparations is suggested to determine whether the enzymatic activity observed was the result of two distinct enzymes or a single enzyme, such as a non-specific phosphodiesterase. Chromatographically pure preparations which are active on DNA, RNA and RNA-core, the polynucleotide which is refractory to pancreatic ribonuclease activity, might indicate the presence of a single enzyme. Further justification for a single entity could be obtained from analyses of chromatographic behavior, kinetic properties, pH optima and the effects of temperature and specific cations.

SUMMARY

Since DNA base compositions of recognized species of lactobacilli ranged from 32.4 to 51.9 moles % GC, compositional heterogeneity within the genus Lactobacillus was apparent. Comparison of thermal denaturation data from this study with results from previous reports (9-13) revealed several differences. Significant variations were noted for L. leichmannii, L. delbrueckii and L. lactis. Additional differences were observed also for L. helveticus, L. jugurti, L. cellobiosus and L. salivarius. With the exception of L. acidophilus Farr and L. bulgaricus ATCC 12278, the data substantiated results obtained by buoyant density measurements (33) as well as by chemical analyses (34).

On the basis of GC content, the lactobacilli were placed in three groups similar to those proposed by Gasser and Sebald (34) and Gasser and Mandel (33). Further distinctions within each group were apparent when the results of DNA-RNA hybridizations were assessed and a classification scheme, consisting of six homofermentative and two heterofermentative subgroups, was proposed. The hybridization data also further magnified the extent of compositional heterogeneity within the genus Lactobacillus.

The possibility of a phylogenetic relationship between the genus Bacillus and selected species of lactobacilli was discussed also. An

obvious lack of DNA homology among the species examined appeared to preclude any possibility of a common phylogenetic origin. Nevertheless, evolutionary development may have followed a number of independent routes, each resulting in extensive changes in polynucleotide sequence from a primary DNA of a particular structure.

Several species of lactobacilli, B. coagulans and S. inulinus produced detectable amounts of extracellular nuclease. All species capable of depolymerizing DNA also hydrolyzed RNA. Further investigation with purified enzyme preparations was suggested to determine whether the enzymatic activity observed was the result of two specific enzymes or a single entity, such as a non-specific phosphodiesterase.

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APPENDIX

APPENDIX I

[5-^{3}H] Cytidine 5'-triphosphate, 250 μc
Specific activity, 13.5 c/mmole
Solution, 50% ethyl alcohol
Salt form, tetralithium

Sample calculation:

13,500 mc/mmole = 13,500 μc/μmole
250 μc
13,500 μc/μmole = 0.018 μmoles = 18 mμmoles

Molecular weight of CTP = 483.2
1 mμmole = 483.2 mμg
100 mμmoles = 48,320 mμg

Stock solution: 100 ml of distilled water plus 4.8 mg of CTP
Add 1 ml (100 mμmoles) of CTP stock solution plus 0.5 ml of distilled water to vial containing tritiated substrate (CTP-^{3}H). The concentration of ethyl alcohol is reduced from 50 to 17%. The substrate is lyophilized and diluted with distilled water so that 0.01 ml contains approximately 20 mμmoles.

Above procedure reduced the specific activity of CTP-^{3}H to approximately 1.35 c/mmole.

1 μc = 2.22×10^6 dpm at 10% efficiency
1 μc = 2.22×10^5 cpm
0.01 ml = 20 mμmoles = 50 μc
$(50)(2.22 \times 10^5) = 1.1 \times 10^7$ cpm

Calculations for [5-^{3}H] uridine 5'-triphosphate, [8-^{3}H] adenosine 5'-triphosphate and [^{3}H] guanosine 5'-triphosphate were carried out as described above.