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Classification and DNA base ratios of propionibacteria

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INTRODUCTION

The propionic-acid bacteria form a scientifically and commercially interesting group. Since antiquity, their natural occurrence in milk has been responsible for the development of particular attributes in certain ripened cheeses. They produce propionic and acetic acids and carbon dioxide from a variety of carbon sources, including lactate. In Emmentaler-type cheese, this ability provides for a useful relationship with lactic-acid bacteria. These two acids are important elements in the characteristic flavor and aroma, and carbon dioxide is responsible for the development of the eyes of this cheese. The use, in pure culture, of propionic-acid bacteria in commercial cheesemaking has provided a valuable means of control to the industry.

Bacteria with the ability to produce propionic acid were isolated from dairy products and described early in this century. The genus, Propionibacterium, including eight species, was proposed by Van Niel (105) in 1928. Since that time, 11 additional species and one variety have been proposed. Bergey's Manual of Determinative Bacteriology, 7th edition (10), recognizes all of the original eight and three of the additional species. These eleven species are separated and identified on the basis of morphological, carbohydrate fermentation, pigmentation, and gross cultural char-

acters. A number of the separations, including the division into the two main groups, are defined by more than one set of characters, a convention which implies that the characters used together to define a single separation always appear in concert. This notion is admittedly absurd. In fact, many of the additional species described since Van Niel were defined by combinations of multiple characters not allowed by the 7th edition of Bergey's Manual.

A number of compatible dichotomous characters such as those used to separate the main groups of the genus Propionibacterium might be expected to occur in a random fashion with respect to each other in a given strain. The propionibacteria provide a suitable and convenient arena in which this randomness can be tested. The purpose of this study has been to examine a set of such characters and determine the level of agreement or lack of agreement among them. Deoxyribonucleic acid base ratios have been determined to supplement the phenotypic data.

The findings resulting from this study should have value in the selection of characters for the classification of propionibacteria. This approach should find application in other bacterial taxa as well.

REVIEW OF LITERATURE

Lamanna and Mallette (49) state that the purpose of taxonomy is to categorize plants and animals according to their natural relationships. At the same time, the value of a classification system is tied to its usefulness--the identification of an organism on the basis of characters of clinical or economic importance (89). In the 17th century, Locke (52) said, "The boundaries of the species, whereby men sort them, are made by men." More recently, the notion of a species as a biological fact-of-life has been described as an "act of faith" by Mandel (58) and as "man-made" by Cowan (22).

Mandel (58) suggested three forms of systematics (alpha, beta, and gamma) which are necessary to tie the descriptive properties of bacteria to the "principles of evolutionary relationships." He observed that until this process is completed, which he optimistically predicted could be realized by the end of the decade, pragmatic classifications will be necessary and desirable. Colwell (16) has called for a multidimensional taxonomy, incorporating information from the molecular to the ecological levels.

Cowan (21, 22) has proposed what he has termed the 12 principles of heretical taxonomy. He criticizes the imposition of a hierarchial system which suggests that one set of

characters is more important than another and the practice of defining a species on the basis of a single strain (22).

Historical Development of the Genus *Propionibacterium*

In 1907, von Freudenreich and Orla-Jensen (12, 107) and Orla-Jensen (12, 72) described a group of propionic acid-producing bacteria and named three species, *Bacterium acidi propionici a*, *Bacterium acidi propionici b*, and *Bacillus acidi propionici*. Two years later, Orla-Jensen (73) first proposed the creation of a separate genus for propionic-acid bacteria suggesting *Propionibacterium* for its name. No action was taken on his proposal at that time, and subsequent reports of work with propionic-acid bacteria placed proposed new species in the genus *Bacterium*. In 1909, Troili-Petersson (104) proposed the designation *Bacterium acidi propionici c* for a propionic acid-producing organism which she had isolated from cheese and soil. A year later, Thöni and Alleman (103) reported having isolated a propionic acid-producing bacterium from Emmentaler cheese and proposed *Bacterium acidi propionici* var. *fuscum* as its name. They also reported the cause of red spots in Emmentaler cheese as a pigmented propionic-acid organism for which the name *Bacterium acidi propioni* Var. *rubrum* was proposed.

In 1921, the American bacteriologist, Sherman (90),

reported that the eyes and characteristic flavor of Swiss cheese were the result of the action of propionic acid-producing bacteria, present in large numbers in this cheese variety. He isolated an organism similar to Bacterium acidi propionici a, designated it Bacterium acidi propionici d, and demonstrated the practicality of its use in pure culture to insure the proper ripening of Swiss cheese.

In 1928, in his classic publication, The Propionic Acid Bacteria, Van Niel (105) repeated Orla-Jensen's proposal of Propionibacterium as the generic name for propionic-acid bacteria, named eight species within the genus, and offered a key for their identification. Some of the species described by previous workers retained their identity and found places and new names in Van Niel's scheme.

His key divided the species among three main groups, separated on the basis of morphology and the extent and pigmentation of growth on an agar stab. Propionibacterium freudenreichii and P. shermanii, previously known as Bacterium acidi propionici a and B. acidi propionici d respectively, formed one group, having in common a coccoid cell morphology, a colorless and scanty growth on the surface of an agar stab, and the absence of the ability to ferment sucrose and maltose. These two species were separated on the basis of the ability of the latter to ferment lactose.

The second group, characterized morphologically as short

rods, on an agar stab by distinct and pigmented growth, and by the ability to ferment sucrose and maltose, contained five of the eight new species, P. jensenii (previously known as B. acidi propionici b), P. peterssonii (previously known B. acidi propionici c), and P. techicum. These species had a yellow pigmentation while P. thoenii and P. rubrum, similar to the previously described B. acidi propionici var. rubrum, had a deep red pigmentation. P. technicum possessed the unique ability to ferment polysaccharides, such as starch, and was so named because Van Neil contemplated its possible use as a "technical" source of propionic acid.

The third group contained a single species, P. pentosaceum (previously known as Bacillus acidi propionici). It was characterized morphologically as a long rod, irregular in shape, locally swollen, and branched. Stab surface growth was distinct and colorless, and the ability to ferment sucrose and maltose was again present.

A key to the genus Propionibacterium first appeared in Bergey's Manual of Determinative Bacteriology in the Third Edition (4). The genus was placed in the tribe Propionibacterieae of the family Bacteriaceae of the order Eubacteriales. Although the same eight species proposed by Van Niel were recognized, different and fewer criteria for separation were used. These criteria included the presence or absence of pigment formation, the color of pigment when

formed, and carbohydrate fermentation patterns. P. thoenii, which Van Niel (105) separated from P. rubrum on the basis of its lack of the ability to ferment raffinose and mannitol, was now reported as possessing this ability and the two species were separated solely on the basis of their propionic: acetic acid ratios, 5:1 for the former and 3:1 for the latter. The dried and wrinkled appearance of P. peterssonii growth was ignored, and the species was separated from P. jensenii only on the basis of the occasional ability of the latter to ferment raffinose. Domke (26) reported the propionic:acetic acid ratios of 25 strains of propionibacteria, including two strains of P. thoenii. No strain had a ratio greater than 2.54:1, and almost half of them, including the two P. thoenii strains, had ratios of less than 2:1. These ratios were in contrast to the values of Van Niel (105).

Werkman and Kendall (112) argued against the extensive use of pigment formation as a classification criterion and proposed a scheme using carbohydrate fermentation and nitrate reduction as key identifying characters. Van Niel's (105) P. jensenii var. raffinosaceum was proposed as a separate species, P. raffinosaceum, on the basis of its ability to ferment raffinose. It was separated from P. rubrum on the basis of pigment color. This is the only reference to pigmentation in the key. P. thoenii was not recognized as a species.

In 1932, Hitchner (38) proposed P. zeae and P. arabinosum as additional species. These were included in a key published the following year by Werkman and Brown (110). P. thoenii also was accorded renewed special status, making eleven species in all. Cell morphology and characteristics of cultural growth were still not included as criteria. The general agreement among morphological, physiological, and serological classifications was considered remarkable. It was implied that the different classifications served only to confirm one another, and that the use of only one ought to be considered sufficient. The key included catalase production as a character separating P. arabinosum, which was negative, from P. technicum. This was in contradiction to previous classifications and reports (4, 111, 112) which considered catalase production a character common to all propionibacteria. Hitchner (39) suggested that the position that catalase production is a character common to all propionibacteria ought to have been modified.

The fourth edition of Bergey's Manual (5) offered a key following the same scheme as Werkman and Brown (110), however, only nine species were recognized. P. arabinosum and P. zeae were not recognized, and catalase production was not used as a character. Dorner and Thöni (27) recommended combining P. shermanii and P. freudenreichii because their investigation showed that typical strains of the two species

differed only in their ability to ferment lactose. However, the fifth edition of Bergey's Manual (6) retained the two as separate species. The fifth edition of this manual also placed the genus in the family Lactobacteriaceae and dropped the designation of the tribe Propionibactericae. The key provided criteria including morphological forms, carbohydrate fermentation patterns, and general cultural characteristics to distinguish between the species. Although P. arabinosum was recognized as a species, catalase production was not used as an identifying character.

With the use of more than one character to define a given separation in the key, the possibility that at least one, but not all, of the characters would be present in a given strain presented itself. Sakaguchi et al. (84) isolated six strains which could not be fitted into the keys then extant and proposed five new species: P. amylaceum, P. japonicum, P. orientum, P. coloratum, and P. globosum and one variety; P. amylaceum var. aurantium. These were fitted into the keys of both Werkman and Brown (110) and of the fifth edition of Bergey's Manual (6). Janoschek (42) faced a similar problem, and his proposed key had few multiple criteria for single separations. He added three new species, P. casei, P. sanguineum, and P. pituitosum, to the 11 recognized by the fifth edition of Bergey's Manual (6).

Prevot (78) criticized the classification of propionibacteria in the family Lactobacteriaceae, pointing out that they lacked the ability to produce lactic acid. However, the sixth edition of Bergey's Manual (9) retained the species in that family which, in turn, was placed in the sub-order, Eubacteriineae, of the order Eubacteriales. Only the 11 species recognized by the previous editions, and none of those proposed by Sakaguchi et al. (84) or Janoschek (42) were recognized, and the key to the species was little changed. The seventh edition (10) placed the genus in a new order, Propionibacteriaceae, but retained recognition of the same 11 species. The key for identification contained some modifications but followed the same general outline as that of the previous edition. The keys which have been published for the classification of propionibacteria are reproduced in the Appendix.

Kurmann (47, 48) has demonstrated that carbohydrate fermentation data can be inconsistent. He advanced the possibility that the reports of earlier workers contained many inaccuracies, and criticized the classification system used for propionibacteria for its heavy dependence upon characters of carbohydrate fermentation.

Numerical taxonomy

The techniques of numerical taxonomy were first applied to bacteria by Sneath (96). Critical treatments of the issues involved have been published by Leifson (51) and by Lysenko (53).

Antila and Gyllenberg (2) were the first to apply the principles of numerical taxonomy to the traditional propionibacteria. Studying 32 characters with 85 strains, they recommended that P. arabinosum be divided into two subgroups, that P. casei be recognized as a distinct species, and that P. jensenii and P. peterssonii be regarded together as a common species. They found that P. freudenreichii, P. shermanii, P. casei, and P. arabinosum formed one general group, P. technicum and P. pentosaceum another; P. peterssonii, P. jensenii and P. raffinosaceum (those forming orange pigment) a third, and the red-pigmented strains a fourth. Interesting departures from the scheme of Bergey's Manual (10) were the separation of P. arabinosum from P. pentosaceum and the separation of P. technicum from the other orange pigment-formers.

A numerical analysis of 82 strains of streptococci, lactobacilli, and propionibacteria, including 25 strains of the last, by Seyfried (88) supported the argument that lactobacilli and propionibacteria do not belong in the same family. An interesting separation of P. shermanii and P.

freudenreichii from the other species of propionibacteria also was obtained. As the result of an investigation of the 38 characters in 56 strains of propionibacteria, Malik (56) and Malik et al. (57) suggested the consolidation of P. shermanii with P. freudenreichii and of P. arabinosum with P. pentosaceum. An interesting placement of P. rubrum and P. thoenii into separate groups also was made.

DNA composition of propionibacteria

Sebald et al. (87) reported GC% values obtained by alkaline hydrolysis and paper chromatography ranging from 66.4 to 67.8 for six NCIB (National Collection of Industrial Bacteria, Aberdeen, Scotland) strains of traditional propionibacteria. A seventh, P. arabinosum NCIB 5958 had a value of 70.4. This is the earliest reported work of this nature with propionibacteria (37).

Mandel (58) has suggested that DNA base composition is a useful and necessary criterion for assaying relatedness. Jones and Sneath (44) state that a GC% difference of from 2-5% is necessary to imply a special difference, which differences of less than 2% are usually not taxonomically significant. It also follows that similar GC% values do not necessarily prove genetic homogeneity.

There have been a number of recent studies in which taxonomic groupings based upon either phylogenetic or numerical methods have been compared with the DNA base ratios of the

same groupings. The validity of the separation of Micrococcus from Staphylococcus as a separate genus has been verified in work done by Silvestri and Hill (91) and by Bohacek et al. (8). The validity of the classifications within the family Pseudomonaceae has been examined by DeLey et al. (25), Mandel (59), Palleroni et al. (76), and Stanier et al. (100); those of coryneform bacteria by Yamada and Kazuo (114); and of beta-bacteria and streptobacteria by Cantoni et al. (15).

Studies in which phena derived from numerical analysis have been corroborated by DNA GC%'s have been reported by Slosarek (95) with Mycobacterium, Colwell (16) with Vibrio, and Blokhima and Levanova (7) with various species of enterobacilli, Flavobacterium, and Pseudomonas.

Johnson and Cummins (43), examining the DNA base ratios of 29 strains of traditional propionibacteria by thermal denaturation, obtained a range of from 64 to 68 GC% with a directional difference between P. freudenreichii and P. shermanii, as a group; and the balance of the species as another. The GC% of the former averaged about 65. The GC% of the latter averaged about 67. Supporting their findings with DNA hybridization and cell-wall structure data, they proposed the consolidation of the traditional (or classical) propionibacteria into four species. These are P. freudenreichii, P. thoenii, P. jensenii, and P. acidi-propionici.

With the exception of the last, each coincides with the main group or sub-group of the seventh edition of Bergey's Manual (10) which includes the traditional species of the same name. P. acidi-propionici includes the traditional species of P. arabinosum and P. pentosaceum. The greatest differences were observed between the strains of the proposed P. freudenreichii species and the balance of the strains combined.

Type and reference strains

A compilation of type and reference strains of propionibacteria in the major culture collections as of 1965 was published by Sneath and Skerman (97). Authentic strains for all of the species recognized by the seventh edition of Bergey's Manual (10), except for P. freudenreichii and P. raffinosaceum and cotypes for P. arabinosum, P. technicum, and P. zeae, were included. The ninth edition of the American Type Culture Collection Catalog of Strains (1) (ATCC) lists as least one strain for each of the traditional species except P. raffinosaceum. These include most, but not all, of the strains listed by Sneath and Skerman (97). A strain of P. intermedium (ATCC 14072) also is available. No strains of P. raffinosaceum had been added to the ATCC collection as of mid 1971 (Lessel, E. F. 1971. Private communication. American Type Culture Collection, Rockville, Maryland).

Anaerobic coryneforms

Douglas and Sunter (28) observed that strains of Corynebacterium acnes produced propionic acid and proposed the transfer of the species to the genus Propionibacterium. A similar proposal was made by Moore and Cato (71), who also observed that strains of the species fermented lactate. Work by Voss (108) provided the basis for the separation of a large number of Corynebacterium acnes isolates, including ATCC strains 6919, 1621, and 11827, P. acnes, into two distinct groups. The average GC% of the two groups were 60.5 and 63.9.

Johnson and Cummins (43), studying 80 strains of anaerobic coryneforms, including two of the ATCC strains used by Voss (108), divided them into three groups with a GC% ranging from 58 to 64. They support their position that the anerobic coryneforms should be placed in the genus Propionibacterium by the fact that propionic acid is the major acid of fermentation and because of certain similarities in cell-wall carbohydrates and lipids.

Preparation of Microbial DNA

Cell disruption for DNA isolation

Marmur (62) described a method using bile salts and lysozyme to disrupt cells. This method was the first one described which has application across a broad range of

of microbial species. Cells resistant to lysozyme, including cells of strains of streptococci (77, 94) and lactobacilli (69, 70), have been lysed with preparations of the lytic factor from Streptomyces albus. An enzyme isolated from Pseudomonas aeruginosa has been used to achieve lysis of Staphylococcus aureus (46). Streptomyces albidoflavus lytic enzymes have been used in combination with lysozyme for the lysis of algal cells (99). Cells of coryneform bacteria, normally resistant to lysozyme, have exhibited increased sensitivities when grown in media with increased levels of glycine (114). Sakaguchi and Mori (85) have grown pediococci in penicillin G to weaken the cell walls and make the cells more susceptible to enzymatic lysis. Bacterial spores have been sensitized to lysozyme by the addition of disulfide bond-reducing agents (34). Cells of gram-negative strains have exhibited lysozyme-sensitivity in the presence of EDTA (80, 81). Physical methods which have been employed against cells refractory to enzymatic lysis include the French pressure cell with algal cells (23, 29, 99), grinding with a mortar and pestle (62), or with glass beads (95), and mechanical cell homogenization (43). These physical methods generally yield DNA preparations which are of lower molecular weight and are more difficult to recover. Edelman et al. (29) have shown, with algal cells, that the buoyant density of DNA from a given

strain is the same, whether the DNA is obtained through enzymatic lysis or disruption with the French pressure cell.

Isolation and purification

The method of Marmur (62) separates protein from DNA by the use of sodium perchlorate and successive shakings with a mixture of chloroform and isoamyl alcohol. RNA is removed by digestion with RNase, and a preparation of high purity, if required, can be obtained by precipitation with isopropanol. The resulting material is biologically active and has an average molecular weight in the region of 10^7 daltons depending upon the method employed for cell disruption.

The use of phenol to remove contaminating protein by shaking (45) and by holding the mixture at the temperature at which the aqueous mixture and phenol share a common density (65) have been reported. The latter method uses dropwise addition to establish a uniform dispersion of phenol in the mixture. Massie and Zimm (64) reported a method utilizing dialysis with successive additions of pronase, RNase, and pronase resulting in a high-molecular weight (10^8 to 10^9) DNA preparation.

Methods for the preparation of nucleic acids of a high degree of purity include banding in a cesium chloride gradient (113) and their precipitation as cadmium salts (36).

Determination of the Base Ratios of Bacterial DNA

Mandel and Marmur (60) have described a method for the determination of base ratios of DNA using its thermal denaturation profile. They also have summarized the origin and development of the technique.

Mandel et al. (61) have summarized the development of isopycnic banding in a CsCl density gradient, a technique first described by Meselson et al. (67), and have detailed a method of its use in the determination of the buoyant density and base ratios of DNA. The use of the reference marker - usually DNA of known buoyant density, although Richard et al. (82) reported the successful use of an inert chemical marker - is required. DeLey (24) compared the results of using this technique with those obtained from thermal denaturation studies and found a high degree of correlation between the two methods. Isopycnic banding is not only a valuable analytical tool, but it also is useful in preparative work where DNA's of high purity are required (113).

Fredericq et al. (32) studied the spectra of oligonucleotides over a range of pH values and demonstrated a near-linear relationship between the ratio of the extinction coefficients at 260 and 280 nm and the percent adenine

or thymine. Data from this method have been used in conjunction with and have been corroborated by data from thermal denaturation studies (8).

Other methods used for the determination of bacterial DNA base ratios were reviewed by Hill (37). The methods included hydrolysis and chromatography and spectroscopic determination of the relative percentage of adenine by its reactivity toward bromine reagents. Recently, the application of thin layer (14) and cyclodextrin gel (41) chromatography to the base analysis of bacterial DNA have been reported.

Estimation of DNA Molecular Weight

It has been shown (67, 101) that macromolecules banded in a density gradient by ultracentrifugation have a Gaussian distribution with the variance varying as the inverse of the molecular weight. Sueoka et al. (102) pointed out that intermolecular heterogeneity with respect to base distribution will inflate the variance of the band, however, Rolfe and Meselson (83) and Meselson and Stahl (66) have shown that bacterial DNA's are relatively homogeneous. The variance of a band of propionibacterial DNA may therefore be a reliable estimator of its molecular weight.

Sueoka et al. (102) reported an empirical relationship

of the variance of the distribution of DNA molecules in a cesium chloride density gradient to the molecular weight of the DNA as follows:

$$MW = \frac{239000}{\sigma^2}$$

where σ is expressed in millimeters and the conditions include centrifugation at 44770 rpm at 25 C for 24 hr.

MATERIALS AND METHODS

Media

Sodium lactate broth:

A medium was prepared containing the following/1000 ml in distilled water: Trypticase (Baltimore Biological Laboratories, Cockeysville, Maryland), 10 g; yeast extract, 10 g; sodium lactate, 10 g; and K_2HPO_4, 0.25 g, and adjusted to pH 7.2 with 5 N NaOH and autoclaved (121 C for 15 min).

Trypticase soy broth:

A medium was prepared containing 30 g of Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Maryland) in 1000 ml of distilled water and autoclaved.

Glucose broth:

A medium with the same composition and pH as Sodium lactate broth but with 10 g of glucose substituted for 10 g of sodium lactate/1000 ml of medium was prepared and autoclaved.

Sodium lactate agar:

A medium with the same composition and pH as Sodium lactate broth plus 15 g of agar/1000 ml of medium was prepared, dispensed into culture tubes, and autoclaved.

Basal medium:

A medium was prepared containing the following/900 ml in distilled water: Trypticase, 10 g; yeast extract, 10 g; K_2HPO_4, 0.25 g; and brom thymol blue (1.6% solution in ethanol), 0.9 ml. The pH was adjusted to 7.4 by the addition of KOH, and the medium was dispensed into culture tubes in 9-ml amounts and autoclaved.

Blank medium:

Basal medium was diluted 9:10 with distilled water, dispensed into culture tubes in 10-ml amounts and autoclaved.

Test media (sucrose, lactose, raffinose, sorbitol, and rhamnose):

For each carbohydrate, a solution containing 10% (w/v) carbohydrate in distilled water was sterilized by filtration through a filter of 0.45 μ mean pore size (Millipore Filter Corporation, Bedford, Massachusetts). Amounts of 1 ml each were dispensed aseptically into culture tubes containing 9 ml of basal medium.

Test medium (starch):

A suspension containing 10% starch in distilled water was heated until the starch dissolved. The solution was immediately dispensed in 1-ml amounts into culture tubes, each containing 9 ml of unsterilized basal medium, and the

tubes and their contents were autoclaved.

Test media (cellobiose and salicin, conventional method):

For each carbohydrate, a suspension containing 10% (w/v) carbohydrate in distilled water was dispensed in 1-ml amounts into culture tubes containing 9 ml each of unsterilized basal medium, and the tubes and contents were autoclaved.

Test media (cellobiose and salicin, alternative method):

Cellobiose and salicin were each weighed into separate, previously cotton-stoppered, beaker-capped, and sterilized 250-ml Erlenmeyer flasks. Ethyl ether was added to each flask to capacity, and the flasks were placed in a bench drawer and the ether allowed to evaporate at room temperature. Sterile distilled water was added aseptically to each flask to bring the concentration of the carbohydrate to 10% (w/v), and the resulting suspension was added aseptically in 1-ml amounts to culture tubes containing 9 ml each of basal medium.

Materials for Cell Disruption and DNA Isolation

Saline-EDTA:

A solution was prepared to contain 0.15 M NaCl and 0.1 M ethylene diamine tetraacetic acid (EDTA), and adjusted to pH 8.0 with a 5 N NaOH prior to dilution to final volume.

Saline-EDTA (4.7l strength):

A solution was prepared by dry-mixing NaCl and EDTA in the same molar ratios as previously described, followed by the addition of 5 N NaOH to pH 8.0 and complete solution of EDTA. The final strength was determined by measuring the final volume.

Egg white lysozyme (3 x crystalline, Miles Laboratories, Madison, Wisconsin):

Lytase (Baltimore Biological Laboratories, Cockeysville, Maryland):

Pancreatic ribonuclease (crystalline, Nutritional Biochemicals, Cleveland, Ohio):

Sodium lauryl sulfate (25%, SLS):

A weighed quantity of sodium lauryl sulfate was moistened by steaming. A slurry was then prepared using a minimal amount of water, and the covered container was held at 60 C until the detergent had dissolved. The volume was adjusted with distilled water to make a 25% (w/v) solution.

Sodium perchlorate (5 M):

Chloroform: isoamyl alcohol:

To prepare this solution, 24 volumes of chloroform were mixed with one volume of isoamyl alcohol.

Concentrated saline citrate:

A solution was prepared to contain 1.5 M NaCl and 0.15 M sodium citrate at pH 7.0 ± 0.2.

Standard saline citrate:

A volume of concentrated saline citrate was diluted 1:10 with re-distilled water.

Dilute saline citrate:

A volume of concentrated saline citrate was diluted 1:100 with re-distilled water.

Materials for DNA Assay

Calf thymus DNA:

Calf thymus DNA (Nutritional Biochemicals, Cleveland, Ohio) was dissolved in 5 mM NaOH to a final concentration of 400 µg/ml.

Standard saline citrate:

Perchloric acid (1 N):

Diphenylamine reagent:

Three parts of diphenylamine were dissolved in 200 parts of redistilled acetic acid (w/v). Three parts (v) of concentrated H_2SO_4 were added. Immediately prior to use, one volume of acetaldehyde solution (16 mg/ml) was added to 200

volumes of reagent.

Materials for Analytical Ultracentrifugation

CsCl stock solution:

Optical grade CsCl (Harshaw Chemical Co., Solon, Ohio) was dissolved in 0.02 M Tris buffer (pH 8.5) at the rate of 13 parts (w) of CsCl to 7 parts (v) of buffer, as described by Mandel et al. (61).

Distilled water:

Standard saline citrate:

Reference DNA:

Reference DNA from Escherichia coli B was prepared as described in the sections entitled "Preparation of Cells" and "Cell Disruption and DNA Isolation."

Propionibacterial Strains

The strains used in this investigation are shown in Table A-1. The donor or source of each strain is shown, as well as the strain and species designations given that strain by the donor or isolator. The strains were propagated in sterile Sodium lactate broth. Cultures were incubated at 32 C to first visible turbidity and stored at 7 C. A strain of E. coli B, obtained from Dr. P. A. Pattee of the

Department of Bacteriology, Iowa State University, provided DNA for use as a buoyant density reference. The strain was propagated in Trypticase soy broth at 37 C and stored at 7 C.

Gas-Liquid Chromatography of Spent Culture Media

A culture of each strain was grown in Sodium lactate broth at 32 C for 2 days, acidified to pH 3 with 0.5 ml of 5 N HCl, and centrifuged to obtain the cell-free spent medium.

An Aerograph Model A-600-B gas chromatograph (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) equipped with a hydrogen flame detector and a Honeywell Electronic 15 strip chart chromatography recorder was used for the analysis of the spent media. A 6-ft stainless steel column, prepared according to the procedure outlined by Mahadevan and Stenroos (54), was used. Purified nitrogen gas was the carrier. For each acid, a factor for the conversion of integration units to moles was derived from the chromatographic patterns of five separate injections of a standard contining 5.00×10^{-2} M acetic acid and 6.75×10^{-2} M-propionic acid. The column temperature was about 190 C, and the carrier gas pressure was about 90 psig.

Carbohydrate Fermentation Screening

Culture tubes of basal, blank, and test media were screened for sterility by incubation at 32 C for 7 days. For each strain, a tube containing basal medium and one tube of each of the test media, including only cellobiose and salicin by the conventional method, were inoculated with 3 drops of active, growing Sodium lactate broth culture and overlaid with a small quantity of a melted, sterile mixture of Amogel (American Oil Company, Chicago, Illinois) and paraffin wax. The inoculated media were incubated at 32 C and examined daily for color change over a period of 20 days. In the event that the indicator dye changed to complete yellow, the strain was regarded as positive with respect to that carbohydrate, and the number of days which had elapsed was noted. A strain-carbohydrate combination was not regarded as positive if the color change stopped short of completion.

Certain strains for which the fermentation of cellobiose and salicin were regarded as questionable were inoculated into test media prepared by the alternative method and examined in the same manner.

Observation of Growth with Liquid Media

Sodium lactate broth in a screw-capped culture tube was inoculated for each strain with 2 or 3 drops of active, growing culture and incubated at 32 C for 10 or 11 days. At intervals, during this period, the cultures were examined for turbidity. If turbidity was observed, and there was a zone of clear media above the turbid zone, the depth of the clear zone was measured in mm. Beginning on the day on which turbidity was first observed, the rate at which the boundary between the zones settled was determined and expressed in mm/day.

At the end of the incubation period, the cultures were examined and grouped according to the observed presence or absence of brown and gray colors and were examined for ropiness and flocculence.

For each strain, a culture tube of Glucose broth was inoculated with 2 or 3 drops of active, growing culture and incubated at 32 C for 24 hr. At the end of the incubation period, the cultures were immediately examined for ropiness and flocculence.

For each strain, a culture tube of Sodium lactate broth was inoculated with 2 or 3 drops of active, growing culture and incubated at 32 C until definite growth was evident. The cultures were then held at 7 C for about 3 wk and examined

for ropiness and flocculence. If either character was observed with any of the cultures of a strain, that strain was regarded as possessing that character.

Observation of Growth on Agar Stab

Sterile sodium lactate agar in a culture tube was inoculated for each strain by stabbing from a sodium lactate broth culture. With the screw caps slightly loosened to permit aeration, the stabs were incubated at 32 C for 13 days.

Surface growth was examined for its extent and color. Arrival at a description of the extent of growth was made by placing the stabs in order according to the amount of growth and making an arbitrary division between those judged to be devoid of surface growth (A), those showing slight surface growth (S), and those showing definite surface growth. Those showing definite surface growth were characterized as exhibiting either the normal pattern of growth——circular form, convex elevation, and entire margin——(P), a dome-shaped pattern of growth (D), or growth with a wrinkled or dried-up appearance (W). The color of the surface growth, when present, was noted and characterized as either grayish cream (G), cream (C), yellow (Y), orange (O), deep red (R), or white (W).

The color of the sub-surface growth was noted and

characterized as grayish (G), cream (C), yellowish (Y), deep red (R), or reddish (r).

Determination of Cell Morphology

Cells were grown in Glucose broth at 32 C for 18 to 24 hr and, after having been fixed to slides, stained by the Hucker modification of the Gram stain (20). The cells were examined microscopically and the gross morphology of each strain was determined. Cells were described as either short, small, spherical rods (S); larger, diptheroid-appearing rods (D); as one of the involution-like forms — long, irregular rods (R) or as large, swollen spheres (L).

Determination of Medium, Temperature, and Incubation Time for Bulk Cultures

To obtain the greatest possible yield of DNA, it was intended that cells be harvested from bulk culture in or near the final stages of the logarithmic phase of growth. To aid in the selection of a suitable medium temperature and time of incubation, four strains were chosen for examination of growth patterns. For each strain, two flasks of Trypticase soy broth and two flasks of Sodium lactate broth were inoculated with 0.25% of a 24-hr culture which had been adjusted with sterile medium to A_{540} = 1.07, the absorbance of the least turbid inoculating culture. Cultures of each medium

were incubated at 32 C and 21 C. The absorbance of 540 nm of an aseptically drawn aliquot was determined at intervals for each culture.

Determination of Lysozyme Sensitivity

To determine which strains would be sensitive to lysozyme under the conditions of the Marmur (62) procedure, separate 190-ml amounts of Glucose broth were inoculated with 1% 24-hr culture of each strain. Cells were grown at 32 C for 24 hr and harvested by centrifugation at 7970 X G. The cells were washed once with saline-EDTA, and a suspension containing 250 mg washed cells/ml in saline-EDTA was prepared. The suspension was heated to 60 C for 10 min and cooled to ambient temperature. Duplicate tubes of the heated cell suspension were prepared. Lysozyme was added to one of the duplicates to a final concentration of 500 µg/ml following the adjustment of the concentration of cells in both duplicates to about 100 mg/ml with saline-EDTA. Following overnight incubation at 37 C, SLS, prepared by diluting the 25% solution with two volumes of saline-EDTA, was added to each duplicate to a final SLS concentration of about 2%, and both duplicates were heated to 60 C for 10 min. Following heating, the suspensions were cooled to ambient temperature, and the sensitivity of the cells to the treatment was determined by visually comparing the duplicates with respect to

opacity and viscosity. An obvious difference in either property between the duplicates was evidence of sensitivity. Questionable pairs were compared by phase contrast microscopy.

Metcalf and Deibel (68) have reported successful lysis of cells of a normally lysozyme-resistant strain of Streptococcus faecalis by incubation of a cell suspension with lysozyme in distilled water followed by the addition of solutes. The applicability of this approach to study of lysis of strains of propionibacteria shown to be resistant to the conditions of the Marmur (62) procedure was explored.

Cells of 35 strains shown to be resistant to lysozyme in saline-EDTA were grown and harvested as previously described and washed once in distilled water. Following washing, a suspension of cells for each strain containing about 150 mg of washed cells/ml in distilled water was prepared, and the suspension was heated as previously described. Duplicate tubes of material were prepared, and enough lysozyme was added to one duplicate for a final concentration of 1.2 mg/ml after adjustment with distilled water of the concentration of cells in both duplicates to about 120 mg/ml. Following overnight incubation at 37 C, saline-EDTA (4.7$\times$ strength) was added to both duplicates to bring the concentration of NaCl and EDTA to about 0.15 M and 0.1 M respectively. SLS diluted with saline-EDTA as previously described was added to bring the

concentration of SLS to 2%, and the suspensions were heated at 60 C for 10 min. The duplicates were compared, and the sensitivity of each strain to lysozyme in distilled water was determined in the same manner as previously described.

Because a number of strains remained resistant to lysozyme in spite of the success of incubation in distilled water, lysis by the combined actions of lysozyme and Lytase, a preparation of the lytic factor from Streptomyces albus, was attempted. A modification of the method of Miller et al. (70) was used. Duplicate suspensions of cells from 31 strains previously shown to be resistant in saline-EDTA were prepared in the manner previously described. Incubation mixtures were prepared in pairs with one containing 500 mg of lysozyme and 0.15 ml of Lytase/ml, and both containing about 100 mg of cells/ml. Incubation was at 37 C overnight. SLS was added to each duplicate suspension to a final concentration of 2%, and the material was heated to 60 C for 10 min. Because the discoloration imparted to the one duplicate by the Lytase might have distorted findings based upon direct observation, all duplicate pairs were examined using phase contrast microscopy.

Preparation of Cells

Quantities of wet, packed cells of strains of propionibacteria selected for DNA studies were produced from bulk cultures in Sodium lactate broth or Glucose broth. Cultures were inoculated with 1% growing 24-hr culture, incubated at 32 C for 24 hr, and harvested at 12100 x G using a Sorval Continuous-flow KSB system (Ivan Sorvall Inc., Norwalk, Connecticut). The resulting cell paste was suspended in saline-EDTA and heated to 60 C for 10 min to destroy deoxyribonuclease activity. The suspension was centrifuged at 34800 x G and resuspended in saline-EDTA in amount sufficient to make a suspension containing about 100 mg cells/ml.

Cells from strains that produced large amounts of extracellular slime were suspended once in saline-EDTA without pouring off any of the slime. The slime and cells were mixed together thoroughly, heated at 60 C for 19 min, and centrifuged at 34800 x G. At this point, it was assumed that the bulk of the cells had been pelleted on the bottom of the tube and the slime was poured off. Slime adhering to the surface of the cell pellet was washed off with distilled water from a polyethylene squeeze bottle, resulting in a relatively slime-free cell paste. These cells were then suspended and handled in the same manner as previously described for cells from other strains.

Cell suspensions for which immediate handling was not intended were frozen by immersion in an ethanol bath at -20 C and stored at that temperature. Prior to subsequent handling, the suspensions were thawed by immersion in a 32 to 37 C water bath.

E. coli B cells were grown at 37 C for 12 hr and harvested, heated, washed, suspended, and stored in the same manner as were those from propionibacteria.

Cell Disruption and DNA Isolation

Suspensions of propionibacterial cells shown to be sensitive to lysozyme in saline-EDTA were handled by the method of Marmur (62) with modifications. Lysozyme was added to the suspension to a concentration of about 400 µg/ml, and the suspension was incubated overnight. Following incubation, sodium lauryl sulfate was added to bring the final concentration to 2%, and the mixture was heated to 60 C, held at that temperature for 10 min, and cooled to ambient temperature.

Sodium perchlorate (5 M) was added to bring the final concentration to 1 M in the mixture, and one volume of chloroform-isoamyl alcohol was added. This mixture was shaken for 30 min (270 cycles/min through 3.7 cm), and the phases were separated by centrifugation at 240 x G for 30 min. The

aqueous layer was recovered by pipetting, and the DNA was precipitated by the addition of two volumes of 95% ethanol in the following manner: The aqueous mixture containing the DNA was placed in a beaker of sufficient capacity to hold three times the volume of the mixture. Ethanol was added by forcing a thin stream down and against the inside of the beaker just above the surface of the mixture, facilitating maximum mixing with a minimum of agitation. The crude DNA, which precipitated as a white, fibrous material, was recovered by spooling on a thin piece of scored rodding. The spool was stored in 95% ethanol to await additional handling.

Some of the crude DNA precipitates obtained in this manner were further purified according to the method of Marmur (62), omitting, however, solution of the precipitate obtained following treatment with ribonuclease in dilute saline citrate and acetate-EDTA and the precipitation with isopropanol. Washing in progressively increasing concentrations of ethanol was, likewise, omitted. The final precipitate obtained was stored in 95% ethanol for additional handling.

A cell suspension of E. coli B was treated with sodium lauryl sulfate and heated without prior lysozyme treatment. The cell lysate and resulting precipitate were handled in the same manner as those of propionibacteria.

Methods attempted for the disruption of cells of strains found to be resistant to lysozyme in saline-EDTA included incubation with lysozyme in distilled water, passage through a French pressure cell, and grinding in a mortar with a pestle and with alumina or glass powder (200 mesh) (Fisher Scientific Co., Fair Lawn, New Jersey).

Incubation with lysozyme in distilled water

Following harvesting, cells were washed once with distilled water, heated to 60 C for 10 min, centrifuged at 34800 x G, and resuspended in distilled water to 15 g wet, packed cells/ml. Lysozyme was added (400 µg/ml), and the mixture was incubated at 37 C overnight. Following incubation, saline-EDTA (4.71 strength) was added to bring the ionic strength of the mixture up to the level of standard saline-EDTA, and the mixture was handled using the modification of the Marmur (62) procedure.

French pressure cell

Cell suspensions were standardized to 0.10 g wet, packed cells/ml, chilled in an ice bath, and forced through a French pressure cell at 1300 atm of pressure. Unbroken cells and cell debris were removed by centrifugation at 12100 x G for 10 min. The supernatant was handled according to the modification of the Marmur (62) procedure beginning with the

addition of SLS but not including the heating of the mixture.

Grinding (alumina)

Wet, packed cells (2-3 g) were placed in a chilled mortar. Enough alumina was mixed with the cells to make a slightly moist, friable paste, and the mixture was ground in the cold with a chilled pestle for 5-10 min. The mixture was suspended in enough cold saline-EDTA to make a suspension containing about 10 g cells/ml, and the suspension was centrifuged at 12100 x G for 5 min to remove alumina and cell debris. The supernatant was handled in the manner previously described for the French pressure cell.

Grinding (ground glass)

The same procedure was followed, except that ground glass (500 mesh) was substituted for alumina, and the ground mixture was suspended in a minimal (2-3 ml) amount of saline-EDTA. Sodium lauryl sulfate and sodium perchlorate were not added to the supernatant, but it was shaken with chloroform:isoamyl alcohol and centrifuged and the nucleic acids were precipitated in the same manner as previously described.

Assay of DNA Preparations

DNA preparations were assayed by a modification of the method by Burton (13). Calf thymus DNA (400 µg/ml) was used as a standard. Test tubes were acid cleaned, and a series of tubes containing standard DNA in amounts ranging from 0 to 80 µg was prepared. Standard saline citrate was added to bring the volume to 0.50 ml/tube. The assay mixtures to contain propionibacterial DNA were prepared in semi-serial dilutions (10^{0}, $10^{-0.5}$, 10^{-1}, $10^{-1.5}$, etc.), each tube containing 0.50 ml. Perchloric acid (0.50 ml, 1 N) was added to each tube, and the tubes were capped and heated to 70 C for 15 min. After the mixtures had been cooled, 2.0 ml of diphenylamine reagent was added to each, and they were capped and incubated at 30 C for 16 hr. The optical density (600 nm) was measured with a Spectronic 88 colorimeter (Bausch and Lomb Inc., Rochester, New York).

Amounts of DNA in the bacterial DNA preparations were determined from a standard plot constructed from the optical densities of the standard DNA series.

Analytical Ultracentrifugation of DNA

DNA extracted from the strains studied was analyzed for guanine plus cytosine content (GC%) by isopycnic banding in a CsCl density gradient using the method described by Mandel

et al. (61). A Spinco Model E Analytical Ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, California) equipped with a 2-cell An-H titanium rotor and a monochrometer was used. The cells were equipped with Kel-F centerpieces and negative wedges, and the assembled cells were torqued to 130 in-lb. Each sample solution was made to contain approximately 2.0 μg of propionibacterial DNA and was adjusted to a refractive index of 1.4000 ± 0.0005 with distilled water using an ABBE-3L Refractometer (Bausch and Lomb Inc., Rochester, New York). Each run was conducted at 44000 rpm and at about 25 C. No effort to refine the temperature control was made in order to avoid the possibility of convection currents in the cell interior resulting from the repeated application and removal of heat.

At the beginning of each run, a series of exposures of each cell was taken to reveal any anomalies or artifacts that might have existed. At the end of about 20 hr of operation, exposures of 10, 20, and 40 sec were taken of each cell, the plate was developed, and the centrifuge was shut down and cleaned. To verify that 20 hr was a sufficient length of time, one early run was exposed at 20 hr as described, and the run was then permitted to continue for about another 20 hr. Although the bands obtained following the longer run were slightly narrower, they did not provide

any more precision in the location of the band center, so subsequent runs were limited to about 20 hr.

Determination of DNA Base Ratios

Triplicate tracings were made of each sample on K & E 46 1513 paper (Keuffel and Esser Co., offices in all principal cities) from exposed and developed film of each sample using an Analytrol tracing densitometer (Beckman Instruments, Inc., Spinco Division, Palo Alto, California). With the stretch of the rotor assumed to be 0.012 cm at 44000 rpm (3), 7.312 cm was used as the radius of the inner knife edge of the reference hole in computing banding radii. Triplicate tracings made of a run with the counterbalance gave a magnification factor reciprocal (MF = distance on tracing/ actual distance in cell) of 0.11212. The buoyant density of the DNA in a given sample was computed using the expression of Mandel et al. (61):

$$\rho = \rho_o + 4.2\omega^2 (r^2 - r_o^2)(10^{-10}) g/ml$$

where:

ρ = buoyant density of the propionibacterial DNA

ρ_o = buoyant density of the reference DNA

ω = angular velocity in radians/sec = 4406.8

r = banding radius of propionibacterial DNA

r_o = banding radius of reference DNA.

This expression became:

$$\rho = 1.710 + (8.156)\ (10^{-3})\ (r^2 - r_o^2).$$

The percent guanine plus cytosine (GC%) was computed using the expression:

$$GC\% = \frac{\rho - 1.6600}{0.00098} \qquad (61).$$

Measurements of the tracings were estimated to the nearest 0.01 cm. A variation of this amount would result in a variation of about 0.14 in the final computed GC%. The mean of the measured distances on the triplicate tracings of each sample was used to compute the buoyant density and GC% for that sample.

Estimation of Molecular Weight

Values of T were plotted for the function:

$$T = 10^{-A} = 10^{\left[-k\left(\frac{e^{-x^2/2}}{\sqrt{2\pi}}\right)\right]}$$

for $0.90 \leq x \leq 1.84$ and for values of k such that when $x = 0$, $A = 1.000$, 0.5247, 0.300, 0.2500, and 0.2000. Values for the ordinate of the Normal Curve of Error (40) were used

to compute A and T. The linear segment of the curve for each value of $\underline{k}$ was extended to its intersection with the base line, T = 1, and the value of the quantity $[(MF)2I]^{-1}$ was plotted against A_{max} (the value of A when x = 0, see Table 1 and Figure 1) where:

MF = magnification factor

I = the intercept of the extension of the linear segment of the plot with the base line (T = 1).

Table 1. Values for A_{max}, I, and $[(MF)2I]^{-1}$ obtained from listed values for $\underline{k}$

$\underline{k}$	A_{max}	I	$[(MF)2I]^{-1} \times 10^2$
2.550	1.0000	2.455	2.284
1.314	0.5247	2.313	2.424
0.753	0.3000	2.210	2.537
0.627	0.2500	2.178	2.574
0.502	0.2000	2.151	2.606

Maximum absorbence, A_{max}, was obtained from an Analytrol trace of each sample by using the expression:

$$A_{max} = -\log(\underline{b}/\underline{a})$$

where $\underline{a}$ is the distance from the baseline to the unexposed line (see Figure 2) and $\underline{b}$ is the distance from the peak tip

Figure 1. Plot of A_{max} vs. $[(MF)2I]^{-1}$

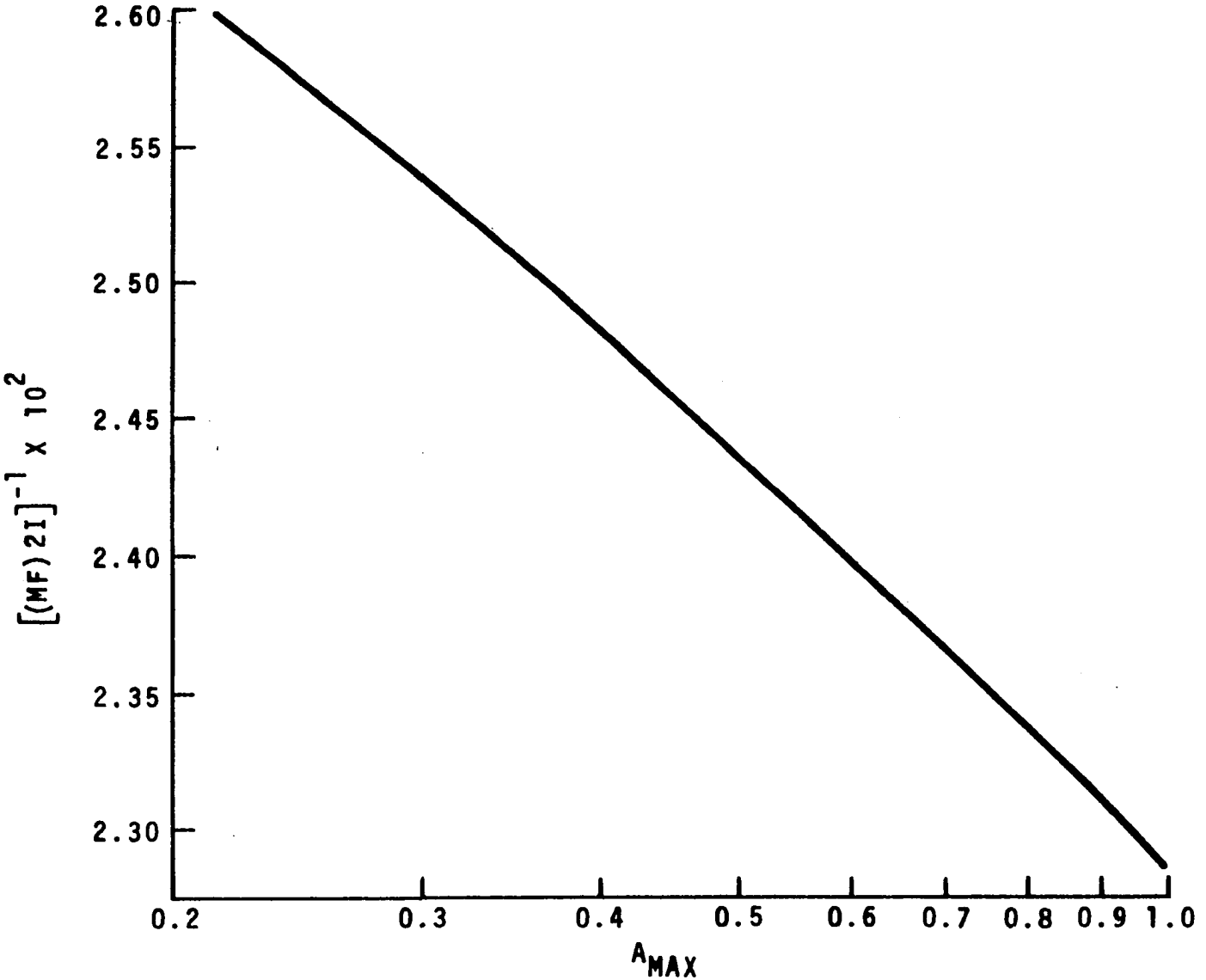
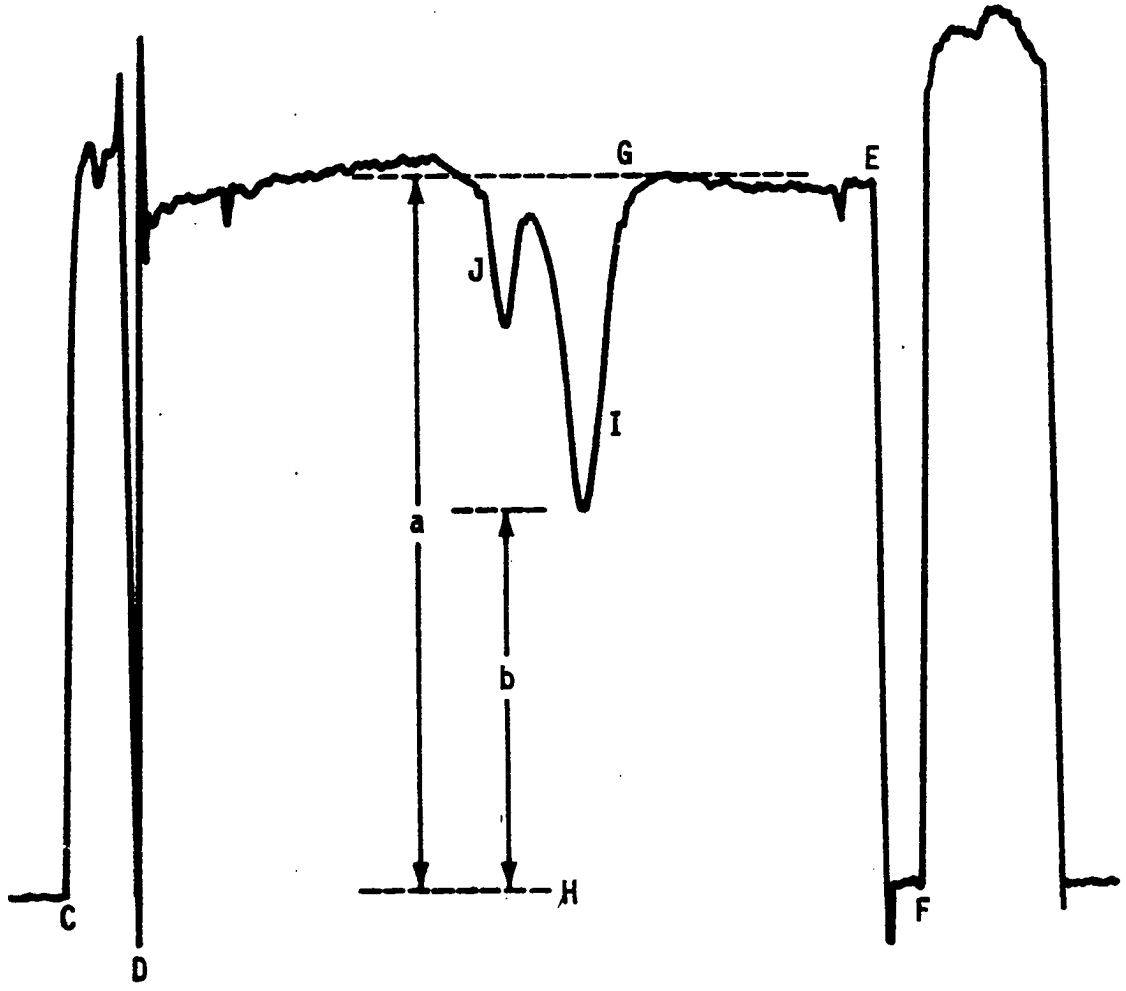

2.60
2.55
2.50
2.45
2.40
2.35
2.30
$[(MF)2I]^{-1} \times 10^2$
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
A_{MAX}

Figure 2. Typical Analytrol trace of centrifuge cell at equilibrium



C = Top of cell
D = Meniscus
E = Bottom of cell
F = Inner edge of reference hole
G = Base line
H = Unexposed line
I = Propionibacterial DNA peak
J = Reference DNA peak

$$A_{max} = -\log (b/a)$$

to the unexposed line. The value of σ for each sample was estimated using the expression:

$$\sigma = \frac{w}{(MF)2I}$$

where:

$\underline{w}$ = width in cm of the peak base between the points of intersection of the extensions of the linear segments of each side

and:

$[(MF)2I]^{-1}$ = the value corresponding to the value of A_{max} (see Figure 1). In cases where A_{max} lay outside the range of the plot, $[(MF)2I]^{-1}$ was estimated by graphical extrapolation.

A corrected value of σ, σ_{corr}, was obtained by multiplying σ by $\underline{r}/\underline{r}_{ave}$ where $\underline{r}$ = banding radius of the sample and $\underline{r}_{ave}$ = the mean banding radius for all samples. The estimated molecular weight of the DNA in a sample was calculated by using the expression:

$$MW = \frac{239000}{\sigma^2_{corr}} \times \frac{44770}{44000} = \frac{247000}{\sigma^2_{corr}}$$

where σ is given in mm. The amount of propionibacterial DNA in each sample was estimated by assuming that the average amount per sample was 2.00 μg and by using the expression:

$$\mu g \text{ DNA} = \frac{2A_{max}\sigma}{\overline{A_{max}\sigma}}$$

where $\overline{A_{max}\sigma}$ is the mean for all values of $\overline{A_{max}\sigma}$.

Analysis of Phenotypic Characters

Species determination

Each strain was assigned to a species according to the key in the seventh edition of Bergey's Manual (10) on the basis of the characters determined in this investigation. In each case where multiple characters used to define a single separation were not in agreement, the placement directed by each character was considered, and the strain was given tentative assignment in each of the possible species.

Sums of insimilitudes

The frequency with which the characters considered to be typical of P. freudenreichii and P. shermanii occurred in agreement with each other was examined in a manner similar to that reported by Véron (106). Given n characters which are expected to separate a collection of m strains into two taxa, with respect to the kth character:

$$A_{i,j,k} \equiv \text{Event} \left\{ \begin{array}{l} \text{kth character absent and the} \\ \text{ith character present} \\ \text{in the jth strain} \end{array} \right\}$$

and:

$$P_{\underline{i},\underline{j},\underline{k}} \equiv \left\{\begin{matrix}\underline{k}\text{th character present and the} \\ \underline{i}\text{th character absent} \\ \text{in the } \underline{j}\text{th strain}\end{matrix}\right\}$$

then:

$$\sum_{\underline{i}=1}^{n} \sum_{\underline{j}=1}^{m} A_{\underline{i},\underline{j},\underline{k}}$$

is defined as the sum of negative insimilitudes, and

$$\sum_{\underline{i}=1}^{n} \sum_{\underline{j}=1}^{m} P_{\underline{i},\underline{j},\underline{k}}$$

is defined as the sum of positive insimilitudes.

$$\sum_{\underline{i}=1}^{n} \sum_{\underline{j}=1}^{m} (A_{\underline{i},\underline{j},\underline{k}} + P_{\underline{i},\underline{j},\underline{k}})$$

is defined as the combined sum of insimilitudes for the $\underline{k}$th character.

Paired comparisons

For each character, and using a table of confidence intervals for the binomial distribution (75), a $\geq$ 95% confidence interval was constructed about the fraction, π_t, of the total number of strains exhibiting that character. Within each group of strains exhibiting a given character, a $\geq$ 95% confidence interval for each of the remaining characters

was constructed about the fraction, π_s, of the number of strains exhibiting the second character. The value, π_t, for each character and the limits of its confidence interval were each multiplied by 50 and expressed as the number of strains in a group of 50 "predicted" to exhibit that character and the confidence limits of that "predicted number." An "actual number" and its confidence limits were determined in the same manner for each character within the group of strains exhibiting a given character.

The hypothesis of stochastic independence of one character from another was tested in two ways. If the predicted number computed from π_t lay outside the confidence limits of the actual number computed from π_s, the difference was considered significant at the first level. If the confidence interval of the predicted value lay outside the confidence limits of the actual value, the difference was considered significant at the second level.

Phenotypic characters and GC%

The difference between the mean GC% for strains exhibiting a given character and that for strain not exhibiting the character was tested for each character by "Student's" t-test (75).

RESULTS AND DISCUSSION

Gas-Liquid Chromatography of Spent Culture Media

All strains produced both acetic and propionic acid and satisfied these criteria for inclusion in the genus Propionibacterium. The molar ratios of propionic acid to acetic acid for most of the strains lay between 1.90 and 1.13. Strains with acid ratios lying outside this range included P15 at 0.94, P28 at 0.54, P30 at 0.91, P44 at 0.97, P63 at 0.65, and P98 at 0.83.

An idealized metabolism of lactate to propionate and acetate, yielding a 2:1 ratio would result in a net production of 2 moles of ATP and a net deficit of 1 mole of reduced nucleotide (55). Van Niel (105) stated that with lactate as a substrate, the molar ratio of the production of propionic acid to acetic acid was generally lower than 2:1, while with glucose, it was generally higher.

In 1930, Foote et al. (31) brought together the reports of organisms which, to that point in time, had been observed to produce propionic acid. These included anaerobic spore formers, yeasts, molds, staphylococci, and streptococci. Carbon sources included a wide range of sugars, acids, fats, alcohols, nitrogenous compounds, and plant and animal products. More recently, Buchanan and Pine (11) reported the production

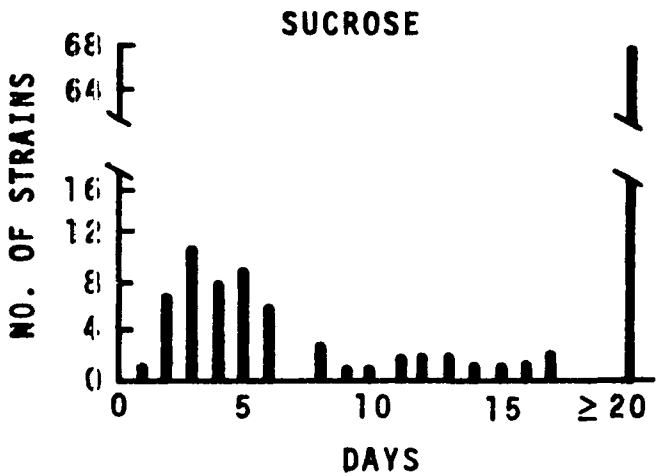
of propionic acid, acetic acid, and carbon dioxide from glucose by an anaerobic actinomycete. These reports of the rather broad occurrence of the ability to produce propionic acid give reason to question the validity of the proposals of Douglas and Sunter (28) and of Moore and Cato (71) that certain corynebacteria, because of their demonstrated ability to produce propionic acid, be classified in the genus Propionibacterium. The traditional propionibacteria represent a distinct population by both phenotypic and genetic yardsticks, and at least have "squatter's rights" to sole possession of the genus.

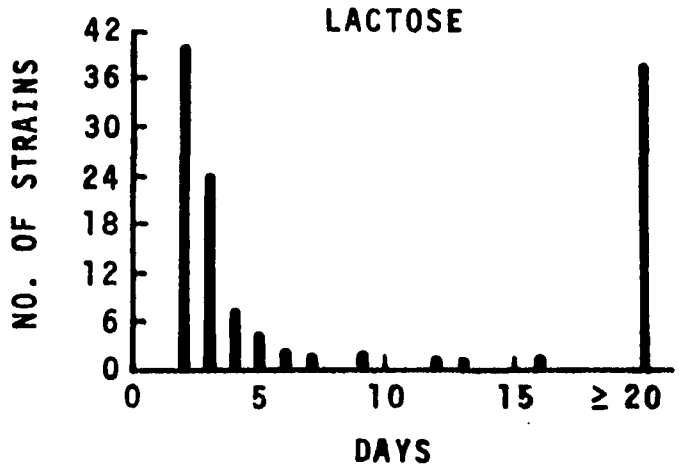
Carbohydrate Fermentation

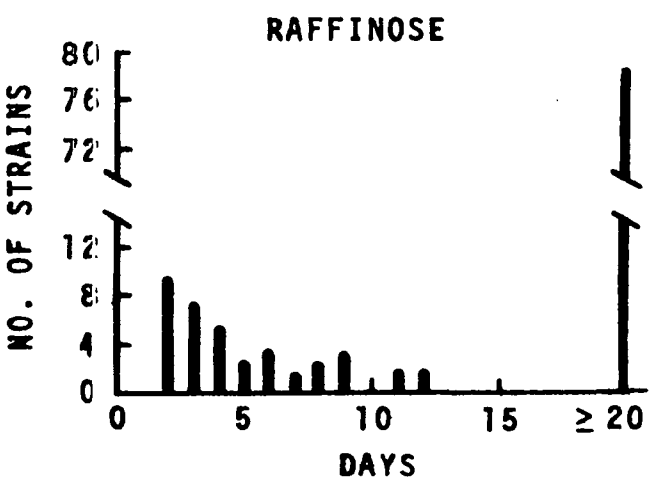
The general results of the carbohydrate fermentations, including only cellobiose and salicin by the conventional method, and including duplicate tests of some strains, are shown in Figures 3 and 4. In none of the test media did a color change, which had failed to reach completion before 20 days, reach completion when held for a period of time afterwards. The results for each strain with each carbohydrate are shown in Table A2.

At the beginning of the carbohydrate fermentation tests, the species designation given by the donor or the isolator of each strain was assumed to be authentic, based upon the Key

Figure 3. Number of strains reaching a complete color change by the indicated number of days


SUCROSE
NO. OF STRAINS
68
64
16
12
8
4
0
0
5
10
15
≥ 20
DAYS


LACTOSE
NO. OF STRAINS
42
36
30
24
18
12
6
0
0
5
10
15
≥ 20
DAYS


RAFFINOSE
NO. OF STRAINS
80
76
72
12
8
4
0
0
5
10
15
≥ 20
DAYS

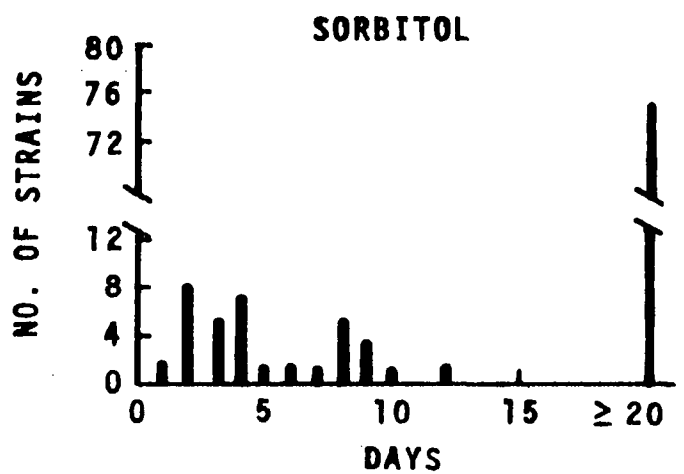
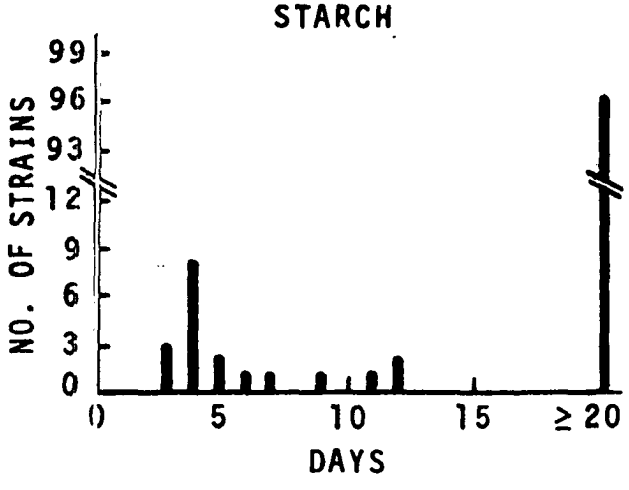
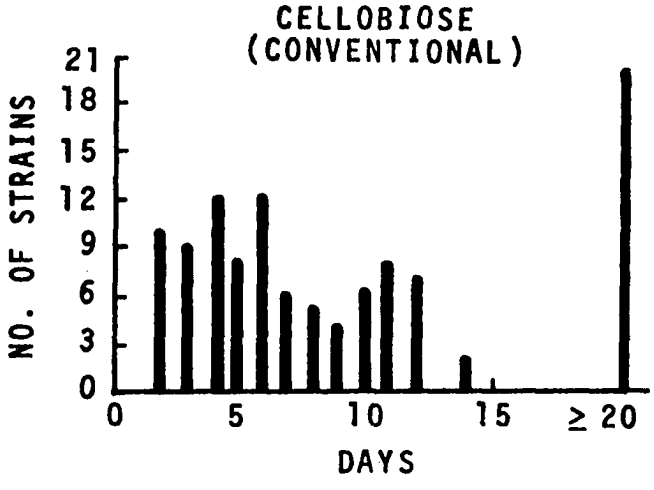
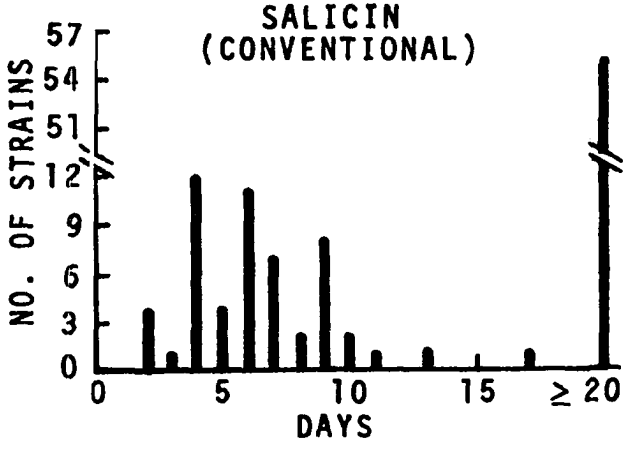
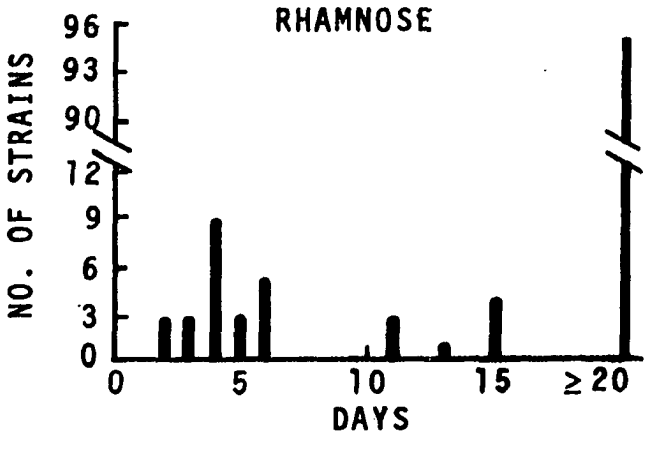

SORBITOL
NO. OF STRAINS
80
76
72
12
8
4
0
0
5
10
15
≥ 20
DAYS

Figure 4. Number of strains reaching a complete color change by the indicated number of days


STARCH
NO. OF STRAINS
99
96
93
12
9
6
3
0
0
5
10
15
≥ 20
DAYS


CELLOBIOSE
(CONVENTIONAL)
NO. OF STRAINS
21
18
15
12
9
6
3
0
0
5
10
15
≥ 20
DAYS


SALICIN
(CONVENTIONAL)
NO. OF STRAINS
57
54
51
12
9
6
3
0
0
5
10
15
≥ 20
DAYS


RHAMNOSE
NO. OF STRAINS
96
93
90
12
9
6
3
0
0
5
10
15
≥ 20
DAYS

to the species of the genus Propionibacterium in the seventh edition of Bergey's Manual (10). The results of the fermentation tests with the carbohydrates directly involved in identification were examined with particular interest. These results were compared with those obtained by Malik (56), who has reported his work with strains P1 through P56 of this same collection. Discrepancies were observed with four strains which were found to ferment sucrose (sucrose +) in this study and which Malik reported as not fermenting sucrose (sucrose -), three strains which were lactose + by this study but were lactose - by Malik, and four strains which were lactose - by this study, but were lactose + by Malik. The first seven discrepancies might be explained by the fact that Malik terminated the fermentation period at 14 days, while in this investigation the period was extended to at least 20 days and was based upon an observed lack of additional activity on the part of strains which had, to that point, failed to produce a complete color change. The other four might have been due to a less rigorous definition of a completed fermentation on the part of Malik. A disadvantage of this technique is the lack of standard approach with respect to the degree of color change required for a positive finding and to the length of incubation time.

Where carbohydrate fermentation results were in disagreement with those expected from the key (10), the tests

were repeated. The first results were corroborated by the second in the cases of seven sucrose - strains, nine sucrose + strains, five lactose - strains, four lactose + strains, one raffinose - strain, one raffinose + strain, two starch - strains, one starch + strain, two rhamnose + strains, and one sorbitol + strain. In three cases, an initial positive result was followed by a negative result. These were P16 with sucrose, and P23 and P49 with lactose. In these cases, the second result was used as the final finding. In no case was an initial negative result followed by a positive result. These results demonstrate the general reproducibility and reliability of carbohydrate fermentation tests. The most reasonable, although least desirable, explanation for the inconsistent results is that the test media may have been contaminated.

Growth Characters with Liquid and Solid Media

The observations made of growth with liquid and solid media are given in Table A3.

Cell Morphology

The cells of all strains were Gram positive. The results of the examination for cell morphology are summarized in Table A4. In the case of many strains, the observed form

lay between either the short, spherical rod and the diptheroid-like rod or the diptheroid-like rod and the long, irregular rod. In each of these cases, a judgment was made and the strain was placed in one of the traditional morphological groups.

Table A4 has a central column and appropriate side columns for each traditional morphological form. A typical form is indicated for the exhibiting strain by an x in the central column for that form. The case where a strain exhibited a form most resembling but not typical of a traditional form is indicated by an $\underline{x}$ in the side column nearest the column set for the other form suggested by the strain's morphology.

Growth Patterns in Liquid Media

The rate and amount of growth in liquid media of each of four strains are shown in Figures 5, 6, 7, and 8. There appeared to be little difference in the total amount of growth obtained, comparing the two incubation times when Sodium lactate broth was used. The culture incubated at 32 C did, however, approach the end of the logarithmic phase of growth in about 24 hr, while that incubated at 21 C took about four times as long.

To avoid the long period of growth which would be

Figure 5. Growth of strain P8

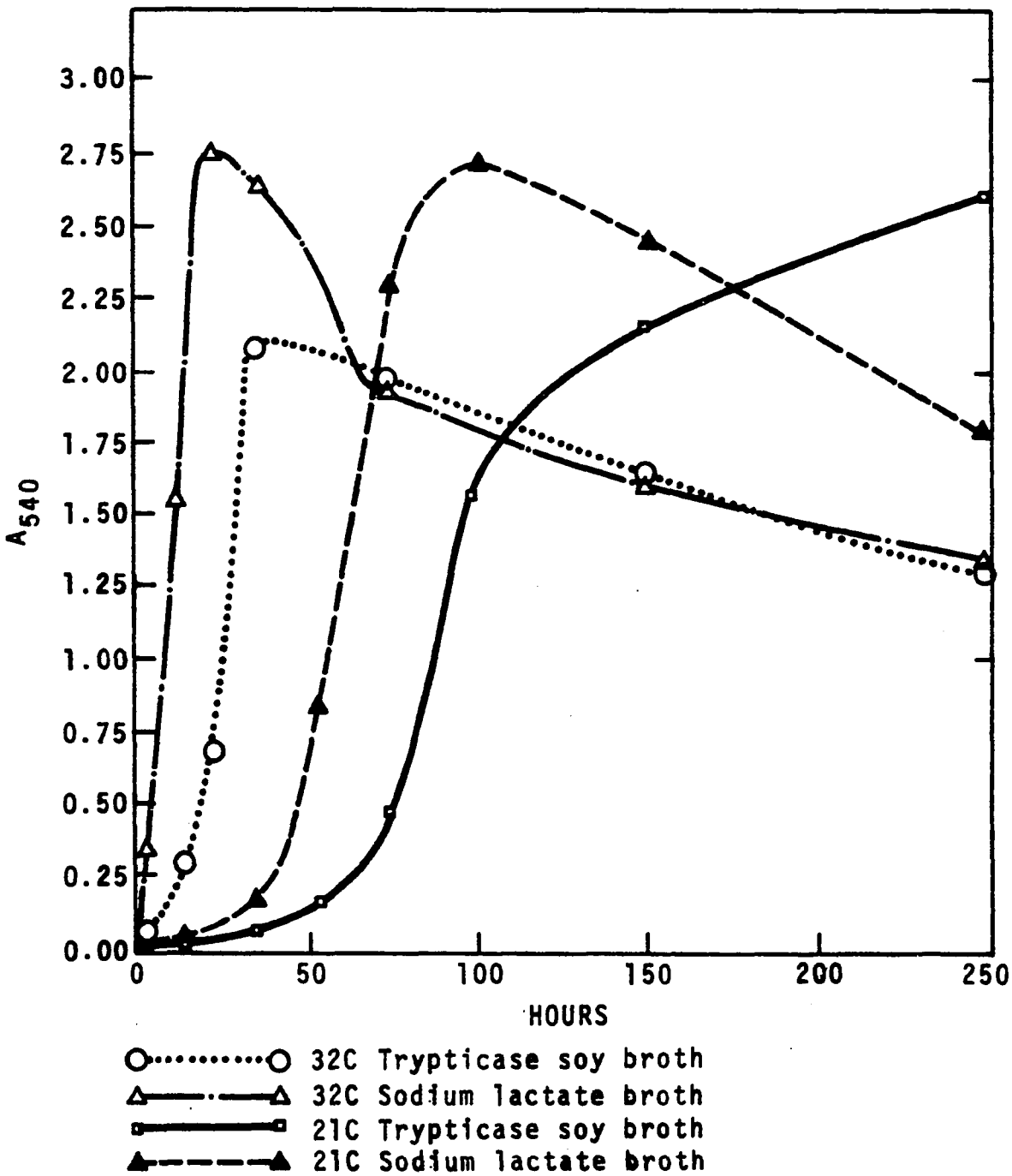

3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
A_{540}
0
50
100
150
200
250
HOURS
32C Trypticase soy broth
32C Sodium lactate broth
21C Trypticase soy broth
21C Sodium lactate broth

Figure 6. Growth of strain P12

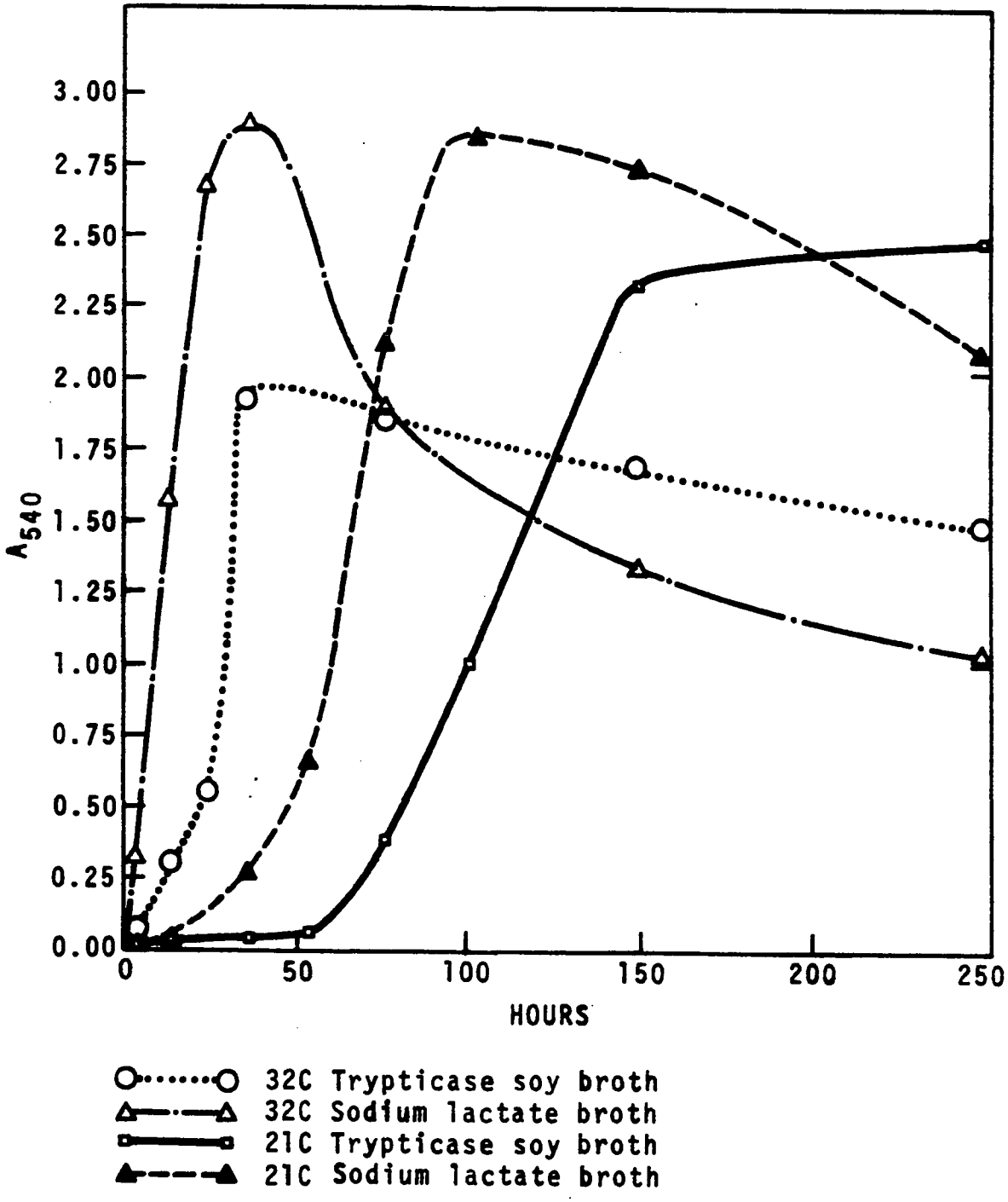

3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
A_{540}
0
50
100
150
200
250
HOURS
32C Trypticase soy broth
32C Sodium lactate broth
21C Trypticase soy broth
21C Sodium lactate broth

Figure 7. Growth of strain P16

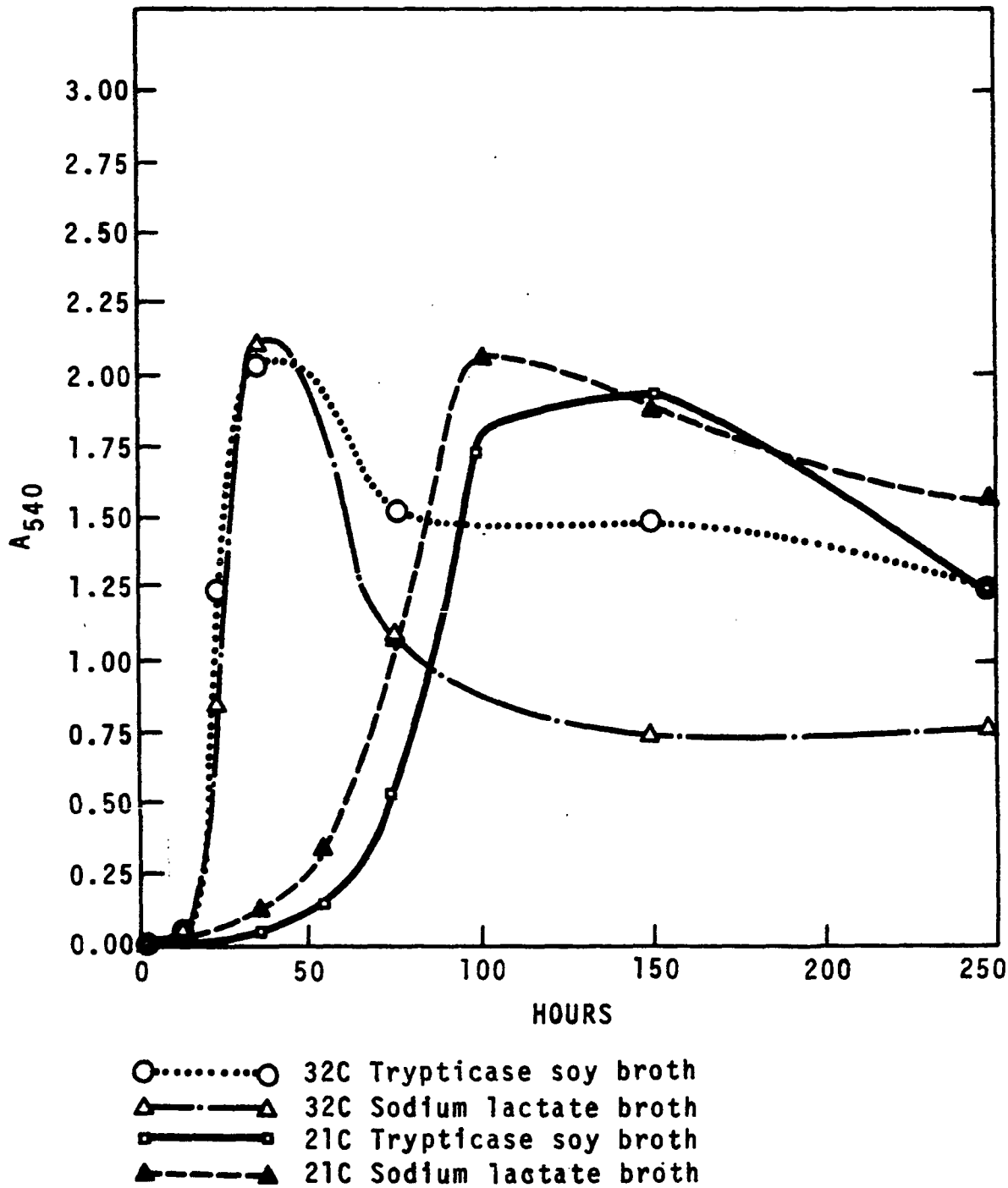
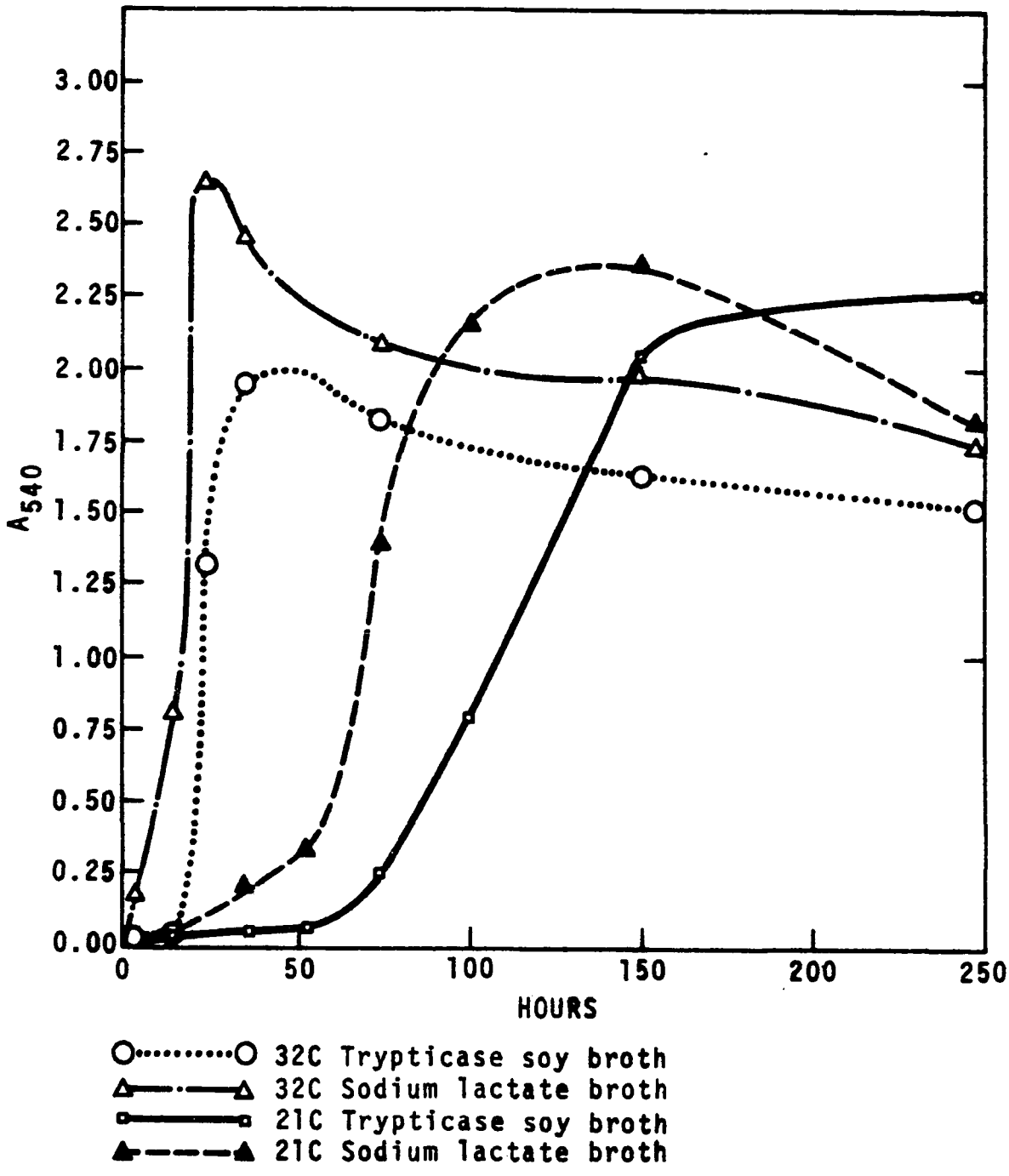

3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
A_{540}
0
50
100
150
200
250
HOURS
32C Trypticase soy broth
32C Sodium lactate broth
21C Trypticase soy broth
21C Sodium lactate broth

Figure 8. Growth of strain P23


3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
A540
0
50
100
150
200
250
HOURS
32C Trypticase soy broth
32C Sodium lactate broth
21C Trypticase soy broth
21C Sodium lactate broth

required in cultures incubated at the lower temperature, 32 C was selected as the incubation temperature for cultures from which DNA would be prepared.

It was observed in trial harvesting runs with strain P12, that considerable slime was present in cultures grown at 21 C, while those grown at 32 C were virtually slime-free also was demonstrated by Skogen (93). The presence of slime lowered the efficiency of the harvesting and washing of cells and provided an additional reason for growing cells at the higher temperature.

In each case, the amount of growth in Sodium lactate broth was equal to or greater than that in Trypticase soy broth. Sodium lactate broth was initially selected as the medium for cell growth in bulk culture. At a point later in the investigation, Glucose broth was substituted for Sodium lactate broth. There was no substantial difference observed in the growth patterns and cell yields of the strains grown on the less expensive carbon source with those grown on sodium lactate.

Lysozyme Sensitivity

The results of the screening for lysozyme sensitivity under the conditions of the Marmur (62) procedure and in distilled water are summarized in Table 2. If the duplicate pair of a strain showed obvious differences in opacity or

Table 2. Lysozyme sensitivity of propionibacterial strains[a]

Strain	In SEDTA	In water	Strain	In SEDTA	In water	Strain	In SEDTA	In water
P1	++		P42	-	?	P80	++	
P2	-	++	P44	-	++	P81	?	++
P3	+		P45	++		P82	?	
P4	-	++	P46	-	-	P83	+	
P5	-	-	P47	++		P84	-	++
P6	+		P48	++		P85	-	++
P7	++		P49	++		P86	-	-
P8	++		P50	-	+	P87	-	-
P9	++		P51	+		P88	-	++
P10	-	+	P52	-		P89	-	
P11	++		P53	-	++	P90	++	
P12	++		P54	-	++	P91	++	
P13	++		P55	++		P92	++	
P14	++		P56	++		P93	++	
P15	-	+	P57	++		P94	++	
P16	++		P58	++		P95	++	
P17	?	+	P59	+		P96	++	
P18	++		P60	+		P97	++	
P19	++		P61	+		P98	-	
P20	-	-	P62	++		P99	++	
P21	-	+	P63	-	+	P100	++	
P22	++		P64	++		P101	++	
P23	++		P65	++		P102	++	
P24	++		P66	++		P103	++	
P25	-	+	P67	+		P104	++	
P26	-	++	P68	-	+	P105	-	-
P28	-	-	P69	-	++	P106	?	+
P30	-	+	P70	++		P107	-	+
P31	++		P71	++		P108	-	++
P32	+		P72	++		P109	+	
P33	++		P73	++		P110	++	
P35	++		P74	-	++	P121	-	
P37	++		P75	++		P122	-	
P38	-	+	P76	++				
P39	+		P77	+				
P40	+		P78	-	++			
P41	-	+	P79	+				

[a]SEDTA = Saline EDTA;
++ = Highly sensitive;
+ = Moderately sensitive;
- = Not sensitive.

viscosity, the degree of sensitivity is indicated with two plus signs. Less obvious, but none-the-less observable, differences are indicated by a single plus sign. In the latter case, most strains exhibited only partial lysis as observed by phase contrast microscopy. In either case, the strain was characterized as sensitive. For the purpose of the analysis of the data, strains with questionable sensitivity in saline-EDTA were regarded as not sensitive.

Strains assigned to species P. freudenreichii and P. shermanii by their donors or isolators or by this investigator were generally sensitive to the Marmur (62) approach to enzymatic lysis, with only 5 of the 65 exhibiting absolute resistance. Strains assigned to the remaining species were generally resistant, with only 10 of 42 strains either sensitive or exhibiting only questionable resistance. Thirty-two of the lysozyme-resistant strains were incubated in distilled water. Twenty-four of these were sensitive to lysis under this set of conditions, however, no clear pattern emerges from this information. One explanation for the lysozyme resistance in Saline-EDTA of strains sensitive in distilled water is competition by small ions with the enzyme for reactive sites on the cell-wall (68). This would suggest that the possible cell wall structural differences between the two main groups responsible for the general difference in sensitivity to lysozyme are subtle and complex. One specific

difference which has been reported is that the peptidoglycan diaminopimelic acid of strains belonging to P. freudenreichii and P. shermanii is of the meso configuration while that of strains belonging to the other species is of the L configuration (43).

All strains examined for sensitivity in the lysozyme-Lytase system were resistant. Repeated heating, as suggested by Miller et al. (70), failed to bring about lysis of any of the cells so tested.

Cell Disruption and DNA Isolation

Cells of all of the lysozyme-sensitive strains chosen for DNA analysis gave adequate yields of crude precipitate from treatment by the modification of the Marmur (62) procedure.

Incubation with lysozyme in distilled water resulted in a satisfactory yield of DNA from cells of only one strain, P25. Although the common evidences of cell lysis, increased viscosity and reduced turbidity, were usually present, repeated attempts to obtain high-quality DNA from cells of other strains met with failure, and use of the method was discontinued. Residual deoxyribonuclease activity, freed from EDTA inhibition, may be the reason for this lack of success.

Only occasional success was realized from the attempts

at cell disruption with the French pressure cell and by grinding with alumina. For a given suspension, only one passage through the pressure cell was attempted because of the possibility, with a second passage, of excessive shear degradation of the DNA freed by already-disrupted cells. For most strains, probably only a small fraction of the cells were disrupted by the single passage. The DNA thus liberated could have been rapidly degraded due to its low concentration (62). The lack of success from grinding with alumina can be partially explained by assuming that the alumina adsorbed a significant amount of freed DNA. If only a small fraction of the cells were disrupted by the grinding, this might have accounted for the entire loss of yield. The argument with respect to degradation of DNA in low concentrations also would hold. In the centrifuge supernatants from pressure cell disruption and alumina grinding, a cloudy precipitate was observed upon the addition of $NaClO_4$. The possibility that DNA was being occluded with what was assumed to be a protein precipitate also was considered.

To minimize the possible losses of DNA to these suspected causes, glass powder (Fisher Scientific Co., Fair Lawn, New Jersey) was substituted for alumina. Following grinding, the glass powder-cell paste mixture was resuspended in only a minimal amount of saline-EDTA, and sodium lauryl

sulfate and $NaClO_4$ were not added to the centrifuge supernatants of the cells. Adequate amounts of crude precipitate were obtained from cells of all strains with which this modified method of grinding and isolation was attempted.

The amount of grinding could be a critical element in determining the amount of a yield or even the fact of a yield. An insufficient quantity of cells might be disrupted by incomplete grinding, while shear degradation would result from excessive grinding. The physical consistency of the grinding mixture also could play a role in the efficiency of grinding. A too-viscous or too-dry mixture would absorb and shield the cells from much of the grinding energy, while a not-viscous-enough or too-moist mixture would result in a lowered efficiency due to a smaller quantity of cells/unit volume and the loss of grinding mixture through its adherence to inaccessible places on the mortar or pestle. The latter effect would be repeated grinding of some parts of the mixture and no grinding at all and the loss of other parts. Judging the physical consistency of the grinding mixture and the amount of grinding necessary to give the best possible yield is an art developed with practice. Generally, a slightly-moist, friable paste and grinding for not more than 5 min gave best results. Additional yield could be salvaged by washing the glass powder-cell paste mixture with a minimal amount of saline-EDTA a second time and handling the

centrifuge supernatant in the same manner as previously described.

The methods used to disrupt cells and isolate DNA and the yields of DNA for each strain, based upon the amount in the final preparation are summarized in Table 3. The mean yields of DNA obtained using lysozyme with extensive purification, lysozyme with limited purification, grinding with alumina, grinding with ground glass, and the pressure cell were 1.04, 1.54, 0.44, 0.36, and 0.48 mg/g wet packed cells respectively. These may be compared with the yield of 1 to 2 mg/g wet packed cells suggested as a general possibility using the method by Marmur (62). As previously stated, the low yields obtained by grinding with glass powder could have been improved by a second wash but since initial yields were sufficient, scavenging of unrecovered DNA was not usually attempted.

DNA Base Ratios

Fifty-three strains were selected from which DNA was isolated and analyzed. The GC%'s are shown in Table 3.

DNA was prepared by two different methods for two propionibacterial strains and reference strain E. coli B. Mixtures of reference and propionibacterial DNA's in different combinations with respect to the method of preparation

Table 3. Methods of preparation, GC%'s, yields, and estimated molecular weights of propionibacterial DNA's

Strain	Method of Preparation	GC%	Yield (mg/g wet, packed cells)	Estimated molecular weight ($\times 10^6$)	Estimated amount of propionibacterial DNA in sample (µg)
P1	Lysozyme/limited purification	65.6	>0.63	2.88	1.86
P2	Pressure cell	66.9	0.35	5.55	1.30
P2	Grinding/alumina	67.7	0.27	3.57	1.38
P4	Grinding/ground glass	66.1	0.24	3.77	1.55
P5	Grinding/ground glass	66.8	0.32	4.47	1.55
P7	Lysozyme/lim. purif.	65.7	>0.61	2.65	2.30
P8	Lysozyme/extensive purification	65.9	0.52	3.95	1.91
P10	Grinding/ground glass	67.2		4.88	2.14
P11	Lysozyme/lim. purif.	65.9	0.86	8.95	2.55
P12	Lysozyme/lim. purif.	65.2	2.6	6.84	2.07
				&8.15	&2.08
P12	Grinding/alumina	65.3	0.94	6.92	1.93
				&5.60	&2.02
P13	Lysozyme/ext. purif.	65.9	0.38	2.31	1.77
P14	Lysozyme/ext. purif.	66.2	0.57	2.11	1.33
P15	Grinding/ground glass	66.8		4.29	1.19
P16	Lysozyme/ext. purif.	66.2	0.85	2.60	3.21
P18	Lysozyme/ext. purif.	68.0	0.32	2.59	2.19
P19	Lysozyme/ext. purif.	65.7	2.02	2.98	3.07
P20	Grinding/ground glass	67.1	0.071	5.39	1.26
P22	Lysozyme/ext. purif.	65.6	1.08	3.56	1.80
P23	Lysozyme/ext. purif.	65.5	0.36	5.20	1.60
P24	Lysozyme/ext. purif.	65.7	1.70	5.65	2.81
P25	Lysozyme/dist. water	67.5		8.64	2.24
P26	Grinding/ground glass	66.9	0.69	7.06	0.97
P28	Grinding/ground glass	66.8	0.22	6.37	1.59
P31	Lysozyme/ext. purif.	66.0	1.91	3.15	3.08
P33	Lysozyme/ext. purif.	66.2	1.83	2.55	2.44
P37	Lysozyme/ext. purif.	65.0	0.20	5.01	20.3
P38	Grinding/ground glass	67.1	0.182	6.05	2.09
P39	Lysozyme/lim. purif.	66.3	3.6	4.80	2.93
P44	Grinding/ground glass	67.1		6.24	2.44
P45	Lysozyme/ext. purif.	65.1	0.78	7.22	2.74
P46	Grinding/ground glass	67.4	0.071	4.05	2.03
P47	Lysozyme/ext. purif.	65.7	2.19	3.80	1.51
P48	Lysozyme/ext. purif.	65.8	0.84	4.40	2.00
P50	Pressure cell	67.5	0.93	3.32	1.50
P52	Grinding/ground glass	67.3	0.134	2.04	1.97
P54	Grinding/ground glass	67.0	0.33	7.37	3.13

Table 3 (Continued)

Strain	Method of Preparation	GC%	Yield (mg/g wet, packed cells)	Estimated molecular weight ($\times 10^6$)	Estimated amount of propionibacterial DNA in sample (µg)
P55	Lysozyme/lim. purif.	66.8	2.7	4.88	2.03
P63	Grinding/ground glass	66.7	0.27	9.29	1.27
P65	Lysozyme/lim. purif.	65.8	1.40	11.44	1.86
P66	Grinding/ground glass	65.3	0.86	4.51	1.66
P68	Grinding/ground glass	66.5	0.36	6.84	2.04
P69	Grinding/ground glass	66.7	0.96	5.01	1.69
P74	Grinding/ground glass	67.2	0.191	7.12	1.66
P76	Lysozyme/lim. purif.	65.8	0.67	6.92	2.49
P77	Lysozyme/lim. purif.	65.3	1.23	11.76	3.09
P78	Pressure cell	67.0	0.165	7.89	1.22
P85	Grinding/ground glass	67.2		7.89	1.65
P89	Grinding/ground glass	65.2	0.69	3.52	1.94
P95	Lysozyme/lim. purif.	67.6	0.70	4.88	2.52
P96	Lysozyme/lim. purif.	65.8	0.60	7.62	2.46
P98	Grinding/ground glass	65.3		5.24	1.81
P106	Grinding/alumina	67.2	0.114	5.39	1.95
P107	Grinding/ground glass	67.3		5.60	1.97
P108	Grinding/ground glass	67.5	0.138	2.64	1.08

were analyzed to discover any effect that the method of preparation might have had upon buoyant density. The results of these runs are shown in Table 4. The bulk of the preparations analyzed were obtained by either lysozyme or by grinding, the two methods which showed the closest agreement in this limited comparison. The only lysozyme-resistant strain from which a usable quantity of DNA was obtained by incubation with lysozyme in distilled water, P25, gave a GC% in the same region as those from the

Table 4. Buoyant densities and GC%'s of DNA's prepared from the same strains by different methods and using reference DNA prepared by two different methods

Strain	Method of preparation	Method of preparation - reference DNA	$(\rho-1.72)10^3$	GC%
P12	Lysozyme	SLS	3.86	65.2
P12	Grinding	SLS	3.95	65.3
P2	Pressure cell	SLS	5.58	66.9
P2	Grinding	SLS	6.33	67.7
P12	Lysozyme	Pressure cell	4.44	65.7
P12	Grinding	Pressure cell	4.49	65.8

other lysozyme-resistant strains obtained by grinding.

Mandel et al. (61) stated that the standard error of the mean GC% obtained by equilibrium banding is 0.7%. These comparisons thus provide no evidence that the method of preparation has an effect upon the buoyant density of the resulting DNA.

Analysis of Phenotypic Characters

Those characters which define P. freudenreichii and P. shermanii (Main Group I) as opposed to the balance of the traditional propionibacterial species (Main Group II) in the Key to the species of genus Propionibacterium (10)

include sucrose -; small, spherical cells; and little or no surface growth on a Sodium lactate agar stab. In addition, ropiness and gray-colored growth, both in liquid media, are described as being uniquely characteristic of species in Main Group I. It was observed, in the course of this investigation, that strains identified as belonging to the species in Main Group I were generally sensitive to lysozyme and tended to exhibit a dull reddish color (not to be confused with the deep red of the pigmented strains) in the sub-surface growth of a Sodium lactate agar stab.

It was hypothesized that the inability to ferment sucrose, small and spherical cell morphology, slight growth or the absence of growth on a stab surface, grayish growth in broth, ropy growth in broth, lysozyme sensitivity, and a reddish stab interior were stochastically independent characters. The results of the test of this hypothesis are shown in Tables 5 through 11, and a graphical representation of the levels of significance between paired characters is shown in Figure 9.

In addition to the seven already-described characters, an arbitrary universal character and an arbitrary non-existent character are included in a tabulation of the sums of insimilitudes shown in Table 12. The positions, on a linear scale, of the characters with respect to combined sums of insimilitudes are shown in Figure 10.

Table 5. Paired comparisons of "actual numbers" with "expected number" of sucrose negative propionibacterial strains in the groups of total strains exhibiting the listed characters (expected number = 27, confidence interval = 22.5-32.5)

Group-defining character	Sucrose negative strains		Differences from "expected number" - significance	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Stab surface growth slight or absent	33	26.0-39.5	Yes	No
Stab interior growth reddish	41	34.5-45.5	Yes	Yes
Small, spherical cells	42	35.5-46.5	Yes	Yes
Ropy in liquid media	29	21.5-36.0	No	No
Gray in liquid media	35	28.0-41.0	Yes	No
Lysozyme sensitive	42	35.5-46.5	Yes	Yes

Table 6. Paired comparisons of "actual numbers" with "expected number" of propionibacterial strains with slight or absent stab surface growth in the groups of total strains exhibiting the listed characters (expected number = 22, confidence interval = 17.5 -27.5)

Group-defining character	Strains with slight or absent stab surface growth		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Sucrose negative	27	19.5-34.0	No	No
Stab interior growth reddish	33	26.0-39.5	Yes	No
Small, spherical cells	27	19.5-34.0	No	No
Ropy in liquid media	24	17.0-31.5	No	No

Table 6 (Continued)

Group-defining character	Strains with slight or absent stab surface growth		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Gray in liquid media	25	18.0-32.0	No	No
Lysozyme sensitive	25	18.0-32.0	No	No

Table 7. Paired comparisons of "actual numbers" with "expected number" of propionibacterial strains with reddish interior stab growth in the groups of total strains exhibiting the listed characters (expected number = 30, confidence interval = 25.0-35.0)

Group-defining character	Stains with reddish interior stab growth		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Sucrose negative	45	39.0-48.5	Yes	Yes
Stab surface growth slight or absent	44	38.0-47.5	Yes	Yes
Small, spherical cells	42	35.5-46.5	Yes	Yes
Ropy in liquid media	31	23.5-37.5	No	No
Gray in liquid media	34	27.0-40.0	No	No
Lysozyme sensitive	42	35.5-46.5	Yes	Yes

Table 8. Paired comparisons of "actual numbers" with "expected number" of propionibacterial strains with small, spherical cells in the groups of total strains exhibiting the listed characters (expected number = 29, confidence interval = 24.0-34.0)

Group-defining character	Small, spherical cells		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Sucrose negative	44	38.0-47.5	Yes	Yes
Stab surface growth slight or absent	34	27.0-40.0	Yes	No
Stab interior growth reddish	41	34.5-45.5	Yes	Yes
Ropy in liquid media	32	25.0-38.5	No	No
Gray in liquid media	34	27.0-40.0	Yes	No
Lysozyme sensitive	41	34.5-45.5	Yes	Yes

Table 9. Paired comparisons of "actual numbers" with "expected number" of propionibacterial strains with ropy growth in liquid media in the groups of total strains exhibiting the listed characters (expected number = 36, confidence interval = 31.5-40.5)

Group-defining character	Strains with ropy growth in liquid media		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level ?	2nd level ?
Sucrose negative	38	31.0-43.5	No	No
Stab surface growth slight or absent	40	33.0-45.0	No	No
Stab interior growth reddish	38	31.0-43.5	No	No
Small, spherical cells	40	33.0-45.0	No	No
Gray in liquid media	40	33.0-45.0	No	No
Lysozyme sensitive	39	32.0-44.0	No	No

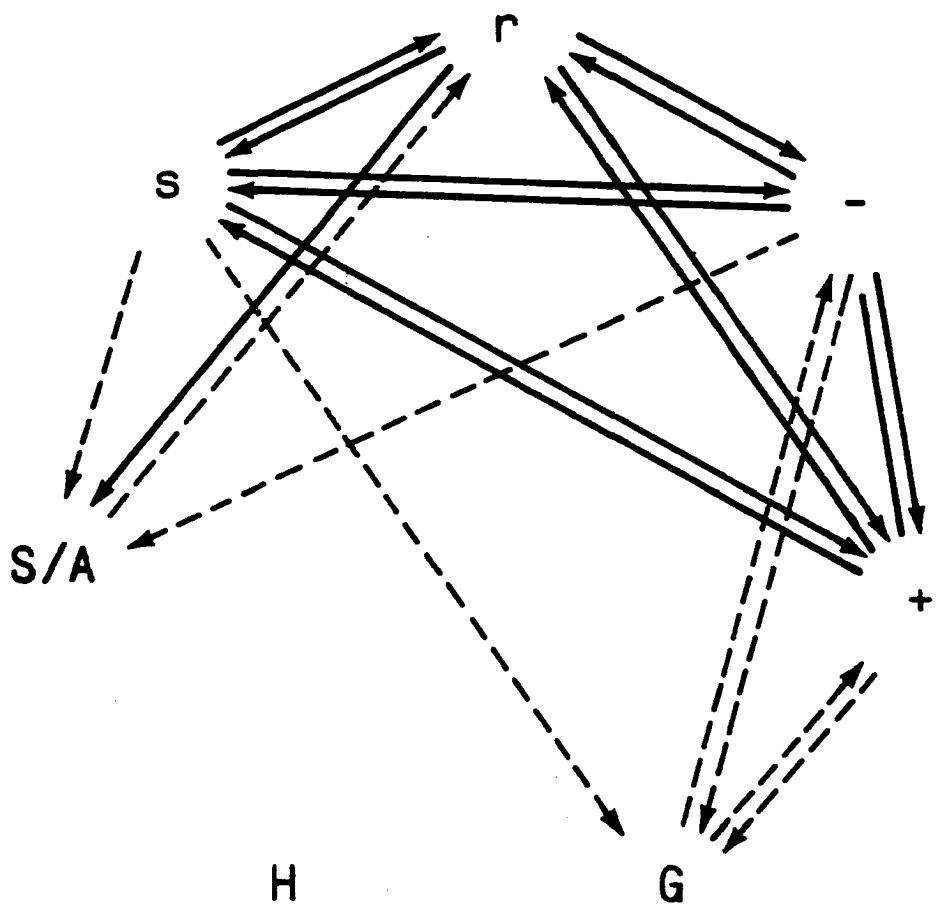
Table 10. Paired comparisons of "actual numbers" with "expected number" of propionibacterial strains with a gray color in liquid media in the groups of total strains exhibiting the listed characters (expected number = 26, confidence interval = 20.5-30.5)

Group-defining character	Strains with a gray color in liquid media		Significance of differences from "expected number"	
	"Actual" Number	Confidence Interval	1st level ?	2nd level ?
Sucrose negative	33	26.0-39.5	Yes	No
Stab surface growth slight or absent	29	21.5-36.0	No	No
Stab interior growth reddish	29	21.5-36.0	No	No
Small, spherical cells	30	22.5-36.5	No	No
Ropy in liquid media	28	20.5-35.0	No	No
Lysozyme sensitive	33	26.0-39.5	Yes	No

Table 11. Paired comparisons of "actual numbers" with "expected number" of lysozyme sensitive propionibacterial strains in the groups of total strains exhibiting the listed characters (expected number = 31, confidence interval = 26.0-36.0)

Group-defining character	Lysozyme sensitive strains		Significance of differences from "expected number"	
	"Actual Number"	Confidence Interval	1st level?	2nd level ?
Sucrose negative	48	43.0-50.0	Yes	Yes
Stab surface growth slight or absent	34	27.0-40.0	No	No
Stab interior growth reddish	43	36.5-47.0	Yes	Yes
Small, spherical cells	44	38.0-47.5	Yes	Yes
Ropy in liquid media	33	26.0-39.5	No	No
Gray in liquid media	40	33.0-45.0	Yes	No

Figure 9. Significance of differences between "expected" and "actual" numbers of propionibacterial strains exhibiting paired characters. A dotted line indicates significance at first level. Arrow points toward group-defining character



(r) Stab interior growth reddish

(s) Small, spherical cells

(-) Sucrose negative

(S/A) Stab surface growth slight or absent

(+) Lysozyme sensitive

(H) Ropy in liquid media

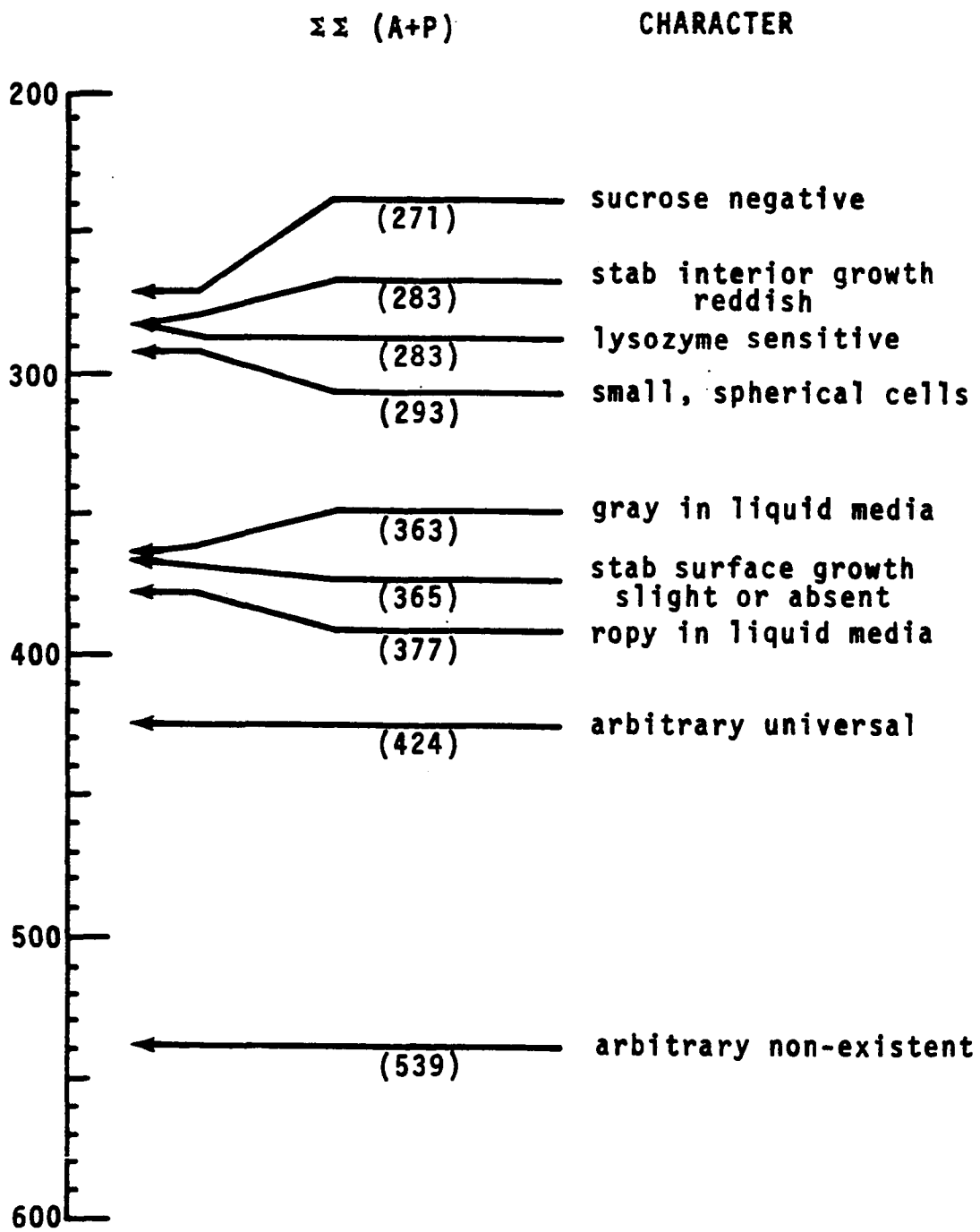
(G) Gray in liquid media

Table 12. Sums of insimilitudes for characters used in separating main groups I and II

Character	$\sum_{i=1}^{9} \sum_{j=1}^{107} A_{ijk}$	$\sum_{i=1}^{9} \sum_{j=1}^{107} P_{ijk}$	$\sum_{i=1}^{9} \sum_{j=1}^{107} (A_{ijk}+P_{ijk})$
Sucrose negative	136	135	271
Stab surface growth slight or absent	236	129	365
Stab interior growth reddish	123	160	283
Small, spherical cells	137	156	293
Ropy in liquid media	107	270	377
Gray in liquid media	217	146	363
Arbitrary universal	0	424	424
Arbitrary non-existent	539	0	539

Of the seven characters studied, no two appeared in complete concert, however, two of the three used by the seventh edition of Bergey's Manual (10) to define the separation between Main Groups I and II; sucrose - and small, spherical cells; along with lysozyme sensitivity

Figure 10. Combined sums of insimilitudes for seven real and two arbitrary characters on a linear scale


ΣΣ (A+P)
CHARACTER
200
300
400
500
600
(271)
sucrose negative
(283)
stab interior growth reddish
(283)
lysozyme sensitive
(293)
small, spherical cells
(363)
gray in liquid media
(365)
stab surface growth slight or absent
(377)
ropy in liquid media
(424)
arbitrary universal
(539)
arbitrary non-existent

and reddish interior stab growth; showed a significantly high frequency of simultaneous occurrence. The third character used to separate the main groups, the absence or presence of only slight surface growth on an agar stab, together with gray and with ropy growth in liquid media, showed only weak and generally insignificant agreement with the other four characters, and practically no agreement among themselves.

It is reasonable to suppose, given a set of compatible dichotomous characters, that a population in possession of any given combination of these characters exists in nature. It follows that populations possessing one combination of characters will exist in greater numbers than populations possessing other combinations. These differences in numbers can result from ecological influences. The determination of more than one character by a single genetic influence offers another explanation. If an array of $2^{\underline{n}}$ positions is visualized, where $\underline{n}$ is the number of characters under consideration, a suitable population of strains would be expected to form a distribution pattern on the array. With a large population, every position would be occupied, but the pattern would be far from uniform, some positions containing proportionately greater numbers of strains than could be explained by random distribution.

The strains and characters used in this study provide

a microcosm demonstrating the plausibility of this model. There were 91 unoccupied positions, 1 position with 11 strains, and 1 position with 16 strains. If the 107 strains had been distributed in a random fashion among the 128 positions, the expected number of empty positions would have been 46.3. The expected number of positions having 11 or more strains is less than 1.8×10^{-8}. The fit of the actual number of positions with the expected number of positions containing k number of strains according to the Poisson distribution (30) is shown in Table 13. The general

Table 13. Fit of actual number of positions with expected number of positions containing k strains

Number of strains (k)	Actual number of positions containing k strains (N_k)	Expected number of positions containing k strains [$N_p(k, 107/128)$]
0	91	46.3
1	17	38.7
2	9	16.2
3	2	4.52
4	1	0.944
5	2	0.157
6	3	2.19×10^{-2}
7	1	2.62×10^{-3}
11	1	1.62×10^{-11}
16	1	1.26×10^{-17}

agreement among the characters is apparent, but since a species historically has usually been established on the basis of information from a small number of strains, positions with a high number of strains may not necessarily coincide with established taxa or genotypes.

For each of the seven phenotypic characters, the results of the test for differences between the mean GC% for strains exhibiting the character and the mean GC% for strains not exhibiting the character are shown in Table 14 and illustrated in Figure 11. The same pattern that was established with the sums of insimilitudes and with paired comparisons emerges. These data suggest that the traditional propionibacteria fall into two genotypes with GC%'s of approximately 66 and 67, and with the former generally characterized as sucrose negative; lysozyme sensitive; small, spherical cells; and with reddish interior stab growth. The difference between the mean GC%'s of the two genotypes, although less than the 2% suggested by Jones and Sneath (44) as necessary to imply special differences, is none-the-less significant. The pooled standard error of the GC%'s of the two genotypes ranges from 0.27, if they are separated using the sucrose - character to 0.66, if gray color in liquid media is used. These are compared to a value of 0.7 which Mandel et al. (61) obtained from 44 repeat runs of

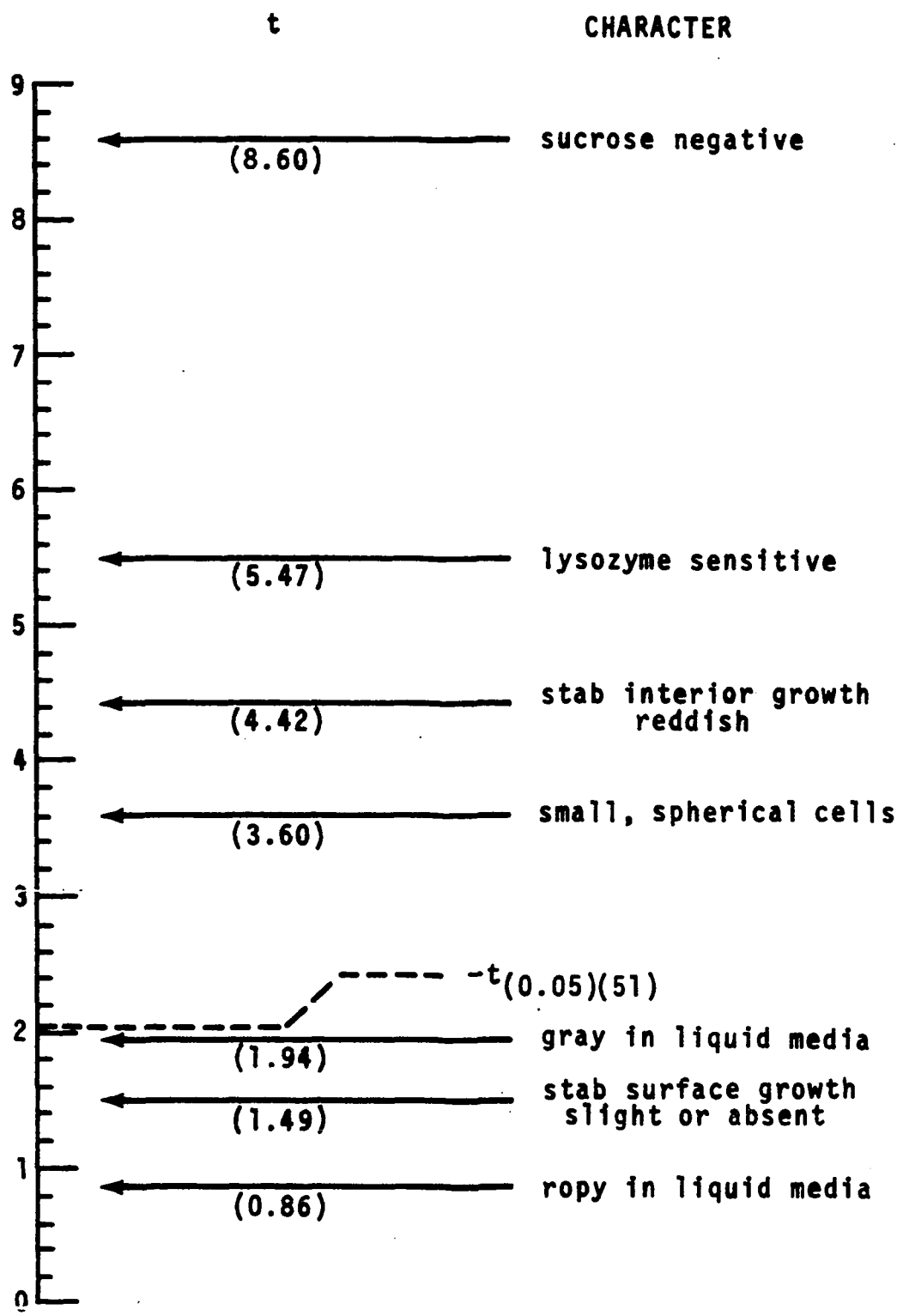
Table 14. Mean GC%'s for strains exhibiting and for strains not exhibiting given characters and significance of differences between means

Character	Mean GC%		$\bar{X}_2-\bar{X}_1$	$S_{\bar{X}_2-\bar{X}_1}$	t[a]
	Strains exhibiting character ($\bar{X}_1$)	Strains not exhibiting character ($\bar{X}_2$)			
Sucrose negative	65.80	67.03	1.23*	0.143	8.60
Lysozyme sensitive	65.91	66.91	1.00*	0.174	5.47
Stab interior growth reddish	65.97	66.81	0.84*	0.190	4.42
Small, spherical cells	66.05	66.77	0.72*	0.200	3.60
Gray in liquid media	66.20	66.62	0.42	0.216	1.94
Stab surface growth slight or absent	66.31	66.46	0.15	0.224	1.49
Ropy in liquid media	66.35	66.58	0.23	0.267	0.86

[a] $t_{(0.05)(51)} = 2.007$ (75).

*Significant at 95% level.

Figure 11. Representation of the levels of significance of the difference between the mean GC% of propionibacterial strains exhibiting the indicated character and of strains not exhibiting the indicated character


t
CHARACTER
9
8
7
6
5
4
3
2
1
0
(8.60)
sucrose negative
(5.47)
lysozyme sensitive
(4.42)
stab interior growth
reddish
(3.60)
small, spherical cells
$-t_{(0.05)(51)}$
(1.94)
gray in liquid media
(1.49)
stab surface growth
slight or absent
(0.86)
ropy in liquid media

a single DNA preparation. This precision is made more remarkable by the fact that the 53 runs were made with DNA from all different strains. The overall range was from 65.0 to 68.0 GC%. These results are in general agreement with previously reported work and specifically with Johnson and Cummins (43) with regard to the values obtained for the genotypes associated with Main Groups I and II. Only five strains for which GC%'s were determined exhibited all seven characters associated with Main Group I, including P19. This particular strain provides an interesting anomaly, having the highest GC% (68.0) of all strains examined.

Species Designation

The species designation based upon characters determined in this investigation, together with stab interior color, lysozyme sensitivity, and GC%, are shown for each strain in Table A5. The placement of a strain in either Main Group I or II was based only upon cell morphology and the ability to ferment sucrose, and not upon the amount of agar stab surface growth.

This exercise illustrates the difficulty encountered when multiple criteria are used to make a particular separation. An exhaustive number of strains need not be examined in order to discover one which exhibits one character placing it in one group and another character placing it in the

other. If one is determined to assign the recalcitrant strain to a single taxon within the existing hierarchy, the dilemma may be resolved in a number of ways.

One approach is to single out one character and use it alone to determine to which group the strain will be assigned. This character may be selected arbitrarily, on the basis of subjective judgement, or as a result of a predetermined decision-making process. It can be argued that to select the character with the most extensive display of significant differences between "expected" and "actual" numbers in paired comparisons (Figure 9) or with the least combined sum of insimilitudes (Figure 10) would be to select the character least likely to be "wrong." Using this rationale in the particular case of the three characters used to separate the Propionibacterium Main Group I from Main Group II; sucrose -; small, spherical cells; and stab surface growth slight or absent; along with the other characters included in the analysis; sucrose - is the character of choice. This choice is supported by the results from the tests of significance of differences between the mean GC% of strains exhibiting and strains not exhibiting each of the characters.

The choice of sucrose - as the character determining assignment in either Main Group I or Main Group II does

present the possibility of an interesting dilemma. If a sucrose + strain exhibits small, spherical cells as its morphology; it is placed in Main Group II; but one has no means for determining to which of the sub-groups within this main group it should be assigned, since morphology is the sole criterion for this assignment and neither alternative includes small, spherical cells. This was, in fact, the case with four of the strains included in this study, P11, P38, P54, and P68. If one is intent upon assigning these to a single species, the common sense approach prevails, and the strain is placed in Main Group I in spite of its failure to exhibit the sucrose - character.

The character judged to be most important from an economic, medical, or scientific standpoint may be selected. A particular strain may then legitimately be classified in several different taxa by as many different workers who may be interested in the strain for a variety of reasons.

An alternative to selecting a single character is to place the strain in the group determined by a "majority vote" of the characters considered as defining the separation. This may be carried a step farther by weighting the influence upon the assignment decision for each character. This weighting may be based on the worker's particular needs and interests or upon predetermined rules.

Reflecting phenetic differences is a major objective of

taxonomy (49), but to examine the chromosomal material of each newly-isolated strain in the necessary depth would be prohibitive. It is reasonable to suppose that some conveniently-observed phenotypic characters are more accurate indicators of phenetic differences than other phenotypic characters. The task at hand then becomes one of determining which character or set of characters most closely correlates with these phenetic differences.

Johnson and Cummins (43) showed that their four proposed species of traditional propionibacteria are separated by demonstrable differences in DNA hybridization. These four species correspond rather precisely to the principal groups of the currently accepted key. The relatively few characters which have traditionally defined these groups have correlated with phenetic differences rather well.

Molecular Weight

It is argued that the transmittance at 265nm (T_{265}) of the DNA in an infinitesimal band can be measured as the ratio of a (see Figure 2), the distance from the base line to the unexposed line; to b, the distance from the point on the tracing corresponding to the band to the unexposed line. Under this assumption, as a representation of the normal distribution curve, the peak is distorted with a unit of area near the tip of the peak representing a greater amount of DNA

than the same unit near the base. On an undistorted peak, the inflection point along its side would lie a distance of one standard deviation from its axis (98); however, the distortion assumed here would move the inflection point a greater distance from the axis.

It was observed that the peaks did not have an obvious inflection point, but instead appeared to have a linear segment between the curves with opposing senses of concavity. The plots for the function:

$$T = 10^{-A} = 10^{[-\underline{k}(\frac{e^{-\frac{x^2}{2}}}{\sqrt{2\pi}})]}$$

revealed that for each there was indeed a very nearly linear segment in the distorted normal distribution curve. Values for I and for $[(MF)2I]^{-1}$ obtained from these plots are shown in Table 1. A plot of log A_{max} vs. $[(MF)2I]^{-1}$ (see Figure 1) resulted in a smooth curve which was used to graphically determine values for the latter term corresponding to the values of the former obtained by computation from tracing measurements.

The estimated molecular weight and amount of propionibacterial DNA in the centrifuge cell from which the determination was made for each strain are given in Table 3. A comparison of the estimates resulting from separate runs with each of two P12 preparations gives a general indication of

the precision of the method of estimation ($\sigma \simeq 1.2 \times 10^6$) (98). The means of the estimates for DNA's obtained by using lysozyme with extensive purification, lysozyme with limited purification, grinding with alumina, grinding with ground glass, and the pressure cell are 3.81×10^6, 6.81×10^6, 5.37×10^6, 5.34×10^6, and 5.59×10^6 respectively. It appears that there is no real difference in the quality (molecular weight being an index) among the DNA preparations obtained using the various methods, although the yields from the physical methods averaged from only 50% to 30% of the yields using lysozyme.

A significant part of the total DNA freed as a result of physical disruption would probably be too low in molecular weight to be recovered by spooling. Because a sufficient quantity of high-quality material was obtained, recovery of the lower molecular weight fraction was not attempted.

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APPENDIX

Table A1. Propionibacterial cultures used in the investigation

Strain number	Species designation[a]	Strain designation	Source
P1	P. freudenreichii	F24	A
P2	P. pentosaceum	128	B
P3	P. pentosaceum	E14	A
P4	P. thoenii	TH25	A
P5	P. pentosaceum	E214	A
P6	P. freudenreichii	F23	A
P7	P. shermanii	52	A
P8	P. shermanii	57	A
P9	P. arabinosum	129	B
P10	P. rubrum	R9611	A
P11	P. pentosaceum	P31C	A
P12	P. shermanii	58	A
P13	P. shermanii	56	A
P14	P. shermanii	51	A
P15	P. thoenii	TH20	A
P16	P. freudenreichii	F24	A
P17	P. rubrum	R19	A
P18	P. shermanii	59	A
P19	P. shermanii	KP1	C
P20	P. thoenii	TH21	A

[a]Sources: A. Cornell University, Ithaca, N.Y.; B. Iowa State University, Ames; C. Kraft Foods Co., Stockton, Ill.; D. Isolated from imported Finnish Emmentaler cheese: E. Dr. K. W. Sahli, Station Federale D'Industrie Laitiere Liebefeld-Bern, Switzerland; F. American Type Culture Collection, Rockville, Md.; G. Dr. J. J. Campbell, University of British Columbia, Vancouver, Canada; H. Dr. W. Kundrat, University of Munich, Munich, Germany; I. Dr. V. B. D. Skerman, University of Queensland, Brisbane, Australia; J. Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, Calif.; K. Communicable Disease Laboratory, Atlanta, Ga.; L. Isolated from Gruyére cheese imported from France; M. J. L. Johnson, Virginia Polytechnic Institute and State University, Blacksburg; N. Contaminant of P121, O. Origin unknown.

Table A1 (Continued)

Strain number	Species designation[a]	Strain designation	Source
P21	P. rubrum	R6	A
P22	P. shermanii	123	B
P23	P. freudenreichii	F13	A
P24	P. shermanii	KP2	C
P25	P. jensenii	J17	A
P26	P. rubrum	8266	B
P28	P. shermanii	167	B
P30	P. freudenreichii	1291	E
P31	P. shermanii	1294	E
P32	P. shermanii	1298	E
P33	P. shermanii	1409	E
P35	P. peterssonii	1505	E
P37	P. shermanii	6	H
P38	P. peterssonii	13	H
P39	P. freudenreichii	3 or 5	H
P40	P. freudenreichii	3 or 5	H
P41	P. jensenii	14	H
P42	P. arabinosum	10	H
P44	P. jensenii	363	I
P45	P. shermanii	360	I
P46	P. jensenii	E.1.2	J
P47	P. shermanii	E. 11.2	J
P48	P. shermanii	E.11.3	J
P49	P. freudenreichii	E.15.01	J
P50	P. arabinosum	E.7.1	J
P51	P. shermanii	E.11.1	J
P52	P. peterssonii	E.5.1	J
P53	P. peterssonii	E.5.2	J
P54	P. jensenii	E.1.1	J
P55	P. shermanii	E.11.4	J
P56	P. freudenreichii		G
P57	P. freudenreichii		G
P58	P. pentosaceum	ATCC4875	F
P59	P. shermanii	ISU	B
P60	P. freudenreichii	PF39	K

Table A1 (Continued)

Strain number	Species designation[a]	Strain designation	Source
P61	P. shermanii	PS13	K
P62	P. shermanii	PS260	K
P63	P. jensenii	PJ54	K
P64	P. shermanii	PS10	K
P65	P. freudenreichii	PF33	K
P66	P. shermanii	PS9	K
P67	P. shermanii	PS9614	K
P68	P. jensenii	PJ53	K
P69	P. jensenii	PJ23	K
P70	P. shermanii	PS31c	K
P71	P. shermanii	PS12	K
P72	P. shermanii	PS14	K
P73	P. freudenreichii	PF6207	K
P74	P. zeae	PZ99	K
P75	P. shermanii	PS15	K
P76	P. shermanii	PS1	K
P77	P. shermanii	PS49	K
P78	P. arabinosum	PA62	K
P79	P. thoenii	PT52	K
P80	P. shermanii	PS18	K
P81	P. peterssonii	PP798	K
P82	P. shermanii	FIN	D
P83	P. shermanii	SAUER	B
P84	P. shermanii	10	L
P85	P. rubrum	20	L
P86	P. zeae	11	L
P87	P. thoenii	24	L
P88	P. jensenii	22	L
P89	P. freudenreichii	5571	O
P90	P. pentosaceum	5578	O
P91	CNRZ 88		O
P92	CNRZ 90		O
P93	CNRZ 91		O
P94	CNRZ 92		O
P95	P. shermanii	5964	O

Table A1 (Continued)

Strain number	Species designation[a]	Strain designation	Source
P96	P. shermanii	8903	O
P97	P. shermanii	ATCC 8262	F
P98	P. shermanii	ATCC 9614	F
P99	P. shermanii	ATCC 9615	F
P100	P. shermanii	ATCC 9616	F
P101	P. shermanii	ATCC 9617	F
P102	P. shermanii	ATCC13673	F
P103	P. freudenreichii	5932	O
P104	P. freudenreichii	ATCC 6207	F
P105	P. rubrum	ATCC 4871	F
P106	P. zeae	ATCC 4964	F
P107	P. technicum	ATCC 14073	F
P108	P. intermedium	ATCC 14072	F
P109	Dutch A		O
P110	Dutch B		O
P121	P. raffinosaceum	VPI 5163	M
P122	P. raffinosaceum		N

Table A2. Results of carbohydrate fermentation tests[a]

Strain	Sucrose	Lactose	Raffinose	Sorbitol
P1	-	+	-	-
P2	+	+	+	+
P3	+	+	+	
P4	+	-	-	
P5	+	+	+	+
P6	-	-	-	-
P7	-	+	-	-
P8	-	+	+	-
P9	+	+	+	+
P10	+	+	+	-
P11	+	+	-	-
P12	-	+	-	-
P13	-	+	-	-
P14	-	+	-	-
P15	+	+	-	+
P16	-	-	-	-
P17	+	+	+	-
P18	-	+	-	-
P19	-	-	-	-
P20	+	+	-	+
P21	+	+	+	-
P22	-	+	-	-
P23	-	-	-	-
P24	-	+	-	-
P25	+	-	+	+
P26	+	-	+	-
P28	+	+	+	+
P30	+	+	-	-
P31	-	+	-	-
P32	-	+	-	-

[a] + = color reaction to completion in less than 20 days,
- = color reaction not to completion in less than 20 days.

Table A2 (Continued)

Strain	Starch	Cellobiose	Salicin	Rhamnose
P1	-	-	-	-
P2	+	+	+	+
P3	+	+	+	+
P4	-	+	+	-
P5	+	+	+	+
P6	-	-	-	-
P7	-	+	-	-
P8	-	+	-	-
P9	+	+	+	+
P10	-	+	-	-
P11	-	+	-	-
P12	-	+	-	-
P13	-	+	-	-
P14	-	+	-	-
P15	-	+	+	-
P16	-	-	-	-
P17	-	+	+	-
P18	-	+	-	-
P19	-	+	-	-
P20	+	+	+	-
P21	-	+	+	-
P22	-	-	-	-
P23	-	+	+	-
P24	-	-	-	-
P25	-	+	+	+
P26	-	+	+	+
P28	-	+	+	+
P30	-	+	+	-
P31	-	+	-	-
P32	-	-	-	-

Table A2 (Continued)

Strain	Sucrose	Lactose	Raffinose	Sorbitol
P33	-	-	-	-
P35	-	+	-	-
P37	-	-	-	-
P38	+	+	-	-
P39	-	-	-	-
P40	-	-	-	-
P41	+	+	+	+
P42	+	+	-	+
P44	+	-	+	-
P45	-	+	-	+
P46	+	+	+	-
P47	-	+	-	-
P48	-	+	-	-
P49	-	-	-	-
P50	+	+	+	+
P51	-	+	-	-
P52	+	-	-	+
P53	+	+	-	+
P54	+	+	+	+
P55	-	+	-	-
P56	-	-	-	-
P57	-	-	-	-
P58	+	+	+	+
P59	-	+	-	-
P60	+	-	-	-
P61	-	+	-	-
P62	-	+	-	-
P63	+	+	+	+
P64	-	+	-	-
P65	-	+	-	-
P66	-	+	-	-
P67	-	+	-	-
P68	+	-	+	+
P69	+	-	+	+

Table A2 (Continued)

Strain	Starch	Cellobiose	Salicin	Rhamnose
P33	-	-	-	-
P35	-	+	-	-
P37	-	+	-	-
P38	-	+	+	-
P39	-	+	+	-
P40	-	-	+	-
P41	+	+	+	-
P42	+	+	+	+
P44	-	+	+	
P45	-	+	+	+
P46	-	+	+	-
P47	-	-	-	-
P48	-	-	-	-
P49	-	+	+	-
P50	+	+	+	+
P51	-	+	-	-
P52	-	-	-	-
P53	-	+	+	-
P54	-	+	+	-
P55	-	+	+	-
P56	-	+	+	-
P57	-	-	-	-
P58	+	+	+	+
P59	-	-	-	-
P60	-	+	+	-
P61	-	+	-	-
P62	-	+	-	-
P63	+	+	+	-
P64	-	+	-	-
P65	-	+	+	-
P66	-	+	-	-
P67	-	-	-	-
P68	-	+	+	-
P69	-	+	+	-

Table A2 (Continued)

Strain	Sucrose	Lactose	Raffinose	Sorbitol
P70	-	+	-	-
P71	-	+	-	-
P72	-	+	-	-
P73	-	-	-	-
P74	+	-	+	+
P75	-	+	-	-
P76	-	+	-	-
P77	-	+	-	-
P78	+	+	+	+
P79	+	+	+	+
P80	-	+	-	-
P81	+	+	+	+
P82	-	+	-	-
P83	-	+	-	-
P84	+	+	-	+
P85	+	+	+	-
P86	+	+	-	+
P87	+	+	-	+
P88	+	+	-	+
P89	-	-	-	-
P90	+	+	+	+
P91	+	+	-	-
P92	-	-	-	-
P93	-	+	-	-
P94	+	+	-	-
P95	+	+	-	-
P96	-	-	-	-
P97	-	+	-	-
P98	-	+	-	-
P99	-	+	-	-
P100	-	+	-	-
P101	-	+	-	-
P102	-	+	-	-

Table A2 (Continued)

Strain	Starch	Cellobiose	Salicin	Rhamnose
P70	-	+	-	-
P71	-	+	-	-
P72	-	-	-	-
P73	-	+	+	-
P74	+	+	+	-
P75	-	-	-	-
P76	-	-	-	-
P77	-	+	-	-
P78	+	+	+	+
P79	+	+	+	+
P80	-	-	-	-
P81	+	+	+	+
P82	-	+	-	-
P83	-	+	-	-
P84	+	+	+	-
P85	-	+	+	-
P86	+	+	+	-
P87	-	+	+	+
P88	-	+	+	-
P89	-	-	-	-
P90	+	+	+	+
P91	-	+	-	-
P92	-	-	-	-
P93	-	+	-	-
P94	-	+	-	-
P95	-	+	-	-
P96	-	-	-	-
P97	-	+	-	-
P98	-	+	-	
P99	-	+	-	-
P100	-	+	-	-
P101	-	+	-	-
P102	-	+	-	-

Table A2 (Continued)

Strain	Sucrose	Lactose	Raffinose	Sorbitol
P103	-	-	-	-
P104	-	-	-	-
P105	+	+	+	+
P106	+	-	+	+
P107	+	-	+	-
P108	+	+	+	+
P109	-	+	-	-
P110	-	+	-	-
P121	+	-	+	+
P122	+	-	+	+

Strain	Starch	Cellobiose	Salicin	Rhamnose
P103	-	+	-	-
P104	-	+	+	-
P105	-	+	+	-
P106	+	+	+	-
P107	-	+	+	-
P108	+	+	+	-
P109	-	+	-	-
P110	-	+	-	-
P121	-	+	+	-
P122	-	+	+	-

Table A3. Cultural characters of propionibacteria[a]

Strain	Liquid Media									Solid Media		
				Sodium lactate broth for 10 days at 32 C		Sodium lactate broth to definite growth at 32 C and 21 days at 7 C		Glucose broth for 24 hr at 32 C				
	Rate of settling mm/day	Brown	Gray	Ropy	Flocculent	Ropy	Flocculent	Ropy	Flocculent	Surface growth	Surface color	Stab color
P1	-	-	+	+	-	-	-	-	-	P	G	C
P2	6.1	-	+	-	-	+	-	-	-	P	G	C
P3	5.2	+[b]	-[b]	-	-	+	-	-	-	P	G	C
P4	-	-[b]	-[b]	+	-	-	-	+	-	P	R	R

[a] A = stab surface growth absent
S = stab surface growth slight
P = stab surface growth distinct
D = stab surface growth dome-shaped
W = stab surface growth with wrinkled texture

G = stab surface gray
R = stab surface deep red
C = stab surface cream
O = stab surface orange
Y = stab surface yellow

C = stab interior cream
R = stab interior deep red
r = stab interior reddish
Y = stab interior yellow
G = stab interior gray
W = stab interior white

NT = liquid culture not turbid, + = character present, - = character not present.

[b] Exhibited a red color typical of P. rubrum and P. thoenii.

Table A3 (Continued)

		Liquid Media								Solid Media		
				Sodium lactate broth for 10 days at 32 C		Sodium lactate broth to definite growth at 32 C and 21 days at 7 C		Glucose broth for 24 hr at 32 C				
Strain	Rate of settling mm/day	Brown	Gray	Ropy	Flocculent	Ropy	Flocculent	Ropy	Flocculent	Surface growth	Surface color	Stab color
P5	5.0	+	-	-	-	-	-	-	-	P	G	C
P6	0.6	+	-	+	-	-	-	-	-	P	G	r
P7	0.7	+	+	+	-	-	-	+	-	P	G	C
P8	1.3	-	-	-	-	+	-	+	-	P	G	r
P9	4.6	+[b]	-[b]	+	-	-	-	-	-	P	C	C
P10	0.9	-[b]	-[b]	+	-	+	-	+	-	P	R	R
P11	1.1	-	+	+	-	-	-	-	-	P	C	r
P12	3.1	+	+	+	-	+	-	+	-	P	G	r
P13	0.8	+	-	-	-	+	-	+	-	S	G	r
P14	-	+[b]	-[b]	-	-	-	-	-	-	P	G	r
P15	NT	-[b]	-[b]	+	-	-	-	-	-	P	R	R
P16	-	+[b]	-[b]	-	-	-	-	-	-	P	G	r
P17	1.0	-[b]	-[b]	-	-	+	-	-	-	D	R	R
P18	0.4	-	+	+	-	+	-	-	-	A		r
P19	4.8	+[b]	+[b]	+	-	+	-	-	-	S	G	r
P20	NT	-[b]	-[b]	+	-	+	-	-	-	P	R	R
P21	2.8	-[b]	-[b]	+	-	+	-	+	-	P	R	R
P22	1.7	+	+	+	-	+	-	-	-	P	G	r
P23	1.6	+	+	-	-	-	-	+	-	P	C	C
P24	0.7	-	+	+	-	-	-	-	-	P	C	r
P25	5.0	-	+	-	-	+	-	+	-	P	O	Y
P26	4.3	-	+	-	-	+	-	+	-	P	O	G
P28	NT	-	-	-	+			-	-	A		W
P30	12.7	-	-	-	-	+	-	-	-	S	C	C
P31	-	-	-	-	-	-	-	-	-	P	G	r
P32	1.3	+	-	+	-	+	-	-	-	P	G	r
P33	2.8	+	-	+	-	-	-	-	-	P	G	r

Table A3 (Continued)

		Liquid Media								Solid Media		
				Sodium lactate broth for 10 days at 32 C		Sodium lactate broth to definite growth at 32 C and 21 days at 7 C		Glucose broth for 24 hr at 32 C				
Strain	Rate of settling mm/day	Brown	Gray	Ropy	Flocculent	Ropy	Flocculent	Ropy	Flocculent	Surface growth	Surface color	Stab color
P35	1.3	+	+	-	-	-	-	-	-	S	G	r
P37	5.5	+	-	+	-	+	-	-	-	S	G	r
P38	53.0	+	-	-	-	+	-	-	-	P	C	C
P39	5.0	+	+	+	-	-	-	-	-	S	G	r
P40	3.9	+	+	+	-	-	-	-	-	S	G	r
P41	5.0	-	-	-	-	-	-	-	-	W	Y	Y
P42	1.1	-	+	-	-	-	-	-	-	P	G	G
P44	6.7	+	-	-	+	+	+	-	-	W	O	Y
P45	2.4	-	+	-	-	-	-	-	-	S	G	r
P46	5.3	+	-	-	-	-	-	-	+	P	O	Y
P47	5.6	+	+	+	-	+	-	-	-	S	G	r
P48	6.0	+	+	+	-	+	-	-	-	S	G	r
P49	2.8	+	-	+	-	-	-	-	-	S	G	r
P50	6.3	-	+	+	+	+	-	-	-	P	Y	Y
P51	2.7	+	+	+	-	+	-	-	-	S	G	r
P52	9.8	+	+	+	-	+	-	-	+	S	Y	G
P53	6.4	+	-	-	-	+	-	-	-	P	R	R
P54	11.2	+	-	+	-	+	-	-	-	S	C	r
P55	-	+	+	+	-	-	-	-	-	P	C	r
P56	-	+	+	-	-	-	-	-	-	P	G	r
P57	-	+	-	-	-	-	-	-	-	P	G	r
P58	5.4	-	+	-	-	-	-	-	-	P	G	C
P59	0.6	+	-	-	-	+	-	-	-	P	G	r
P60	3.4	+	-	-	-	+	-	-	-	S	G	r
P61	1.6	-	+	+	-	-	-	-	-	P	C	C
P62	3.1	-	+	+	-	-	-	-	-	S	G	r
P63	9.2	+	-	+	-	+	-	-	-	S	O	r

Table A3 (Continued)

Strain	Rate of settling mm/day	Liquid Media								Solid Media		
				Sodium lactate broth for 10 days at 32 C		Sodium lactate broth to definite growth at 32 C and 21 days at 7 C		Glucose broth for 24 hr at 32 C				
		Brown	Gray	Ropy	Flocculent	Ropy	Flocculent	Ropy	Flocculent	Surface growth	Surface color	Stab color
P64	3.7	+	+	+	-	+	-	-	-	P	G	r
P65	4.4	-	+	+	-	-	-	-	-	P	G	r
P66	4.8	+	+	+	-	-	-	-	-	S	G	r
P67	3.5	-	+	+	-	+	-	-	-	S	G	r
P68	3.0	-	+	-	-	-	-	+	-	S	O	Y
P69	2.0	+	-	-	-	+	-	-	-	S	O	Y
P70	8.5	-	+	+	-	-	-	-	-	S	G	r
P71	3.2	+	+	+	-	-	-	-	-	S	G	r
P72	1.3	-	+	+	-	-	-	-	-	P	C	C
P73	NT	+	-	+	-	-	-	-	-	S	G	r
P74	2.2	-	-	-	-	-	-	-	-	P	O	Y
P75	-	+	+	-	-	-	-	-	-	S	G	r
P76	0.9	-	+	+	-	-	-	-	-	P	C	C
P77	2.2	+	+	-	-	+	-	-	-	S	G	r
P78	5.3	+	-	-	-	-	-	-	-	S	C	Y
P79	16.6	+	+	-	-	-	-	-	-	P	G	G
P80	7.0	+	+	-	-	-	-	-	-	S	G	r
P81	7.0	+	-	-	-	+	-	-	-	P	G	Y
P82	NT	+	-	-	-	-	-	-	-	A		r
P83	2.7	+	-	-	-	-	-	-	-	P	G	r
P84	4.4	+	-	-	-	-	-	-	-	S	G	r
P85	8.3	-[b]	-[b]	-	-	-	-	-	-	P	R	R
P86	6.5	+	-	-	-	+	-	-	-	A		r
P87	5.3	+	+	-	-	-	-	-	-	S	G	r
P88	3.2	+	-	+	-	+	-	-	-	A	G	r
P89	NT	+	-	+	-	-	-	-	-	S	G	r

Table A3 (Continued)

		Liquid Media								Solid Media		
				Sodium lactate broth for 10 days at 32 C		Sodium lactate broth to definite growth at 32 C and 21 days at 7 C		Glucose broth for 24 hr at 32 C				
Strain	Rate of settling mm/day	Brown	Gray	Ropy	Flocculent	Ropy	Flocculent	Ropy	Flocculent	Surface growth	Surface color	Stab color
P90	4.4	-	+	+	-	+	-	-	-	P	G	Y
P91	3.7	+	-	-	-	+	-	-	-	S	G	r
P92	4.0	+	+	+	-	-	-	-	-	S	G	r
P93	4.4	+	+	+	-	-	-	-	-	S	G	r
P94	3.4	+	+	-	-	+	-	-	-	P	G	r
P95	3.9	-	+	+	-	-	-	-	-	S	G	r
P96	3.0	+	+	-	-	-	-	-	-	P	C	r
P97	1.0	-	+	+	-	-	-	-	-	P	C	r
P98	-	+	+	+	-	+	+	-	-	A		r
P99	3.6	+	+	+	-	+	-	-	-	S	G	r
P100	NT	+	+	+	-	+	-	-	-	S	G	r
P101	-	+	-	-	-	+	-	-	-	P	G	r
P102	3.4	+	-	-	-	-	-	-	-	S	G	r
P103	3.7	+	-	-	-	+	-	-	-	S	G	r
P104	-	+[b]	+[b]	-	-	-	-	-	-	P	G	r
P105	2.1	-[b]	-[b]	-	-	+	-	-	-	P	R	R
P106	2.1	-	+	-	-	-	-	-	-	P	O	Y
P107	6.1	+[b]	-[b]	-	-	+	-	-	-	P	O	Y
P108	NT	-[b]	-[b]	-	-	+	-	-	-	P	R	R
P109	4.5	-	+	+	-	-	-	-	-	A		r
P110	NT	+	+	+	-	+	-	-	-	A		r
P121	4.2	-	-	-	-	-	-	-	-	P	Y	Y
P122	3.6	-	-	-	-	-	-	-	-	P	Y	Y

Table A1. Cell morphology[a]

Strain	Small, spherical cells	Diptheroid-like rods	Long irregular rods	Large swollen spheres	Strain	Small, spherical cells	Diptheroid-like rods	Long irregular rods	Large swollen spheres
1	X				25		X		
2			X		26		X		
3			X		28		X		
4		X			30	X			
5		X			31	X			
6	X[a]				32	X			
7	X				33	X			
8	X				35		X		
9			X		37	X			
10		X			38	X			
11	X				39	X			
12	X				40	X			
13	X				41		X		
14	X				42			X	
15		X			44		X		
16	X				45			X	
17		X			46		X		
18	X				47	X			
19	X				48	X			
20	X				49	X			
21		X			50			X	
22	X				51	X			
23	X				52		X		
24	X								

[a] A strain exhibiting a form most resembling but not typical of a traditional form is indicated by an X in the side column nearest the column set for the other form suggested by the strain's morphology.

Table A4 (Continued)

Strain	Small, spherical cells	Diptheroid-like rods	Long irregular rods	Large swollen spheres	Strain	Small, spherical cells	Diptheroid-like rods	Long irregular rods	Large swollen spheres
53		X			81			X	
54	X				82	X			
55	X				83	X			
56	X				84			X	
57	X				85		X		
58			X		86		X		
59	X				87		X		
60	X				88	X			
61	X				89	X			
62	X				90			X	
63		X			91	X			
64	X				92		X		
65		X			93	X			
66		X			94	X			
67	X				95		X		
68	X				96	X			
69		X			97	X			
70	X				98				X
71	X				99	X			
72	X				100	X			
73	X				101	X			
74		X			102	X			
75	X				103	X			
76	X				104	X			
77		X			105		X		
78			X		106		X		
79			X		107		X		
80	X				108		X		

Table A4 (Continued)

Strain	Small, spherical cells	Diptheroid-like rods	Long irregular rods	Large swollen spheres
109	X			
110	X			
121		X		
122		X		

Table A5. Summary of species findings, other significant phenotypic characters, and GC%'s

Strain	Species		Phenotypic Characters		
	Donor	Per this investigation	Stab color	Lysozyme	GC%
P1	P. freudenreichii	P. shermanii	cream	sensitive	65.6
P2	P. pentosaceum	P. pentosaceum	cream	resistant	66.9
P3	P. pentosaceum	P. pentosaceum	cream	sensitive	
P4	P. thoenii	P. thoenii[a] P. rubrum[a]	deep red	resistant	66.1
P5	P. pentosaceum	P. zeae	cream	resistant	66.8
P6	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P7	P. shermanii	P. shermanii	cream	sensitive	65.7
P8	P. shermanii	P. shermanii	reddish	sensitive	65.9
P9	P. arabinosum	P. pentosaceum	cream	sensitive	
P10	P. rubrum	P. rubrum	deep red	resistant	67.2
P11	P. pentosaceum	P. zeae[b] P. arabinosum[b,c] P. pentosaceum[b,c] P. shermanii[d]	reddish	sensitive	65.9
P12	P. shermanii	P. shermanii	reddish	sensitive	65.2,65.3
P13	P. shermanii	P. shermanii	reddish	sensitive	65.3,65.9
P14	P. shermanii	P. shermanii	reddish	sensitive	66.2
P15	P. thoenii	P. thoenii	deep red	resistant	66.8
P16	P. freudenreichii	P. freudenreichii	reddish	sensitive	66.2
P17	P. rubrum	P. rubrum	deep red	questionable	
P18	P. shermanii	P. shermanii	reddish	sensitive	68.0

[a] Sorbitol - and raffinose -.

[b] Sucrose +.

[c] Rhamnose - and stab surface unpigmented.

[d] Small, spherical cells.

Table A5 (Continued)

Strain	Species		Phenotypic Characters		
	Donor	Per this investigation	Stab color	Lysozyme	GC%
P19	P. shermanii	P. freudenreichii	reddish	sensitive	65.7
P20	P. thoenii	P. thoenii[d]	deep red	resistant	67.1
P21	P. rubrum	P. rubrum	deep red	resistant	
P22	P. shermanii	P. shermanii	reddish	sensitive	65.6
P23	P. freudenreichii	P. freudenreichii	cream	sensitive	65.5
P24	P. shermanii	P. shermanii	reddish	sensitive	65.7
P25	P. jensenii	P. raffinosaceum	yellow	resistant	67.5
P26	P. rubrum	P. raffinosaceum	gray	resistant	66.9
P28	P. shermanii	P. zeae	white	resistant	66.8
P30	P. freudenreichii	P. shermanii	cream	resistant	
P31	P. shermanii	P. shermanii	reddish	sensitive	66.0
P32	P. shermanii	P. shermanii	reddish	sensitive	
P33	P. shermanii	P. freudenreichii	reddish	sensitive	66.2
P35	P. peterssonii	P. shermanii[b] P. zeae[d]	reddish	sensitive	
P37	P. shermanii	P. freudenreichii	reddish	sensitive	65.0
P38	P. peterssonii	P. zeae[b] P. arabinosum[b,c] P. pentosaceum[b,c] P. shermanii[d]	cream	resistant	67.1
P39	P. freudenreichii	P. freudenreichii	reddish	sensitive	66.3
P40	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P41	P. jensenii	P. raffinosaceum	yellow	resistant	
P42	P. arabinosum	P. pentosaceum	gray	resistant	
P44	P. jensenii	P. raffinosaceum	yellow	resistant	67.1
P45	P. shermanii	P. shermanii[b] P. pentosaceum[d]	reddish	sensitive	65.1
P46	P. jensenii	P. zeae	yellow	resistant	67.4
P47	P. shermanii	P. shermanii	reddish	sensitive	65.7
P48	P. shermanii	P. shermanii	reddish	sensitive	65.8

Table A5 (Continued)

Strain	Species: Donor	Species: Per this investigation	Phenotypic Characters: Stab color	Phenotypic Characters: Lysozyme	GC%
P49	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P50	P. arabinosum	P. pentosaceum[e]	yellow	resistant	
		P. arabinosum[e]			67.5
P51	P. shermanii	P. shermanii	reddish	sensitive	
P52	P. petersonii	P. jensenii	gray	resistant	67.3
P53	P. peterssonii	P. thoenii	deep red	resistant	
P54	P. jensenii	P. zeae[b]	reddish	resistant	67.0
		P. arabinosum[b,c]			
		P. pentosaceum[b,c]			
		P. shermanii[d]			
P55	P. shermanii	P. shermanii	reddish	sensitive	66.8
P56	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P57	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P58	P. pentosaceum	P. pentosaceum	cream	sensitive	
P59	P. shermanii	P. shermanii	reddish	sensitive	
P60	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P61	P. shermanii	P. shermanii	cream	sensitive	
P62	P. shermanii	P. shermanii	reddish	sensitive	
P63	P. jensenii	P. technicum	reddish	resistant	66.7
P64	P. shermanii	P. shermanii	reddish	sensitive	
P65	P. freudenreichii	P. freudenreichii[b]	reddish	sensitive	65.8
		P. zeae[d]			
P66	P. shermanii	P. shermanii[b]	reddish	sensitive	65.3
		P. zeae[d]			
P67	P. shermanii	P. shermanii	reddish	sensitive	
P68	P. jensenii	P. raffinosaceum[f]	yellow	resistant	66.5
		P. arabinosum[g]			
		P. freudenreichii[d]			

[e] Rhamnose + and stab surface pigmented.

[f] Stab interior and surface pigmented.

[g] Rhamnose - and stab surface pigmented.

Table A5 (Continued)

Strain	Species: Donor	Species: Per this investigation	Phenotypic Characters: Stab color	Phenotypic Characters: Lysozyme	GC%
P69	P. jensenii	P. raffinosaceum	yellow	resistant	66.7
P70	P. shermanii	P. shermanii	reddish	sensitive	
P71	P. shermanii	P. shermanii	reddish	sensitive	
P72	P. shermanii	P. shermanii	cream	sensitive	
P73	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P74	P. zeae	P. technicum	yellow	resistant	67.2
P75	P. shermanii	P. shermanii	reddish	sensitive	
P76	P. shermanii	P. shermanii	cream	sensitive	65.8
P77	P. shermanii	P. shermanii[b]	reddish	sensitive	65.3
		P. zeae[d]			
P78	P. arabinosum	P. pentosaecum	yellow	resistant	67.0
P79	P. thoenii	P. pentosaceum	gray	sensitive	
P80	P. shermanii	P. shermanii	reddish	sensitive	
P81	P. peterssonii	P. pentosaceum	yellow	questionable	
P82	P. shermanii	P. shermanii	reddish	questionable	
P83	P. shermanii	P. shermanii	reddish	sensitive	
P84	P. shermanii	P. arabinosum[g]	reddish	resistant	
		P. pentosaceum[g]	reddish		
P85	P. rubrum	P. rubrum	deep red	resistant	67.2
P86	P. zeae	P. zeae	reddish	resistant	
P87	P. thoenii	P. zeae	reddish	resistant	
P88	P. jensenii	P. shermanii	reddish	resistant	
P89	P. freudenreichii	P. freudenreichii	reddish	resistant	65.2
P90	P. pentosaceum	P. pentosaceum	yellow	sensitive	
P91		P. shermanii	reddish	sensitive	
P92		P. freudenreichii[b]	reddish	sensitive	
		P. zeae[d]			
P93		P. shermanii	reddish	sensitive	
P94		P. shermanii	reddish	sensitive	
P95	P. shermanii	P. freudenreichii[b]	reddish	sensitive	67.6
		P. zeae[d]			

Table A5 (Continued)

Strain	Species		Phenotypic Characters		GC%
	Donor	Per this investigation	Stab color	Lysozyme	
P96	P. shermanii	P. freudenreichii	reddish	sensitive	65.8
P97	P. shermanii	P. shermanii	reddish	sensitive	
P98	P. shermanii	P. shermanii[b] P. arabinosum[d]	reddish	resistant	65.3
P99	P. shermanii	P. shermanii	reddish	sensitive	
P100	P. shermanii	P. shermanii	reddish	sensitive	
P101	P. shermanii	P. shermanii	reddish	sensitive	
P102	P. shermanii	P. shermanii	reddish	sensitive	
P103	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P104	P. freudenreichii	P. freudenreichii	reddish	sensitive	
P105	P. rubrum	P. rubrum[h] P. thoenni[h]	deep red	resistant	
P106	P. zeae	P. techicum	yellow	questionable	67.2
P107	P. technicum	P. raffinosaceum	yellow	resistant	67.3
P108		P. rubrum[h] P. thoenni[h]	deep red	resistant	67.5
P109		P. shermanii	reddish	sensitive	
P110		P. shermanii	reddish	sensitive	
P121	P. raffinosaceum	P. raffinosaceum	yellow	resistant	
P122	P. raffinosaceum	P. raffinosaceum	yellow	resistant	

[h]Sorbitol + and raffinose +.

KEY I

Van Niel - 1928 (105)

I. Small streptococci in acidified liquid culture media. Slight and colorless surface growth on agar stab. Sucrose and maltose not fermented.

A. Lactose not fermented.

P. freudenreichii

B. Lactose fermented.

P. shermanii

II. Short rods in acidified liquid culture media. Distinct and pigmented surface growth on agar stab. Sucrose and maltose fermented.

A. Stab surface yellow, stab colorless.

1. Polysaccharides not fermented. Cells not coherent in liquid media.

P. jensenii

2. Polysaccharides fermented. Cells not coherent in liquid media.

P. technicum

3. Polysaccharides not fermented. Cells cohesive in liquid media. Dry, wrinkled colonies on solid media.

P. peterssonii

B. Stab and stab surface reddish brown.

1. Raffinose and mannitol not fermented. Propionic and acetic acids produced in ratio of 5:1.

P. thoenii

2. Raffinose and mannitol fermented. Propionic and acetic acids produced in ratio of 3:1.

P. rubrum

III. Irregular, long rods with local swelling and branching.
Pronounced and colorless surface growth on agar stab.
Disaccharides fermented.
Arabinose and xylose fermented.

P. pentosaceum

KEY II

Bergey's Manual - Third Edition - 1930 (4)

I. Non-pigment formers.

A. Only monosaccharides fermented.
P. freudenreichii

B. Monosaccharides and lactose fermented.
P. shermanii

C. Mono- and disaccharides, arabinose, and xylose fermented.
P. pentosaceum

D. Mono-, di-, and polysaccharides fermented.
P. technicum

II. Pigment formers.

A. Orange.

1. Mono- and disaccharides fermented. Raffinose occasionally fermented.
P. jensenii

2. Mono- and disaccharides fermented. Raffinose fermented.
P. peterssonii

B. Red.

1. Raffinose and mannitol fermented.

a. Propionic and acetic acids produced in ratio of 5:1.
P. thoenii

b. Propionic and acetic acids produced in ratio of 3:1.
P. rubrum

KEY III

Werkman and Kendall - 1931 (112)

I. Sucrose and maltose fermented.

A. Polysaccharides fermented.

P. technicum

B. Polysaccharides not fermented.

1. Xylose and arabinose fermented.
   Nitrates reduced.

   P. pentosaceum

2. Xylose and arabinose not fermented.
   Nitrates not reduced.

   a. Raffinose fermented.

      (1) Yellow pigment.

      P. raffinosaceum

      (2) Red-brown pigment.

      P. rubrum

   b. Raffinose not fermented.
      Mannitol fermented.
      Sorbitrol not fermented.

      (1) Amygdalin and salicin fermented.

      P. peterssonii

      (2) Amygdalin and salicin not fermented.

      P. jensenii

II. Sucrose and maltose not fermented.

A. Lactose fermented.
   Nitrate not reduced.

   P. shermanii

B. Lactose not fermented.
   Nitrate reduced.

   P. freudenreichii

KEY IV

Werkman and Brown - 1933 (110)
[With additions (enclosed by dotted lines)
by Sakaguchi et al. - 1941 (84)]

I. Sucrose and maltose fermented.

A. Polysaccharides fermented.

1. Lactose and arabinose fermented.

a. Rhamnose and trehalose fermented.
Raffinose not fermented.
Catalase negative.

P. arabinosum

b. Rhamnose and trehalose not fermented.
Raffinose fermented.
Catalase positive.

Xylose not fermented.

P. technicum

c. Raffinose not fermented.
Catalase positive.
Xylose fermented.

(1) Orange color in lactose culture base.

P. amylaceum var *aurantium*

(2) No orange color in lactose culture base.

P. amylaceum

2. Lactose and arabinose not fermented.

P. zeae

B. Polysaccharides not fermented.

1. Xylose and arabinose fermented.
Nitrates reduced.

P. pentosaceum

2. Xylose and arabinose not fermented.
Nitrates not reduced.

a. Raffinose fermented.

(1) Yellow pigment.
P. raffinosaceum

(2) Pigment red-brown.
P. rubrum

b. Raffinose not fermented.

(1) Mannitol fermented.
Sorbitol not fermented.

(a) Amygdalin and salicin fermented.
P. peterssonii

(b) Amygdalin and salicin not fermented.
P. jensenii

(2) Mannitol not fermented.
Sorbitol fermented.
P. thoenii

3. Xylose not fermented.
Arabinose fermented.
Nitrates not reduced.
P. japonicum

II. Sucrose and maltose not fermented.

A. Lactose fermented.
Nitrates not reduced.
P. shermanii

B. Lactose not fermented.
Nitrates reduced.
P. freudenreichii

C. Lactose fermented.
Nitrates not reduced.
P. orientum

III. Sucrose fermented.
Maltose not fermented.
Nitrates reduced.

A. Glycerol and erythritol fermented.
P. coloratum

B. Glycerol and erythritol not fermented.
P. globosum

KEY V

Bergey's Manual - Fourth Edition - 1934 (5)

I. Sucrose and maltose not fermented.

A. Lactose not fermented.
Nitrates reduced.

P. freudenreichii

B. Lactose fermented.
Nitrates not reduced.

P. shermanii

II. Sucrose and maltose fermented.

A. Polysaccharides fermented.

P. technicum

B. Polysaccharides not fermented.

1. Xylose and arabinose fermented.
Nitrates reduced.

P. pentosaceum

2. Xylose and arabinose not fermented.
Nitrates not reduced.

a. Raffinose fermented.

(1) Yellow pigment.

P. raffinosaceum

(2) Red-brown pigment.

P. rubrum

b. Raffinose not fermented.

(1) Mannitol fermented.
Sorbitol not fermented.

(a) Amygdalin and salicin fermented.

P. peterssonii

(b) Amygdalin and salicin not fermented.

P. jensenii

(2) Mannitol not fermented.
Sorbitol fermented.

P. thoenii

KEY VI

Bergey's Manual - Fifth Edition - 1939 (6)
[With additions (enclosed by dotted lines)
by Sakaguchi et al. - 1941 (84)]

I. Small streptococci in yeast extract- dextrose media.
Slight surface growth on agar stab.
Stab and stab surface growth dirty cream-colored.
Sucrose and maltose not fermented.

A. Lactose not fermented.

P. freudenreichii

B. Lactose fermented.

P. shermanii

II. Small streptococci in yeast extract - dextrose media (easy to produce elongated cells in acid culture).
Slight surface growth on agar stab.
Stab growth cream-colored.
Sucrose and maltose fermented.

P. japonicum

III. Small streptococci in yeast extract-dextrose media (no change in acid).
Stab surface growth flat, enlarged, and cream-colored.
Sucrose fermented.
Maltose not fermented.

P. globosum

IV. Short, diptheroid-like rods in yeast extract-dextrose media.
Distinct surface growth on agar stab.
Sucrose and maltose fermented.

A. Growth brownish red.

1. Raffinose and mannitol fermented.
Sorbitol not fermented.

P. rubrum

2. Raffinose and mannitol not fermented.
Sorbitrol fermented.

P. thoenii

B. Stab growth cream-colored.

1. Surface growth cream-colored.

a. L-arabinose and rhamnose fermented.

P. zeae

2. Surface growth yellow to orange.

a. Flocculant in liquid media.

P. peterssonii

b. Smooth in liquid media.

(1) Polysacchardies not fermented.

P. jensenii

P. raffinosaceum

(2) Polysaccharides fermented.

P. technicum

P. amylaceum

P. amylaceum var aurantium

V. Short rods.
Stab surface growth distinct.
Cream colored.
Sucrose and maltose not fermented.

P. orientum

VI. Short rods.
Stab surface growth flattened and enlarged.
Pinkish-brown pigment.
Sucrose fermented.
Maltose not fermented.

P. coloratum

VII. Involution forms in yeast extract-dextrose media.
Distinct surface growth on agar stab.
D and L-arabinose fermented.

A. Large, swollen spheres.
Stab surface orange-yellow.
Xylose and rhamnose not fermented.

P. arabinosum

B. Long, irregular rods.
Stab surface cream-colored.
Xylose and rhamnose fermented.

P. pentosaceum

C. Long, irregular rods. Stab surface orange. Xylose fermented.	*P. amylaceum* var *aurentium*

KEY VII

Janoschek - 1944 (42)

I. Agar stab surface cream-colored or white.

A. Catalase positive.

1. Nitrates reduced.

a. Lactose fermented.
Acid from litmus milk.

(1) Maltose fermented.
P. pentosaceum

(2) Maltose not fermented.
P. casei

b. Lactose not fermented.
No acid from litmus milk.
P. freudenreichii

2. Nitrates not reduced.

a. Dextrin fermented.
P. technicum

b. Dextrin not fermented.
P. shermanii

B. Catalase negative
P. arabinosum

II. Agar stab surface yellow or orange.

A. Amygdalin fermented.

1. Raffinose fermented.
P. raffinosaceum

2. Raffinose not fermented.
P. peterssonii

B. Amygdalin not fermented.

1. Raffinose fermented.
P. zeae

2. Raffinose not fermented.
P. jensenii

III. Agar stab and stab surface dark red.

A. Inositol fermented.

1. Yeast lactose broth clear.

a. Sediment not deep red.

P. thoenii

b. Sediment deep red.

P. sanguineum

2. Yeast lactose broth turbid.

P. pituitosum

B. Inositol not fermented.

P. rubrum

KEY VIII

Bergey's Manual - Sixth Edition - 1948 (9)

I. Small streptococci in yeast extract-glucose media.
Dirty cream-colored growth in agar stab.
Slight and dirty cream-colored growth on agar stab surface.
Sucrose and maltose not fermented.

A. Lactose not fermented.
P. freudenreichii

B. Lactose fermented.
P. shermanii

II. Short, diptheroid-like rods in yeast extract-glucose media.
Distinct surface growth on agar stab.
Sucrose and maltose fermented.

A. Growth brownish red.

1. Raffinose and mannitol fermented.
Sorbitol not fermented.
P. rubrum

2. Raffinose and mannitol not fermented.
Sorbitol fermented.
P. thoenii

B. Stab growth cream-colored.

1. Surface growth cream-colored.

a. L-arabinose and rhamnose fermented.
P. zeae

2. Surface growth yellow to orange.

a. Flocculent in liquid media.
P. peterssonii

b. Smooth in liquid media.

(1) Polysaccharides not fermented.
P. jensenii
P. raffinosaceum

(2) Polysaccharides fermented.
P. technicum

III. Irregular cells in yeast extract-glucose media.
Distinct surface growth on agar stab.
D- and L-arabinose fermented.

A. Large, swollen spheres.
Surface growth orange-yellow.
Xylose and rhamnose not fermented.
P. arabinosum

B. Long, irregular rods.
Surface growth cream-colored.
Xylose and rhamnose fermented.
P. pentosaceum

KEY IX

Bergey's Manual - Seventh Edition - 1957 (10)

I. Short chains of small, spherical cells in yeast extract-glucose media.
Little surface growth on stab.
Sucrose and maltose not fermented.

A. Lactose not fermented.

P. freudenreichii

B. Lactose fermented.

P. shermanii

II. Not short chains of small, spherical cells in yeast extract-glucose media.
Distinct surface growth on stab.
Sucrose and maltose fermented.

A. Short, diptheroid-like rods in yeast extract-glucose media.

1. Stab growth brownish red.

a. Raffinose and mannitol fermented.
Sorbitol not fermented.

P. rubrum

b. Raffinose and mannitol not fermented.
Sorbitol fermented.

P. thoenii

2. Stab growth cream-colored.

a. Surface growth cream-colored.

P. zeae

b. Surface growth yellow to orange.

(1) Polysaccharides fermented.

P. technicum

(2) Polysaccharides not fermented.

(a) Cellobiose fermented.

P. raffinosaceum

(b) Cellobiose not fermented.

1. Flocculent in liquid media.
   Acid from salicin.
   P. peterssonii

2. Smooth in liquid media.
   No acid from salicin.
   P. jensenii

B. Irregular cells in yeast extract-glucose media.

1. Large, swollen spheres.
   Stab surface growth orange-yellow.
   Xylose and rhamnose not fermented.
   P. arabinosum

2. Long, irregular rods.
   Stab surface growth cream-colored.
   Xylose and rhamnose fermented.
   P. pentosaceum