Cytological and Deoxyribonucleic Acid-Deoxyribonucleic Acid Hybridization Studies on *Lactobacillus* Isolates from San Francisco Sourdough

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Molecular taxonomic and electron microscopy studies were performed on four bacterial isolates obtained from different sources of San Francisco sourdough (SD). These bacteria were first isolated by Kline and Sugihara who tentatively described them as a previously unreported species of heterofermentative *Lactobacillus*; they suggested the name *Lactobacillus sanfrancisco*. The guanine plus cytosine base composition (%GC) of the deoxyribonucleic acid (DNA) ranged from 38 to 40%. The possible genetic relatedness of these SD isolates to five known species of *Lactobacillus* with comparable GC contents was assessed in the present work by means of DNA-DNA hybridization competition experiments. Little or no DNA homology was observed between the SD bacteria and the known species. The SD bacteria exhibited a high degree of homology (>88%) among themselves, suggesting that the four isolates were identical taxonomically. Also, the electron photomicrographs revealed structures similar to those of gram-positive bacilli. Accordingly, since these SD isolates have the characteristic phenotypic and morphological properties of the genus *Lactobacillus* and are not related genetically to any known species, the tentative characterization by the above workers of these isolates as a new species is substantiated.

The bacteria responsible for the souring action in San Francisco sourdough (SD) French breads were first isolated and characterized by Kline and Sugihara (19), who considered these isolates to be a previously undescribed species of the genus *Lactobacillus*, producing lactic and acetic acids and CO₂ from maltose. Certain unusual properties of these isolates include: a requirement for maltose for rapid and heavy growth; inability to initiate rapid growth at pH values above 6; complete inhibition of growth by sorbic acid; and an unusual degree of nutritional fastidiousness including an absolute requirement for unsaturated fatty acid (Tween 80) and a need for freshly prepared yeast extractives.

In view of these facts, confirmation of their genetic distinctiveness has been attempted through the deoxyribonucleic acid (DNA)-DNA hybridization technique. This approach offers a direct measure of the similarity or homology of the polynucleotide sequences in DNA molecules; therefore the genetic relatedness of the bacteria to known species can be quantitatively determined.

This paper presents results of thermal denaturation (Tₘ) and DNA-DNA hybridization analyses of four strains (B, C, L, and T) of SD bacteria. The hybridization was carried out with five known *Lactobacillus* species selected on the basis of fermentative type (5, 34) as well as the percent guanine plus cytosine (%GC) content of the DNA (11, 12, 27, and 28). The results confirm that the lactobacilli of SD are a new species of *Lactobacillus*. Electron microscopy was used to characterize further the phenotypic properties of these unique organisms.

**MATERIALS AND METHODS**

Organisms and media. The strains used are listed in Table 1. Their identity was confirmed...
according to the criteria established by the Taxonomic Subcommittee on Lactobacilli and Closely Related Organisms (14). The enzymatic method of Mattsson (26) was used to determine the type of lactic acid isomer produced.

The known lactobacilli were grown in MRS broth (9) at their optimum growth temperature. The SD organisms were grown in the medium (SD broth) described previously (19) and incubated in an air atmosphere at 30 C. Cells for DNA extraction were washed twice with saline-ethylenediaminetetraacetic acid (0.15 M NaCl plus 0.1 M EDTA, pH 8) and stored frozen until used.

Cultural properties. To confirm that the four strains of SD bacteria exhibited the same phenotypic properties described by Kline and Sugihara (19), cultural studies were made. Methods of these authors were used as well as those described in the Manual of Microbiological Methods (4).

Electron microscopy. Two types of cultures were prepared for electron microscopy. (a) Actively growing cultures in SD broth were transferred into SD broth and incubated at 30 C under 50 to 60% carbon dioxide tension for 18 to 20 h (to stationary growth phase). Cells were harvested by centrifugation at 15,000 × g for 30 min and washed twice in dilute, 0.01 M potassium phosphate buffer, pH 7.0. These cells were suspended 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 SD broth into fresh medium with incubation for 20 min at 30 C. These cells were treated similarly to those of the stationary-phase cells. The cells were not fixed unless otherwise stated. When fixed, 1% glutaraldehyde in the 0.01 M phosphate buffer was used. After fixation, the cells were washed 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 blotted-dried with chromatographic strips and then observed in a Philips EM2 electron microscope.

For thin sections, early stationary-growth-phase cells were fixed in 5% glutaraldehyde 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 postfixed with 1% osmium tetroxide in the 0.01 M phosphate buffer for 6 to 7 h. Samples were dehydrated in increasing concentrations (30, 50, and 70%) of acetone for 10 min. The cells then 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 cubes were mixed in a plastic (Araldite 6005 and EPON 815)—acetone (2:1) mixture (30) in which 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.

DNA preparation and labeling. DNA extraction was carried out by using a modification of the technique of Marmur (24). As it was difficult to lyse the organisms, a dual enzyme system of lytase (20%, vol/vol; BBL) or lytic factor (5%, vol/vol; 37) plus lysozyme (2 to 4 mg/ml; Sigma Chemical Co.) was used. The suspension was incubated at 37 C and checked for complete 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 by an increase in viscosity. Deproteination (initial and the one after ribonuclease treatment) were carried out with saline-EDTA equilibrated liquid phenol at pH 7 to 8; other deproteinizations (a minimum of four) were carried out with Sevag solution (chloroform-isooamylalcohol, 24:1).

Strain B of SD bacteria was grown in SD broth containing 1 μCi of ^4H-E-6-thymidine per ml for 36 h at 30 C. Cells were harvested, washed twice, and used for extraction of DNA. The specific activity of the labeled DNA, as determined in a dual channel Beckman Tri-Carb liquid scintillation spectrometer, was 4,600 counts per min per μg.

The DNA base composition was determined by using T_m data and the equation of Mandel et al. (23). Escherichia coli K-12 DNA was included in each melting as the internal standard. The difference between the T_m of the DNA of test organism and that of E. coli (ΔT_m) was used for the actual calculation of the DNA base compositions.

Loading membrane filters. Unlabeled strain B DNA was loaded onto the membrane filters (B-6; Schleicher and Schuell Co.) by the method of Gillespie and Spiegelman (13). The DNA in single-strength standard saline citrate (1 × SSC; 0.15 M NaCl + 0.015 M trisodium citrate, pH 7.0 ± 0.2) was diluted with 0.1 × SSC 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) 6 × SSC to give a final DNA concentration of 10 μg/ml. The alkali was neutralized with 2 N NaH_2PO_4, and the denatured DNA was gravity loaded onto membrane filters (prewashed with 6 × SSC) by using slight suction and then washed with 200 ml of 6 × SSC. The filters were dried overnight at 25 C and then incubated at 80 C for 2 to 3 h in a vacuum oven. Filters (6 mm in diameter) were

| Organism                    | Strain no. | Source* |
|-----------------------------|------------|---------|
| Lactobacillus acidophilus   | 4356       | ATCC    |
| L. brevis                   | 473        | NCDO    |
| L. helveticus               | 8018       | ATCC    |
| L. jugurti                  | 521        | ATCC    |
| L. salivarius               | 11742      | ATCC    |

* ATCC, American Type Culture Collection; NCDO, National Collection of Dairy Organisms, Reading, England; WRRL, Western Regional Research Laboratory, Albany, Calif.

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TABLE 1. Identity and source of organisms used

| Organism               | Strain no. | Source* |
|------------------------|------------|---------|
| Lactobacillus acidophilus | 4356       | ATCC    |
| L. brevis              | 473        | NCDO    |
| L. helveticus          | 8018       | ATCC    |
| L. jugurti             | 521        | ATCC    |
| L. salivarius          | 11742      | ATCC    |
| SD+ bacterium          | B          | WRRL    |
| SD bacterium           | C          | WRRL    |
| SD bacterium           | L          | WRRL    |
| SD bacterium           | T          | WRRL    |
| Escherichia coli       | K-12       | ATCC    |
punched out and stored in a vacuum at 4 C until used.

Hybridization experiments. DNA homologies were estimated by DNA competition experiments (15, 21). DNA samples were dialyzed three times against 6 x SSC containing 40% formamide at 4 C for 18 to 24 h. Samples were sheared in a French pressure cell at pressure of 10,000 to 15,000 lb/in². Just before use, the DNA samples were denatured by immersion in slow-boiling water for 10 min and immediately quenched in ice to stop reassociation. The 6-mm filters were incubated at 25 C or 15 C below the Tm in 6 x SSC containing 40% formamide, 1 μg of tritium-labeled SD strain B DNA, and 300 μg of competitor DNA. Each filter contained approximately 12 to 15 μg of unlabeled strain B DNA. In the experiments in which the samples were incubated at 25 C below the Tm, the final volume of hybridization solution was 1.0 ml, whereas in the Tm – 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 incubation, the filters were washed in three to five changes of 2 x SSC 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.

RESULTS

Our cultural studies confirmed (19) that the SD bacteria were gram positive, nonmotile, nonsporeforming rods with an optimum growth temperature of 30 C, but that they were capable of growth over the range from 13 to 40 C. They were catalase negative and formed larger colonies (1.0 to 1.5 mm diameter) when grown for 48 h on agar plates in the presence of some (5 to 15%) CO₂. In an air atmosphere, colonies were generally 0.5 mm in diameter or less, although they grew with uniform turbidity in broth culture in an air environment at 30 C. From these observations it was concluded that the organisms were microaerophilic. They also required fresh yeast extract for growth. Strains B, L, and T produced optically inactive lactic acid, whereas strain C only produced the L+ isomer. The four strains produced acetate, ethanol, and CO₂ in significant amounts and were thus judged to be heterofermentative. A carbon balance study of their maltose metabolism, the only sugar rapidly fermented, will be considered in a subsequent report.

Figure 1 illustrates a negatively stained preparation of SD strain B taken from the stationary growth phase. Cell sizes were 0.5 to 0.7 μm x 1 to 3 μm. Each cell typically possessed one to three large internal membranous structures or mesosomes located at the plane of cell division or at a terminal position and usually exhibited structural anomalies at the membrane wall interface. Hurst and Stubbs (16) 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 Fig. 2, an identical preparation of strain T. When glutaraldehyde fixation was employed, no plasmolysis was observed (Fig. 3). The cell wall of this preparation (strain B) does not stand out, and the only obvious structures are mesosomes.

Figure 4 is a thin section of SD strain L which illustrates the typical thick cell wall characteristic of gram-positive bacteria and the mesosomes located at the plane of cell division and at the sides. The cell wall is 22 to 35 nm thick.

In Table 2, the moles %GC content of the four SD strains are tabulated as calculated from the thermal denaturation curves. In every Tm determination E. coli K-12 DNA was used as the internal standard. The difference between the Tm of E. coli and the Tm of the test organism (ΔTm) was used for calculating the GC content according to equation 5 of Mandel et al. (23). If the equation of De Ley (7) is used, the calculated %GC content is lowered by an additional 2%.

Preliminary experiments were conducted to determine the precise influence of the denaturant formamide on the Tm by melting the reference DNA in 6 x SSC containing 40% (vol/vol) formamide. The 6 x SSC with 40% (vol/vol) formamide lowered the Tm of the DNA samples so that hybridization experiments could be carried out at fairly low temperatures. Experiments were also conducted to determine the amount of leaching during the incubation period at the hybridization temperatures used. To measure this, tritium-labeled DNA was loaded onto membrane filters which were then incubated under the exact conditions used for hybridization. The results indicated that leaching was negligible under these conditions. These findings agreed with the observations of De Ley and Tijtgat (8), and Okanishi and Gregory (33). Results of the homology experiments are shown in Table 3. The hybridization data for DNA from strains C, L, and T showed 93, 98, and 93% homology, respectively, at Tm = 26 C. When hybridization was carried out at Tm = 15 C, the values were 89, 95, and 95% homology with strain B DNA. This suggested that all four SD strains were essentially identical. This also was supported by the cultural, biochemical,
and cytological characters. When competition experiments were investigated using DNA from conventional lactobacilli, the uniqueness of SD bacteria became obvious. Competition by the homofermentative species of comparable GC content was minimal, and at $T_m - 25\, ^\circ C$ may have been nonspecific. This is supported by the still lower degree of competition obtained in the $T_m - 15\, ^\circ C$ experiment. The competition experiments with $L.\ brevis$ DNA, a heterofermentative species, yielded only slightly greater homology (39%) than that found when DNA preparations from the group I homofermentative lactobacilli were tested.

**Fig. 1.** Negatively stained preparation of strain $B$ cells in stationary phase of growth. $\times 35,800$. 
Finally, to evaluate the specific nature of the hybrid material formed, thermal elution experiments were conducted. Figure 5 shows that the thermal elution midpoint of the renatured DNA and the $T_m$ of native DNA differed 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. This is in support of the observations of others (2, 17, 35) on the influ-
FIG. 3. Negatively stained preparation of glutaraldehyde-fixed strain B cells in stationary phase of growth. $\times 59,280$. 

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hybridization could be carried out at lower temperatures, Bonner et al. (1) used formamide. Later McConaughy et al. (22) showed that 1% formamide would lower the $T_m$ by 0.72°C and that both a high specificity and a high rate of renaturation could be achieved by the appropriate choice of temperature, formamide, and salt concentrations. This work was extensively investigated by Okanishi and Gregory (33). In view of this, preliminary experiments were carried out in our laboratory to determine the optimum hybridization conditions. These experiments indicated that $6 \times SSC$ containing 40% formamide was a suitable solvent for the hybridization at incubation temperatures of 40 to 50°C. There was negligible leaching under these conditions. The rationale for the studies of Okanishi and Gregory (33) and De Ley and Tijtgat (8) was to find a method to prevent leaching of DNA from the filter. However, it is apparent that leaching has not been a problem with all workers. For example, Gillespie and Spiegelman (13), Moore and McCarthy (32), and Moore and Hirsch (31) did not experience any leaching problem. Nevertheless, in the present study, all precautions were taken to minimize it.

$T_m$ values allow calculation of only the average %GC of a DNA, and only bacteria having similar GC contents may be related to each other. If there is a difference of 5 to 7 moles %GC content, the probability of two organisms having homology is rather low, indicating that the organisms in question belong to different species (18). With these facts in mind, the species selection of lactobacilli was made for the hybridization studies. Since none of the known heterofermentative lactobacilli have a GC content comparable to those of SD bacteria, the group I homofermentative organisms were used because of the similarity in GC to the SD strains.

**DISCUSSION**

The membrane filter technique of Gillespie and Spiegelman has been modified and used for the determination of DNA homologies by several recent workers (8, 10, 20, 31). Pointing out the significance of the incubation temperature, Marmur and Doty (25) concluded that a temperature of 25°C below the $T_m$ was optimal for the DNA renaturation. Recently McConaughy et al. (22) and Rogul et al. (36) showed that leaching of DNA from membrane filters during the incubation was markedly increased by the high temperature employed for stringent renaturation.

To effect a reduction in the $T_m$, so that the extent of temperature on the percent of mismatched base pairs formed in the renatured DNA.

**TABLE 2. Thermal denaturation point and percentage molar guanine plus cytosine values for four strains of sourdough bacteria and Escherichia coli**

| Organism  | $\bar{T}_m$ | Moles %GC* |
|-----------|-------------|------------|
| SD B      | 6.5 ± 0.3   | 38.1       |
| SD C      | 5.7 ± 0.1   | 39.7       |
| SD L      | 6.2 ± 0.4   | 38.7       |
| SD T      | 6.4 ± 0.2   | 38.3       |
| E. coli K-12 |           | 51.0       |

* Calculated by using the equation of Mandel (23):

$$\% G + C_s = \% G + C_{E. coli} + 0.0199 \left( T_{m_s} - T_{m_{E. coli}} \right) \times 100.$$ 

* SD; Sourdough.
Table 3. Hybridization data obtained at thermal denaturation point -25 C and -15 with sourdough bacterium strain B as homologus DNA

| Organism          | $T_m$ - 25 C | $T_m$ - 15 C |
|-------------------|-------------|-------------|
|                   | Depression | Homology | Depression | Homology |
| DNA G count/min   | (%)        | (%)        | (counts/min) | (%)        |
| SD* B             | 1,025      | 100       | 3,937       | 100       |
| SD C              | 952        | 93        | 3,493       | 89        |
| SD L              | 1,007      | 98        | 3,744       | 95        |
| SD T              | 956        | 93        | 3,749       | 95        |
| *Lactobacillus acidophilus ATCC 4556 | 240 | 23 | 907 | 23 |
| L. salivarius ATCC 11742 | 310 | 30 | 476 | 12 |
| L. jugurti ATCC 521 | 377 | 37 | 234 | 6 |
| L. helveticus ATCC 8018 | 352 | 35 | 1,247 | 32 |
| L. brevis NCDO 473 | --- | --- | 1,541 | 39 |

*SD, Sourdough.

Fig. 5. Thermal melting curves of sourdough strain B native and reassociated ($T_m$ - 25 C, left; $T_m$ - 15 C, right) DNA.

The average GC content of the SD bacteria was between 38 and 39 moles %GC. With this in mind, the choices for homology studies with heterofermentative lactobacilli were L. viridescens and L. brevis with GC contents of 42.7 and 45%, respectively. L. buchneri, another heterofermentative lactobacillus, was not considered as it is now considered a nomen dubium by the International Committee on Nomenclature of Bacteria (29). The other two beta-bacterium species, L. cellobiosus and L. fermenti, are about 12 moles %GC higher, and even on a theoretical basis, they cannot have any common polynucleotide sequences and, therefore, cannot be taxonomically related to the SD bacteria (6).

The very high homologies obtained among the four SD bacteria were not surprising. They have almost identical morphological, cultural, and biochemical characters.

The 23 to 37% homology shown between the four group I homofermentative lactobacilli and the SD bacteria indicates the capacity of the hybridization method to differentiate between genetically unrelated organisms with similar GC content. This becomes more obvious by the still lower percentage of homology (6 to 32%) obtained between these organisms under the more stringent conditions of incubation temperature. The slightly higher percent homology (39%) obtained with the heterofermentative L. brevis suggests that the SD bacteria may have evolved from heterofermentative lactobacilli.

The electron microscopy survey reveals the features of these typically gram-positive bacilli with the thick cell wall (Fig. 4) and the presence of mesosomes (Fig. 1). Typical cell sizes for stationary-phase SD broth cultures were 0.5 to 0.7 μm x 1 to 3 μm. The cell wall was 22 to 35 nm in thickness. It was previously reported (16) that both lactic streptococci and lactobacilli have well-defined large mesosomes. As is normally the case, the mesosomes of the SD bacteria are usually found at the plane of cell division or the cell terminus. Figure 2 demonstrates the continuity of mesosomes with the cell membrane. The parallel finger-like intrusions substantiate the concept of Hurst and Stubbs (16), that the membrane is attached to the wall at regular intervals. These membranous intrusions are probably an artifact, since they are not present when the cells are prefixed with glutaraldehyde (Fig. 3).

The classification of Lactobacillus organisms based on their ability to ferment carbohydrate into primarily lactic acid (homofermentative) or lactic acid and other products (heterofermentative) appears unrealistic. Only a few more enzymes would be required to produce acetate, ethanol, and CO₂ from the carbohydrate. These enzymes could be coded for by a
small number of genes, constituting a small portion of the entire genome of the organism. Furthermore, only a fraction of the entire genome is considered when we classify organisms on the basis of selected phenotypic characters such as those used for lactobacilli. In some cases, these considerations are too restricted and arbitrary to be used as a criterion for speciation. However, the routine use of DNA-DNA hybridization to identify species is time consuming and expensive. Thus, for the immediate future, classical methods of taxonomy using phenotypic properties will continue to be used, especially when a utilitarian advantage is offered as in the case of industrially important organisms. Nevertheless, DNA-DNA hybridization provides the means for a better understanding of the relatedness between microorganisms and is especially useful in assigning species names to newly isolated bacteria such as the SD bacteria examined in the present study. It is of interest to consider the evolution of SD bacteria, especially to determine from what known species of *Lactobacillus* they have originated. The SD bacteria appear to be more closely related to the homofermentative types by their GC contents. In this regard, Wittenberger et al. (38) and Brown and Wittenberger (3) have shown that *S. faecalis*, a known homofermentative organism, has the enzyme capabilities of carrying out heterofermentative fermentation of glucose. They have also shown that the heterofermentative pathway is repressed by fructose-1,6-diphosphate. It is conceivable, therefore, that the SD bacteria were originally homofermentative but, through mutation and selection in the dough environment, they have lost the fructose-1,6-diphosphate control of the heterofermentative pathway.

Hybridization and the other data indicate that the SD bacteria are unique and unrelated to any previously described lactic acid bacteria. They deserve consideration as a new heterofermentative species in the genus *Lactobacillus*. We therefore support the suggestion of Kline and Sugihara (19) that the organism be named after the city of San Francisco; these authors (Sugihara et al., manuscript in preparation) have designated strain L as the type species which is on deposit in the U.S. Department of Agriculture, Northern Regional Research Laboratory, Peoria, Ill.

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