A TAXONOMIC STUDY OF THE *SPIRILLUM LIPOFERUM* GROUP, WITH DESCRIPTIONS OF A NEW GENUS, *AZOSPIRILLUM* GEN. NOV., AND TWO SPECIES, *AZOSPIRILLUM LIPOFERUM* (BEIJERINCK) COMB. NOV. AND *AZOSPIRILLUM BRASILENSE* SP. NOV.

by

Jeffrey James Tarrand

Thesis submitted to the Graduate Faculty of the
Virginia Polytechnic Institute
and State University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Microbiology

APPROVED:

Dr. N. R. Krieg, Chairman

Dr. R. M. Smibert

Dr. J. L. Johnson

August, 1977

Blacksburg, Virginia

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Noel R. Krieg, whose advice, interest, encouragement, and financial aid helped me through every phase of this study.

I am also especially grateful to Dr. John L. Johnson for his patience and help in teaching me the DNA homology techniques and for making available to me many of the materials and apparatus in his laboratory. I also thank Dr. Tracy D. Wilkins for his help and ideas regarding the determination of acidification of sugar media by means of his replicator method. I am grateful to Dr. Robert M. Smibert for his many valuable suggestions and interest in my project.

I thank Dr. Robert A. Paterson, Head of the Department of Biology, for the opportunity to perform this graduate study and for his interest and support.

I also am grateful for the financial support provided by the Rockefeller Foundation.

Finally, I wish to acknowledge the encouragement, patience, and financial support from my mother.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In 1974, Döbereiner and Day (18) reported the isolation of a nitrogen-fixing bacterium from the roots of tropical grasses. The organism was identified as Spirillum lipoferum, an organism previously described by Beijerinck in 1925 (7). Although this organism had been recognized for many years, and had been included in Bergey's Manual of Determinative Bacteriology up to 1957 (9), its association with plants was not known until 1974, and, indeed, even its ability to fix nitrogen had been debatable. The main reason for the success of Döbereiner and Day in isolating the organism and demonstrating its ability to fix nitrogen was their use of a semi-solid nitrogen-free medium, since the organism possessed an oxygen-sensitive nitrogenase which was inactivated under aerobic conditions and rendered the organisms obligately microaerophilic under nitrogen-limiting conditions.

Since 1974, S. lipoferum has been isolated from the roots of a number of different types of plants, including maize and wheat, and also from a variety of geographical regions. Because of its potential ability to fix nitrogen in association with the roots of non-legumes, great interest has been exhibited in the organism by soil microbiologists and agronomists.

My contact with S. lipoferum arose as a result of a letter sent by Dr. Noel R. Krieg to Dr. Döbereiner in 1975 requesting information

about S. lipoferum that could be included in a review of the genus Spirillum. Dr. Döbereiner indicated that she wished someone to perform a thorough taxonomic study of the organism and sent a strain to Dr. Krieg for examination. The organism did not appear to have a typical spirillum-like morphology, and Dr. Krieg indicated that the organism might not be similar to members of the genus Spirillum or Aquaspirillum, and that a comprehensive study of the organism should be done. Dr. Döbereiner visited our department in 1975 for further discussions, and this eventually led to contact with the Rockefeller Foundation, which was greatly interested in problems related to the world's food production, especially in tropical regions and in Third World countries. As a result of this contact, the Rockefeller Foundation provided funding for a taxonomic study of S. lipoferum to be directed by Dr. Krieg.

The overall purpose of this project was to promote precise communication between investigators working with S. lipoferum, as this is one of the major goals of bacterial taxonomy. To achieve this goal, the specific objectives of the project were as follows:

(i) to determine by the use of DNA base composition studies, and especially by the use of DNA homology experiments for the direct comparison of bacterial genomes, how many species are represented by the various strains of the S. lipoferum group;

(ii) to learn what phenotypic characteristics can, by correlation with the genetic data, serve to distinguish such species;

(iii) to provide a general characterization of the organisms, and

(iv) to investigate the problem of which genus would be the most appropriate for the organisms.

REVIEW OF THE LITERATURE

I have chosen to divide the Review of the Literature into three main sections, as follows: Previous Studies of Spirillum lipoferum, Bacterial Nitrogen Fixation, and Use of DNA Base Compositions and DNA Homology Experiments in Bacterial Classification.

Previous Studies of Spirillum lipoferum

This section is divided into the following categories: Historical, Occurrence and ecology of S. lipoferum, Physiology of S. lipoferum, and Plant-inoculation experiments with S. lipoferum.

Historical. In 1921-1922, the great soil microbiologist Martinus Beijerinck inoculated on N-free glucose and mannitol solutions heavily with soil from this garden and from a sand bed, and observed extensive development of a spirillum-like bacterium (6,7). Although this organism grew well at first, it was later displaced by competitive growth of nitrogen-fixing Azotobacter and Clostridium. However, if calcium malate or calcium lactate was employed as the carbon source instead of carbohydrates, the organism grew well without competition. From Kjeldahl nitrogen determinations, Beijerinck found that partially purified cultures of the spirillum exhibited increases in nitrogen at the expense of malate, whereas cultures lacking the spirillum failed to exhibit such increases. However, pure cultures of the spirillum failed to grow in the absence of a source of fixed nitrogen. Beijerinck suggested

that the good growth of the spirillum in partially pure cultures might be attributable to their microaerophilic nature (the latter apparently being indicated by microaerotactic band formation in wet mounts). Beijerinck presented drawings of the spirillum cells as cultured in various media (Figure 1). In general, cells cultured in sugar media were plump curved rods, containing many lipid droplets which sometimes distorted the shape of the cells. On malate or lactate agar, the cells tended to be thinner and straighter, while on dilute bouillon agar they exhibited a distinct spirillum shape with one or more helical turns. Because of the ease of cultivation on salts of organic acids, as well as the spirillum shape exhibited under certain conditions, Beijerinck considered the organism to be a member of the genus Spirillum, and also a bridging organism linking the genus Spirillum with the genus Azotobacter (7). He initially named the organism Azotobacter spirillum in 1922 (6), but in 1925 he re-named it Spirillum lipoferum (fat-bearing spirillum).

Later studies by Schröder (47) using isolates from single cells also indicated that S. lipoferum could not fix nitrogen in pure culture. Schröder found that peptone or ammonium salts were required as a nitrogen source, and also that a variety of sugars (including glucose) and organic acids could serve as carbon sources. Her organisms were generally long thick rods, but a few cells having a half-turn were observed. A few strains of S. lipoferum were isolated by Giesberger in 1936 (Ph.D. dissertation, Utrecht Univ., Netherlands), using Beijerinck's methods. Giesberger found that the organisms were almost always rod-shaped or

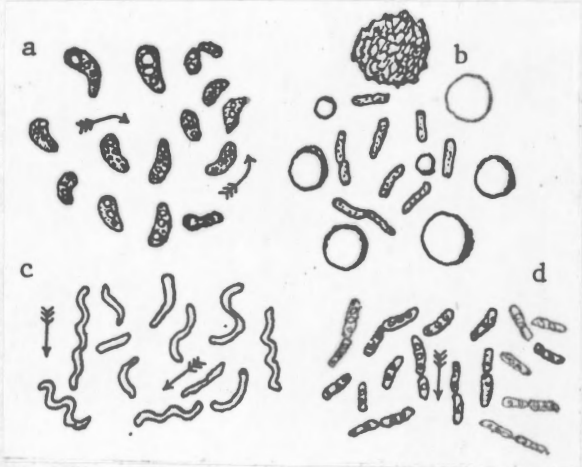

Figure 1. Spirilium lipoferum. a) Junge Kultur in Zuckerlösung (850), Öltropfen in den Zellen. b) Kolonie der Malatagarplatte, die kleinen Kugeln sind durchsichtige Perlen von $CaCO_3$, größere sind trübe und oberflächlich rauh durch Bildung kleinerer Kristalle, wie der obere Sphärit; keine Bewegung (650). c) Stark bewegliche Reinkultur auf verdünntem Bouillonagar mit Spirillengestalt, kein Öl (650). d) Laktatanhäufung. Die stark beweglichen, biegsamen Stäbchen enthalten Fetttropfen (650). (Taken from Beijerinck, M.W. 1925. Über ein Spirillum welches freien Stickstoff binden kann? Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 63: 353-359.)

faintly curved. Because they possessed only a single polar flagellum, Giesberger considered them to be members of the genus Vibrio. In 1963, Becking (3) isolated an organism which he considered to be either a Vibrio or Spirillum, and which was in all probability S. lipoferum. This organism could not grow in a completely nitrogen-free medium and required the addition of 0.01 to 0.005% yeast extract. Under this condition, the organism was capable of assimilating $^{15}N_2$ under aerobic conditions; however, in the broth cultures the organisms became localized below, rather than at, the surface of the medium, suggesting a preference for microaerobic conditions. In general, the cells were only slightly curved, but in peptone media they were spirals. Many intracellular poly-β-hydroxybutyrate granules were present, and the cells possessed a single polar flagellum.

In 1974 Döbereiner and Day (18) reported that root pieces from certain tropical grasses could exhibit active acetylene reducing activity. The ability of such plants to fix nitrogen has been demonstrated directly by use of $^{15}N_2$, and the fixed nitrogen was shown to become incorporated into the plant tissues (17). Döbereiner and Day were able to isolate S. lipoferum from the roots of grasses by placing root pieces into a nitrogen-free semi-solid medium containing sodium malate as the carbon source. After 2 days a white pellicle of organisms formed a few mm below the surface, and after one or two serial subcultures into similar media, almost pure cultures of curved rods were obtained. Purification was achieved by streaking onto malate agar plates containing a low level of yeast extract or other source of fixed nitrogen (aerobic growth did not occur on nitrogen-free agar).

The use of semi-solid media for growth in the absence of fixed nitrogen allowed the organisms to localize at a region where there was sufficient oxygen for respiration but not enough to inactivate nitrogenase. In liquid media, it was necessary to decrease the oxygen level by bubbling gas mixtures containing low levels of oxygen through the cultures. Optimal pO_2 levels for growth in liquid nitrogen-free media have been reported to be 0.01 - 0.02 (15) or 0.006 - 0.02 (38). In media containing a nitrogen source such as ammonium salts, where nitrogenase activity is completely repressed, the organisms grew as aerobes (38).

Occurrence and ecology of the S. lipoferum group. The S. lipoferum group has a wide-spread occurrence in various parts of the world. Schröder (47) reported its occurrence in 74 of 76 soil samples collected in Germany, although recent studies by Döbereiner et al. (19) have indicated that its incidence in root and soil samples from temperate climates is much lower (11 to 17%). According to Döbereiner et al., more than half of the grass root and soil samples collected in tropical countries (four African countries and Brazil) contained abundant S. lipoferum populations, while less than 10% of the samples collected in temperate south Brazil, Kenya, and the United States contained the organisms. In the tropics, the soil of virgin forests seemed to be the poorest environment for S. lipoferum, whereas the soil from Panicum maximum pastures seemed to be the most favorable. It appeared that ecosystems that are not nitrogen-limited were not a selective habitat for S. lipoferum. Soil pH also seemed to be an important factor: the greatest occurrence of S. lipoferum in soil

was found with soils having a pH of ca. 7.3. However, sporadic occurrence was observed even in soils of pH 4.8. In highly acid soils, high numbers of S. lipoferum could best be obtained from surface-sterilized roots, and apparently the roots provided a more tolerable pH for the organisms than did the surrounding soil.

Regarding the types of plant roots from which Döbereiner et al. (19) were able to isolate S. lipoferum, these included forage grasses such as Panicum maximum, Brachiaria mutica, Pennisetum purpureum, Hyparrhenia rufa, Melinis minutiflora, and Digitaria decumbens. The organisms could also be found in the roots of grain crops such as Zea mays, Sorghum vulgare, Triticum aestivum, and Secale cereale. Cultures could also be isolated from legumes belonging to Papilionideae, Mimosoideae and Caesalpinoideae.

In 1974 Da Silva and Döbereiner (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil) examined soil samples from various European countries for the occurrence of S. lipoferum; also roots of various Gramineae, legumes, and tuber plants were examined. In European soils the incidence of the organisms was very low when compared to tropical soils. Only 4 of 50 samples contained the organisms, whereas 68% of Brazilian soils contained the organisms. Soils in Hawaii seemed even more favorable. Soils under grasses contained more of the spirilla than other soils. Washed roots of maize, rice, and forage grasses gave the most active enrichment cultures of spirilla, followed by wheat, sugar cane and tuber plants. Washed legume roots yielded less active enrichment cultures, and surface-sterilized roots gave no enrichment cultures. Roots

from tuber plants, forage grasses, sugar cane and wheat contained some viable spirilla even after 30 sec of surface disinfection, while rice and especially maize produced active cultures even after 1 h of disinfection. The latter observation suggests that S. lipoferum may occur at deep locations in the roots, possibly in the stele. This is in accord with recent results obtained by Patriquin and Döbereiner (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977), who found that even after 6 h of treatment of maize roots with Chloramine-T, the spirilla could still be isolated. Microscopic observations indicated that the bacteria were most common in the inner cortex and in xylem and pith tissues in the stele, and infection seemed to occur initially in secondary roots, spreading into the main roots.

Other investigators have also isolated S. lipoferum from the roots of various plants. In Mexico, Quintero and Garza T (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977) were able to isolate the organisms from the roots of Panicum maximum, Digitaria decumbens, and Saccharum officinarum. In India, Kumari et al. (32) demonstrated the occurrence of S. lipoferum in the roots of several plants: Oryza sativa, Sorghum vulgare, Zea mays, Panicum sp., Setaria, Cynodon dactylon, Amaranthus spinosa, Ficus religiosa, Phyllanthus parviflorus, Portulacea sp., and Xanthium strumarium. Working in the Amazon region of Brazil, Oliveira and Sylvester-Bradley (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977) found low numbers of S. lipoferum in latosols and sandy soils.

Perhaps one of the most significant findings with regard to the potential value of S. lipoferum for agriculture was made by Von Bülow and Döbereiner (53) in 1975. Using a non-destructive C_2H_2-reduction method that permitted maize plants to continue growing and producing seeds in situ despite removal of some root pieces, nitrogenase activities of 74 - 2,167 nmol of C_2H_4/(g of dry roots/h) were found for 10 plants. Selection of maize lines was performed and the best lines showed mean nitrogenase activities of 2,026, 2,313, and 7,124 nmol of C_2H_4/(g of dry roots/h), whereas the original cultivar produced only 313 nmol. S. lipoferum appeared to be primarily responsible for the root nitrogenase activity. It should be noted that recently criticism has been directed against the excised root assay method of assay of nitrogenase activity used by von Bülow and Döbereiner. In this method, roots were assayed by cutting them into small pieces and incubating the pieces for 6 - 10 h in a gas mixture containing 1% O_2, and 12% C_2H_2 in N_2, after which gas chromatography was performed to determine the amount of C_2H_4 produced. As noted by Evans and Barber (22) this technique has been found to lead to an increase in the number of nitrogen-fixing bacteria and therefore to overestimates of rates of acetylene reduction.

A quantitative estimation of the numbers of S. lipoferum associated with the roots of maize has been done by Scott et al. (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brazil, Brazil, 1977), using a most probable number dilution method. During the summer, numbers of S. lipoferum from field grown maize

showed no significant change throughout the life cycle of the plant (ca. 4 X 10^5 baxteria/g wet weight of root). Disinfection of the roots with Chloramine T sharply decreased the numbers of spirilla at 3, 6, and 8 weeks after planting but not at 10 weeks (the latter time was the time during which flowering occurred). This suggested that the number of bacteria deep within the roots may increase before or during flowering. Total bacterial counts for roots were ca. 5.0 X 10^5 - 8.0 X 10^6/g wet weight of root.

The agricultural potential of S. lipoferum has also been suggested by the isolation of the organism from the roots of wheat by Nery et al. (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977). Using the acetylene reduction method with intact soil-plant systems, a potential daily level of nitrogen fixation of ca. 0.8 kg N_2/hectare was estimated. Ninety percent of the enrichment cultures inoculated with root pieces or soil particles yielded S. lipoferum.

The C_2H_2 reduction method is an indirect method for the assay of nitrogenase activity and takes advantage of the low substrate specificity of nitrogenase. The most definitive method for the assay of nitrogenase is the $^{15}N_2$ method, where the potential nitrogen-fixing system is exposed to a gaseous atmosphere containing the isotope; incorporation of the isotope into biological material is subsequently determined the use of a mass spectrometer. Most estimates of nitrogen fixation by S. lipoferum - plant associations have been based on the C_2H_2 reduction method. However, De Polli et al. (17) have confirmed

the occurrence of nitrogen fixation in two tropical grasses by the $^{15}N_2$ method, using intact soil cores containing the roots of Paspalum notatum and Digitaria decumbens. The isotope method also indicated that the fixed nitrogen became incorporated into the plant tissue. Although the fixation of nitrogen by D. decumbens was probably attributable to S. lipoferum, the fixation in P. notatum was probably due to a different organism, Azotobacter paspali - a bacterium associated with the rhizosphere of this grass.

Plant inoculation experiments. The discovery of S. lipoferum - plant roots associations by Döbereiner and her colleagues led to the hope that various non-leguminous crop plants might be subject to inoculation with the spirillum, just as legumes are inoculated with rhizobia. Unfortunately, there is at present no clear evidence that inoculation of plants with S. lipoferum increases the ability of the plants to fix nitrogen under field conditions. In 1976, Smith et al. (51) reported that field pearl millet (Pennisetum americanum) and guinea grass (Panicum maximum) which had been lightly fertilized and inoculated with S. lipoferum produced significantly higher yields of dry matter compared to uninoculated controls that had received only sterile culture medium. However, as has been pointed out by Rogerson (46) and by Evans and Barber (22), no nitrogen fixation rates or nitrogen balances were reported, and it was possible that the control system may also have been inadequate. With regard to the latter, it is now known that various bacteria may produce growth-promoting hormones which increase the dry weight yields of plants but which have no relation to nitrogen fixation. Whether such hormones are produced by

S. lipoferum is not yet known. It is likely that the use of autoclaved cultures of S. lipoferum might provide a better experimental control than the use of sterile culture medium, although it is still possible that the heat treatment might inactivate hormones that might be present.

In 1976, Barber et al. (2) conducted experiments with corn inoculated with S. lipoferum. Corn breeding lines were grown in soil and in greenhouses and inoculated with S. lipoferum (strain Sp 7 or Sp 81). In all experiments, each control plant was treated with an autoclaved suspension of the spirilla. Although the C_2H_2 reducing activity (by the excised root assay) was higher for corn plants inoculated with live cells compared to the controls, the yield and nitrogen content of inoculated plants, and also the C_2H_2 reduction rate by inoculated pot cultivars of corn in situ, provided no evidence of appreciable nitrogen fixation.

Albrecht et al. (1) performed field experiments with corn, using plants treated with sterile culture medium as controls. Although most of the field tests indicated no significant increase in yield from inoculated plants, a few statistically significant increases for inoculated plants did occur; the increase in yield was very low, however.

Although the results of field inoculation experiments to date have been rather disappointing, three items should be borne in mind. (i) The uninoculated control soils frequently have been shown to already harbor S. lipoferum, and this organism is indeed widespread in occurrence. Consequently, spirillum-free controls may in fact

not have been present. (ii) It is not yet known whether strain specificity exists for either the spirilla or for the plants. Such specificity certainly does exist for legumes and members of the genus Rhizobium, and it may exist for spirillum - plant associations as well. (iii) Although inoculation of crop plants has not to date yielded satisfactory results, it should be remembered that nitrogen fixation at a low level has been demonstrated for tropical grasses. Such fixation may prove to be quite important for environments such as tropical soils, which are extremely deficient in nitrogen, whereas similar levels of fixation in richer soils may be relatively unimportant.

Under in vitro conditions in the laboratory, lack of an oxygen protection system for the nitrogenase of S. lipoferum may prove to be a crucial limiting factor, as indicated by experiments by Freitas and Döbereiner (Proc. Int. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977). Unlike the situation with the genus Rhizobium where pleomorphic forms of the bacteria reside intracellularly in root cells of legumes and where oxygen-binding leghaemoglobin serves to mediate the supply of oxygen to the bacteria, S. lipoferum appears to reside mainly intercellularly in the roots and does not cause the formation of nodules containing leghaemoglobin. In the experiments by Freitas and Döbereiner using seedlings of Brachiaria mutica which had been introduced under aseptic conditions into a vermiculite-sand misture, inoculation with S. lipoferum resulted in no detectable level of C_2H_2 reduction in an air atmosphere. However, the spirilla were observed to proliferate on root surfaces

and within the roots. When the pO_2 was decreased to 0.001 to 0.01 atm, C_2H_2 reduction then occurred.

Other in vitro studies of S. lipoferum - plant associations have employed tissue cultures derived from tropical grasses. Vasil et al. (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977) have attempted to establish a forced symbiotic association of callus tissue cultures of sugarcane and S. lipoferum. Growth of the callus cultures and C_2H_2 reduction occurred in cultures lacking any exogenous nitrogen sources. Electron microscopy indicated that the bacteria were living and multiplying in the intercellular space system of the callus tissue. It was also possible to introduce the bacteria directly into protoplasts isolated from the roots or sorghum or suspension cultures of sugarcane, but so far there is no evidence of a functional association or even of the viability of the intracellular bacteria.

With regard to the location of S. lipoferum in plant roots, recent results by Patriquin (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977) indicate that the stele of the roots may harbor a very high number of S. lipoferum cells. It had previously been thought that the organisms were restricted to the root surface and the outer root cortex, but microscopic studies by Patriquin could detect the presence of most of the cells in the stele. The most convincing evidence for the location of the cells in the stele was obtained by use of a 6 h surface sterilization treatment of the roots with Chloramine T. Although such a treatment

would surely have disinfected all but the innermost components of the roots, S. lipoferum could still be isolated from the roots.

Soil fertility in Brazil. In Brazil there are 183 million hectares of vegetation referred to as "cerrado." This huge region is being utilized in an extensive system of cattle production, with the exception of relatively small areas where an intensive agriculture is practiced with results equivalent to those from traditional agricultural areas. Although crops can be grown in the soil of the cerrado, there are great difficulties which must be surmounted first. The soil of the cerrado is very low in plant nutrients - mainly P, N, K, Ca, and Mg - and very high in toxic materials, such as Al. The pH of the soil is very low - 4.5 - 5.0. The soil has only a low holding capacity for nitrates. The acidity of the soil can be corrected by addition of large amounts of lime, and phosphate can be added. However, correction of the nitrogen deficiency would require the application of a great deal of fertilizer, and the latter has a very high cost because in Brazil it must be imported, transported, and distributed. Since nitrogen is the most expensive nutrient and has to be brought to the cerrado region, atmospheric nitrogen fixation would be valuable if it could occur. This accounts for the interest in, and support by, various agencies such as the Energy, Research and Development Administration (ERDA), the National Science Foundation, the U.S. National Academy of Sciences, the Organization of American States, and the United Nations Development Program, of research dealing with nitrogen fixation in the tropics. Although corrective fertilization procedures are being developed to

enable crops to be grown on soils such as those of the cerrado, addition of the necessary amounts of nitrogen may require the establishment of a nitrogen-fixing ecosystem. A great deal of the hope for success of such a landmark project rests on studies now being initiated on biological nitrogen fixation, and much of the emphasis of such studies is being placed on organisms such as S. lipoferum.

Physiology of S. lipoferum. Most physiological studies of S. lipoferum have been done with strain Sp 7 (ATCC 29145), but other strains have been studied with regard to certain aspects of their metabolism. Döbereiner and Day (18) have characterized S. lipoferum as Gram-negative, short, plump, slightly-curved cells having the appearance of a rotating screw while swimming. The organisms have been reported to have a cell diameter of ca. 1.0 m. Döbereiner and Day (18) and Okon et al. (38) found that under nitrogen-free conditions, the organisms were obligately microaerophilic and failed to grow anaerobically or aerobically. In semisolid nitrogen-free medium containing malate as the carbon source, growth was initiated a few mm below the surface, but could eventually extend nearly to the surface. In liquid nitrogen-free malate medium, growth occurred best when a pO_2 of 0.015 atm (15) or 0.008 atm (38) was provided, and the organisms appeared to provide little protection against oxygen for its nitrogenase. When a source of fixed nitrogen was provided, such as NH_4^+, the organism grew as an aerobe.

Strain Sp 7 was reported to grow vigorously in nitrogen-free media on the salts of organic acids, such as succinate, malate, lactate,

and pyruvate, as did strains 4, 13, 51e, 60, 75, 80, 81 and 82 (38). However, strains 59b, RG6xx, and USA 5b were also found to grow with glucose. Using Sp 7, Okon et al. (39) found that succinate supported the highest rates of oxygen uptake by crude cell extracts, but that other tricarboxylic acid cycle intermediates also were oxidized actively. In contrast, glucose, fructose, galactose, glucose-6-phosphate, gluconate-6-phsophate, fructose-6-phosphate, and acetate failed to support oxygen uptake. Fructose 1,6-diphosphate, glyceraldehyde-3-phosphate, and phosphoenol pyruvate were oxidized at a low rate. The results lent support to the idea that the glycolytic and pentose-phosphate pathways of metabolism are of less significance in S. lipoferum than the tricarboxylic acid cycle. Respiratory rates with succinate or other tricarboxylic acid cycle intermediates were ca. 10 to 20% of those in Azotobacter; for succinate, the $QO_2(N)$ was ca. 875 microliters of O_2 per h per mg N. Okon et al. (39) made the interesting observation that there was a mutual response between organisms grown and then tested for oxygen uptake on closely related substrates. For example, cells grown on malate oxidized malate and succinate well, but oxidized lactate and pyruvate to a lesser extent. When the cells were grown on lactate, they oxidized lactate and pyruvate well, but malate and succinate were oxidized to a lesser extent.

Okon et al. (38) reported that S. lipoferum (presumably strain Sp 7) had a doubling time of 20 h under nitrogen-fixing conditions. The doubling time was as low as 2 h in media containing NH_4^+. More

recently, R. H. Burris (Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977) has indicated a doubling time of 5.5 to 7 h under nitrogen-fixing conditions, and a doubling time of 1 h with NH_4^+.

Neyra et al. (36) indicated that all strains of S. lipoferum have an assimilatory nitrate reductase. Many of the strains could reduce nitrate to N_2O and N_2; the remaining strains could reduce nitrate only to nitrite. Neyra et al. (36) noted that S. lipoferum apparently represented the first case of a nitrogen-fixing bacterium that was capable of denitrification. However, in 1976, Strength et al. (52) had reported another nitrogen-fixing bacterium, Aquaspirillum fasciculus, that was also capable of denitrification. Neyra and Van Berkum (35) showed that strain Sp 7 (a denitrifying strain) could grow aerobically with nitrate as a sole nitrogen source (assimilatory nitrate reduction) but could not grow anaerobically with the nitrate. When a crop of cells was first prepared by cultivation under aerobic conditions with nitrate, and then switched to an anaerobic atmosphere containing N_2 and C_2H_2, nitrogenase activity could be induced. This indicated that under certain special conditions, denitrification and nitrogenase activity could occur simultaneously.

Under microaerobic conditions, all strains of S. lipoferum are able to grow in the presence of nitrate but do not fix nitrogen (Megalhaes et al., Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977). Megalhaes et al. were able to isolate spontaneous mutants of S. lipoferum that were

nitrate reductase negative. These mutants might become of great practical interest if they can fix nitrogen in the presence of high nitrate levels in soils and plant roots.

Döbereiner and Day (18) reported that the optimal growth temperature of S. lipoferum (presumably Sp 7) was between 36 and 39°C. They also described the organism as being catalase positive, VP negative, indol negative, and methyl red negative. The organism could not liquefy gelatin or produce H_2S. Urea was hydrolyzed and citrate was used as a sole carbon source.

The specific epithet lipoferum was given by Beijerinck (7) because of the large number of lipid droplets present in the cells. Okon et al. (39) have shown that these "droplets" are granules of poly-β-hydroxybutyrate, (PHB) and that under nitrogen-fixing conditions the cells of Sp 7 contained up to 30% of their dry weight as this polymer. When grown with NH_4^+, only ca. 1.0% of the dry weight was PHB.

Another interesting feature of S. lipoferum is the occurrence of colonies which exhibit a pale pink color (18,38). Okon et al. (38) found this color to appear after ca. 1 week of incubation, but some strains (59b, USA 5b, and RG6xx) developed the pigment only after 5 weeks of incubation.

Nitrogenase in crude extracts of S. lipoferum has been shown by Okon et al. (39) to require ATP, an electron donor of very low O-R potential, Mg^{2+}, and Mn^{2+} for its activity. The Mn^{2+} requirement has also been found necessary for the activity of the nitrogenase of Rhodospirillum rubrum - specifically for activity of the Fe protein

of the nitrogenase complex (39). Like R. rubrum, but unlike other organisms, the Fe protein of the nitrogenase complex of S. lipoferum is almost inactive upon isolation and must be activated by an activating factor that is interchangeable with a similar factor from R. rubrum (Burris et al., Proc. Intern. Symp. Biol. Nitrogen Fixation in the Tropics, University of Brasilia, Brazil, 1977). Furthermore, addition of purified Fe protein from Azotobacter vinelandii, R. rubrum, or Bacillus polymyxa, all restored some degree of nitrogenase activity to S. lipoferum nitrogenase which had had its Fe protein inactivated by storage at -18°C.

Incorporation of NH_4^+ and regulation of nitrogenase synthesis by S. lipoferum appears to follow a similar pattern to that described for the regulation of several other nitrogen-fixing bacteria (39). Such regulation is discussed in the next section.

Bacterial Nitrogen Fixation

Since the most interesting characteristic of S. lipoferum at present is its ability to fix nitrogen in association with plants, some discussion of nitrogen fixation would seem to be pertinent to a thesis dealing with this organism. For a more detailed development of the subject, however, the reader is referred to several comprehensive reviews of nitrogen fixation which have appeared recently (11,13,22,56).

Nitrogen frequently becomes a limiting nutrient in many ecosystems. It is often a limiting factor in human nutrition. The unavailability of nitrogen for many organisms on a planet which has an atmosphere

containing ca. 80% N_2 is explained by the chemical inertness of diatomic nitrogen gas. Such a form of nitrogen is largely inert because the chemical bond joining the two atoms is exceptionally strong. It is a triple bond, and large amounts of energy must be added before it becomes unstable. Even so, the energy added to the triple bond would merely be catalytic in the process of N_2 reduction. The major energy cost when reducing nitrogen by industrial processes is the expense of producing the reductant, H_2 gas, from petroleum or methane.

In Nature, the form of nitrogen which is generally assimilated into plant proteins is nitrogen in the form of NH_4^+. The proteins of dead plants and animals are hydrolyzed by degradative bacteria into amino acids, which are often further dissimilated to ammonia or nitrate. The latter products can be re-used by plants, but denitrifying bacteria compete for the nitrate by reducing it to molecular nitrogen. As a result of denitrification, the reservoir of fixed nitrogen in the soil must be continually replenished.

Small quantities of fixed nitrogen are added to the biosphere each year by inorganic processes such as lightning discharges, volcanic emissions, and ionizing radiation at the Earth's poles. About one-quarter of the Earth's total production of fixed nitrogen comes from NH_3 made industrially by the Haver Process. Most of the Earth's production of fixed nitrogen comes from the action of nitrogen-fixing procaryotes.

The procaryotes which fix nitrogen use solar energy, either directly or indirectly, to reduce atmospheric nitrogen. The best known

of the nitrogen-fixing systems are the nodulated legumes. Rhizobium species with specificities for particular groups of legumes invade the root hairs of plants such as alfalfa, clover, beans, and peas, and induce the formation of root nodules. In addition to legumes, nitrogen is fixed by a variety of microorganisms and biological associations. These include bacteria located in soils, in decaying wood, and on the surfaces of plant roots; free-living blue-green algae (cyano-bacteria) in terrestrial and marine environments; and obligately symbiotic actinomycetes living in association with woody herbs and trees such as Ceanothus, Dryas, Myrica, Purshia, Comptonia, and Alnus. Nodulated legumes are the major nitrogen-fixing plants in agriculture, but most of the nitrogen for maintenance of forests, woodlands, and freshwater and marine habitats is provided by a variety of non-leguminous nitrogen-fixing associations and free-living microorganisms. Recognition of new nitrogen-fixing bacteria or bacteria-plant associations continues to occur each year.

Biological nitrogen fixation contributes ca. 175 million metric tons of nitrogen to the soil each year, with nodulated legumes accounting for nearly half of this amount. The free-living nitrogen-fixing bacteria that lack the capacity for photosynthesis must compete with enormous populations of other microorganisms for the carbon sources which provide energy for their nitrogen fixation. For this reason, free-living non-photosynthetic nitrogen fixers fix much less nitrogen than symbiotic nitrogen fixers or even free-living photosynthetic nitrogen fixers, because the legume provides the carbon sources and a

suitable environment for the functioning of the nitrogenase system. This explains why nitrogen fixation rates for nodulated alfalfa, clover, lupines, and soy beans range from 57 to 600 kg of N per hectare per year, whereas the free-living bacteria such as Azotobacter and Clostridium are estimated to fix only from 0.1 to 0.5 kg per hectare per year.

Largely because of the development of the acetyleve reduction method for detecting nitrogenase activity, a variety of loose associations between nitrogen-fixing bacteria and the roots of several grasses has recently been discovered. Although the quantitative estimates of the rates of nitrogen fixed by such loose associations are debatable, as well as the amount of nitrogen actually contributed to the plants, nevertheless there is little doubt that the bacteria involved in such loose associations fix more nitrogen than free-living non-photosynthetic nitrogen fixers.

The enzyme complex that catalyzes the reduction of N_2 to NH_3 is termed "nitrogenase." All of the nitrogenases so far isolated are remarkably similar in their properties. The complex consists of 2 components, Component I and Component II. Forms of Component I isolated from several sources have molecular weights ranging from 200,000 to 270,000 daltons and contain 1 or 2 molybdenum atoms, 17 to 36 iron atoms, and 14 to 28 acid-labile sulfur groups. Component I, also known as the Mo-Fe protein, consists of 4 subunits, these being of 2 types. Component II is an iron-containing protein with a molecular weight ranging from 55,000 to 67,000, depending on the source of the protein.

Component II, also known as the Fe protein, has 4 iron atoms and 4 acid-labile sulfur groups per molecule. Considering the complete nitrogenase complex, there is uncertainty whether there is a 1:1 or a 1:2 ratio of Component I to Component II. In all nitrogenases so far isolated, both components have been found to be extremely oxygen labile, and anaerobic conditions must be employed throughout the isolation procedures. When Component I from one source is added to a Component II from another source, some fraction of the activity exhibited by the homologous systems is often found. The greatest reconstituted activities shown by such heterologous systems occur when the components are derived from bacteria of the same physiological type - viz., aerobe/aerobe or anaerobe/anaerobe.

Energy is required for nitrogenase activity in the form of (i) a reductant, and (ii) adenosine triphosphate (ATP). During the nitrogenase reaction, energy from ATP and electrons from a ferredoxin (or in one case a flavoprotein) alter Component II in such a way that allows it to become a very powerful reductant, with an E'_o of -400 mv. Component II then transfers electrons to Component I (the Mo-Fe protein), which in turn reduces N_2. Estimates of the ATP requirement for N_2 reduction range from 12 to 24 ATP molecules per molecule of N_2 reduced.

Even though the nitrogenase reaction can proceed <u>in vitro</u> under an atmosphere of pure N_2, approximately 25 to 30% of the total electrons provided to the reaction are expended for the reduction of H^+ to H_2 (nitrogenase also has hydrogenase activity). Unless organisms possess mechanisms for utilizing this H_2, this means that large amounts

of energy are being wasted. It is possible that some of the H_2 could be oxidized to H_2O by a respiratory chain, which would not only provide ATP but which could also help to maintain an anaerobic environment for the intracellular nitrogenase. It is interesting to note that the cowpea and the alder evolve no net H_2, suggesting that the rhizobia in their root nodules may be able to use the H_2 from the nitrogenase reaction effectively. It is possible that rhizobia which evolve no net H_2 might prove to be better strains for agriculture than those which do evolve H_2, because they are less wasteful of energy (22).

The mechanisms for reduction of N_2 and for reduction of H^+ apparently are different, because whereas N_2 reduction is inhibited by carbon monoxide, H_2 evolution is not. Moreover, high concentrations of C_2H_2 inhibit H_2 evolution, but not N_2 reduction. This inhibition of H_2 evolution creates an error when the C_2H_2 reduction method is used to assay nitrogenase activity. Those who believe that diimide and hydrazine are hypothetical intermediates in the N_2 reduction process suggest that H_2 evolution may result from disproportionation of diimide and may be an obligatory step in that process. Reduction of N_2 to diimide is considered to be a thermodynamically unfavorable step in the N_2 reduction process. Production of H_2 from diimide would consequently be expected to help shift the equilibrium toward reduction of N_2.

Ammonia is a repressor of nitrogenase synthesis in all nitrogen-fixing systems so far studied. Also, an active glutamine synthetase (GnS) is necessary for nitrogenase synthesis. Apparently GnS lacking

an attached adenyl group is the active form. High levels of NH_4^+ in the culture medium allow the addition of the adenyl group to GnS and consequent repression of nitrogenase synthesis. Recently, glutamine and also asparagine have also been shown to participate in nitrogenase regulation.

Since all nitrogenase systems are extremely sensitive to oxygen, some organisms have evolved protective mechanisms which allow them to fix nitrogen in aerobic environments. Azotobacter apparently makes use of an extremely high respiratory rate at the cell surface to maintain an anaerobic intracellular environment for its nitrogenase. Other microorganisms produce a gum or slime layer around their cells, which is believed to slow the inward diffusion of oxygen. Most nitrogen-fixing blue green algae (cyanobacteria) localize their nitrogen-fixing apparatus into specialized cells called "heterocysts." These cells have thick walls which limit diffusion of oxygen; also, Photosystem II (which is responsible for oxygen evolution) is not present in the heterocysts. Fixation of nitrogen by the few blue-green algae which do not form heterocysts occurs only at very low oxygen concentrations or under conditions of limited illumination. The most advanced mechanism for oxygen protection in nitrogen-fixing bacteria is probably the rhizobium-legume root nodule. Here, an oxygen-binding protein (leghaemoglobin) and the complex structure of the nodule constitute the main protective devices. In the case of the main subject of this thesis, S. lipoferum, there seems to be relatively little protection offered for the nitrogenase, and

S. lipoferum is able to fix nitrogen only when the level of oxygen in the culture medium is decreased to a very low level. It is possible that the roots of grasses may provide some protection from oxygen, but it is also very probable that oxygen may still be the major limiting factor for nitrogen fixation by spirillum-grass associations.

Use of DNA Base Composition and DNA Homology Experiments in Bacterial Classification

Whether all strains so far identified as S. lipoferum in fact belong to a single species has not been established. Döbereiner et al. (19) noted that certain strains appeared to require yeast extract for growth in nitrogen-free media, such strains generally being isolated from temperate climates. Strains from tropical climates were usually much easier to isolate and did not require yeast extract. A similar distinction between yeast extract-requiring and non-requiring strains was noted by Okon et al. (38); here, it was also noted that yeast extract-requiring strains could also be grown in nitrogen deficient media with glucose as the carbon source, whercas strains not requiring yeast extract did not grow with glucose. Three groups based on denitrification activity have been recognized by Neyra et al. (36): in Group I, strong denitrification occurred with production of gas bubbles and complete or almost complete disappearance of the nitrite formed. All of the remaining strains were placed into Group II, which showed weak denitrifying ability - intermediate or incomplete disappearance of the nitrite formed. All of the strains which required yeast extract were included in Group II.

The essence of bacterial classification is to group like bacteria together. Yet confusion and uncertainty exist among microbiologists as to how much alike two bacterial strains have to be in order to be grouped together into a single species, or even into a single genus, family, or order. In the case of animals and plants, the ways in which the organisms evolved and their consequent genetic relationships form a basis for deciding how the organisms are to be grouped. A phylogenetic classification scheme is based on comparisons of the morphologies of animals or plants, embryological evidence, comparative physiology and biochemistry, genetics, and also on a fossil record. The basic taxonomic unit, the species, for animals and plants is a population of individuals with markedly similar morphology, embroyologic and physiological characters, and whose members in nature breed only with each other and have a common ancestor.

Unfortunately, with bacteria little or no fossil record exists to support a phylogenetic classification. Moreover, bacteria are morphologically much simpler than animals or plants, and it is usually not obvious from looking at them what their relationships are. As for breeding with each other, most bacteria do not multiply by sexual means but instead by asexual binary fission. Therefore, microbiologists are reduced to characterizing bacterial strains as to their cell wall chemistry, cell inclusions and storage products, types of cell metabolism, pigments produced, nutritional requirements, metabolic products, temperature and pH requirements, gaseous needs, antibiotic sensitivities, pathogenicity, habitat, etc. In bacteriology, a species is considered

to be a permanent reference strain, the "type strain," together with all other strains which sufficiently resemble the type strain so as to be included with it in the species. Similarly, a genus consists of a permanent reference species, the "type species," together with all other species which sufficiently resemble the type species so as to be included with it in the genus. Despite this simple concept, and despite a wealth of data concerning bacteria, great difficulties still exist in classifying bacteria. These difficulties stem from the problem of deciding how close the resemblance between two strains should be if they are to be grouped together in a taxon.

Because I have been concerned with a nitrogen-fixing organism, it might seem that this important physiological characteristic might be highly useful for classifying bacteria. Nevertheless, the ability to fix nitrogen is probably not of major taxonomic significance. This is indicated by two considerations: (i) relatively few genes, possibly only two structural genes (and possibly half a dozen regulatory genes) govern the ability to fix nitrogen; yet bacteria possess thousands of genes; (ii) widely diverse bacteria which are clearly not closely related have been found to possess the ability to fix nitrogen. Some of these organisms are listed in Table 1. This list illustrates that to group bacteria together on the basis of an ability to fix nitrogen would result in a chaotic assemblage of very diverse organisms.

There are presently three basic approaches used to classify bacteria. In the classical approach, bacteria are grouped together

TABLE 1. A list of nitrogen-fixing bacteria[a]

Group	Genus	Species
Non-photosynthetic		
Gram negative aerobes	Azotobacter	beijerinckii
		chroococcum
		paspali
		vinelandii
	Azomonas	agilis
		insignis
		macrocytogenes
	Beijerinckia	indica
		mobilis
		fluminensis
		derxii
	Derxia	gummosa
	Aquaspirillum	peregrinum
		fasciculus
	Rhizobium	leguminosarum
		phaseoli
		trifolii
		meliloti
		japonicum
		lupini
	Methylosinus	
	Methylocystis	
	Methylococcus	
	Methylomonas	
	Methylobacter	
Sporeforming rods		
	Bacillus	macerans
		polymyxa

[a]Taken in part from Dalton, H. 1974. Fixation of dinitrogen by free-living micro-organisms. CRC Crit. Rev. Microbiol. 3: 183-220.

TABLE 1. (continued)

Group	Genus	Species
Sporeforming rods		
	Clostridium	butyricum
		pasteurianum
	Desulfotomaculum	orientis
		gigas
Gram negative facultative rods		
	Klebsiella	pneumoniae
	Enterobacter	aerogenes
		cloacae
	Escherichia	coli[b]
Gram negative anaerobes		
	Desulfovibrio	desulfuricans
		vulgaris
		gigas
Actinomycetes and related organisms		
	Mycobacterium	flavum
		roseo-album
		azotabsorptum
	Frankia	alni
		elaeagni
		discariae
		ceanothi
		coriariae
		dryadis
		purshiae
		cercocarpi
		brunchorstii
		casuarinae
Chemolithotrophs		
	Thiobacillus	ferro-oxidans

[b]By genetic transfer of nif from K. pneumoniae

TABLE 1. (continued)

Group	Genus	Species
Photosynthetic, non-oxygen-evolving		
	Rhodopseudomonas	palustris capsulatus gelatinosa spheroides
	Rhodospirillum	rubrum
	Chromatium	vinosum minutissimum
	Chlorobium	thiosulfato-philum
	Ectothiorhodospira	shaposhnikovii
Cyanobacteria		
	Anabeana	
	Anabaenopsis	
	Aulosira	
	Cylindrospermum	
	Nostoc	
	Calothrix	
	Scytonema	
	Tolypothrix	
	Trichodesmium	
	lyngbya	
	Phormidium	
	Plectonema	
	Fischerella	

TABLE 1. (continued)

Group	Genus	Species
Cyanobacteria		
	Hapalosiphon	
	Mastigocladus	
	Stigonema	
	Westiellopsis	
	Gloeocapsa	

or separated according to the intuitive, subjective judgment of an investigator who has worked extensively with a certain group of organisms and who has detailed and comprehensive knowledge of the phenotypic characteristics of the organisms. Although such schemes have usually been supported later by more objective evidence, sufficient errors have been made to lead to the realization that the classical method does not always give definitive results.

In order to provide an objective basis for classification of bacteria, a method known as numerical taxonomy has been developed. In this method, a large number of characteristics are determined for the organisms in question, with each characteristic being given equal weight so as not to introduce bias on the part of the investigator. For any two organisms, the number of positive characteristics which are shared by both organisms, divided by the total number of characteristics (shared + not shared), yields a similarity coefficient. Generally, a computer is required for this method, because the similarity coefficient for each strain as compared to every other strain being considered is difficult to determine manually when 100 to 300 characteristics are being studied for several strains. Although each characteristic is assigned equal weight in order to avoid bias, numerical taxonomy is in fact fully as biased as the classical method. This is because certain characteristics are determined by more genes than other characteristics. For example, the ability to transport and utilize glucose by the Embden-Meyerhof-Parnas pathway of glycolysis involves 14 genes, while the ability to

use lactose involves only two additional structural genes (for the permease and the galactosidase) and a regulator gene. What makes the situation with numerical taxonomy even more difficult is that the number of genes governing many, even most, characteristics is unknown. For example, it is not known how many genes are involved in being an obligate microaerophile, or in formation of spores or cysts, or in having a spiral rather than a straight rod morphology. In such cases, it seems presumptuous to assign equal weights to such characteristics when they may be far from equal in terms of numbers or kinds of genes. Even though up to 300 characteristics may be compared in a numerical taxonomy study, it is not certain that this adequately reflects or samples the full genetic potential of organisms having several thousand genes. Moreover, phenotypic characteristics that are directly on the activity of an enzyme provide no assurance that the enzyme, while functionally similar in two organisms, has the same amino acid sequence. For example, several isozymes of lactic dehydrogenase occur, and although both Staphylococcus aureus and E. coli catabolize lactose, the former employs a lactose-phosphate hydrolase, while the latter has a classical β-galactosidase.

The third approach to bacteria taxonomy employs techniques that allow a direct comparison of bacterial genomes. The usefulness of this approach has been exemplified by studies such as those by Johnson (12,25,26) and by Brenner (10). One of the measurements used to compare bacterial genomes is DNA base composition. The DNA of bacteria consists of a double-stranded molecule. Each strand is

composed of a backbone chain composed of alternating molecules of deoxyribose and phosphate. From each deoxyribose projects a purine base, usually either adenine or guanine, or a pyrimidine base, usually either cytosine or thymine. The two strands are held together by hydrogen bonds between the bases on one strand and the complementary bases on the other strand: adenine is paired with thymine, and guanine is paired with cytosine (54). The percentage of the bases represented by guanine and cytosine, or by adenine and thymine, per mole of the DNA of an organism is characteristic of that organism. This percentage is referred to as the mol% G + C of the DNA. Extensive lists of the mol% G + C values for many bacteria are available (37).

If two strains are closely related genetically, their mol% G + C will be very similar. However, it is quite possible that two completely unrelated strains of bacteria might have exactly the same mol% G + C values, such as 38 and 45, they would certainly not be closely related genetically. Consequently, determination of the DNA base composition provides a firm basis for deciding that strains are <u>not</u> related, but cannot be of definitive value in deciding that they <u>are</u> related.

Determination of DNA base composition (mol% G + C) offers a fundamental but crude basis for comparison of bacterial genomes. A much finer and more definitive comparison can be made on the basis of the sequence of bases in the DNA, as it is this sequence which comprises the genetic specificity of an organism. It is possible that two

bacterial strains may have an identical mol% G + C value, but are yet entirely different in regard to their base sequences. This would indicate no close genetic relationship between the two strains. Alternatively, if a high proportion of the DNA of both strains had the same base sequences, this would indicate a close genetic relationship and common ancestry, and would provide firm, objective evidence that the two strains should be grouped together in a single species.

DNA homology techniques depend on the unique physical properties of DNA. Under certain conditions, such as low salt concentration plus heating or alkalinity, the hydrogen bonds between the complementary strands of the double-stranded DNA collapse and the strands will separate. Then at a somewhat lower temperature and in the presence of a higher salt concentration the complementary strands will reassociate, forming double-stranded DNA having physical and biological properties similar to the original. Using this principle, the relative similarities in the nucleotide sequences between organisms can be compared. Although several methods are available for making these measurements, only the nitrocellulose filter method will be discussed here, since this is a method that has been successfully used in the past (for examples, see references 10, 12, 25, 26, and 44) and also is the method which I have used for analysis of the S. lipoferum group.

(i) Let us assume that we wish to determine the degree of genetic relatedness between two bacterial strains, X and Y. The two strains must have a reasonably similar DNA base composition, no more than ca. 5%

difference in mol% G + C (J. L. Johnson, personal communication) if one expects to find significant DNA homology. Also, the phenotypic properties of the two strains would have to be very similar.

(ii) One of the two strains, e.g., strain X, is chosen as a "reference" strain.

(iii) The DNA is extracted from strain X and denatured by heating at a low salt concentration, causing the two strands of the double-stranded DNA to separate.

(iv) The resulting single strands are caused to adhere to a nitrocellulose filter. This will occur under conditions of high salt concentration.

(v) The cells of strain X are cultured in a medium containing a radioisotope which will be incorporated into the DNA. DNA from strain X may alternatively be labeled in vitro. The DNA is harvested from the cells and subjected to sonic oscillation to break it into small fragments. The fragments are then heated to obtain single strands.

(vi) In the homology experiment, radioactive DNA is used in very low concentrations so that its renaturation with itself will not be significant when compared with its renaturation with filter-bound DNA, or the rate at which heterologous DNA binds with filter-bound DNA and thereby excludes homologous labeled DNA from forming duplexes with the filter-bound DNA (from either strain X or strain Y) and also the amount of DNA on the filter at relatively high concentrations.

(vii) The setup for a DNA homology experiment is depicted in Figure 2. The filter, containing immobilized fragments of X DNA, is incubated in a vessel containing a small amount of radioactive X DNA fragments and a high amount of non-radioactive X or Y competitor. The temperature of incubation is critical, and is usually 25°C below the melting point (Tm) of the DNA to insure a high degree of specificity for duplex formation. (The Tm of the DNA refers to the temperature at which 50% of the hydrogen bonds in double-stranded DNA are dissociated, as measured by an increase in absorbance at 260 nm. There is a linear relationship between the Tm values and the mol% G + C values of the DNA: guanine-cytosine pairs are held together by three hydrogen bonds, whereas adenine-thymine pairs are held together by two hydrogen bonds.)

(viii) If the competitor fragments were not present, the homolgous radioactive fragments would be free to "anneal" (form duplexes) and the filter would acquire a certain amount of radio-activity. If X competitor fragments were present, these would compete with the radio-active X fragments for duplex formation on the filter, and the filter would not become very radioactive. If Y competitor fragments were used instead of X competitor fragments, the competition with the radioactive fragments would depend on the genetic relatedness of strain Y to strain X. For example, if strain Y were genetically very similar to strain X, then the Y competitor fragments would compete strongly with the radioactive X fragments for duplex formation on the filter. The Y competitor would depressed the number of counts recorded for the filter nearly as much as if X competitor had been used. Y

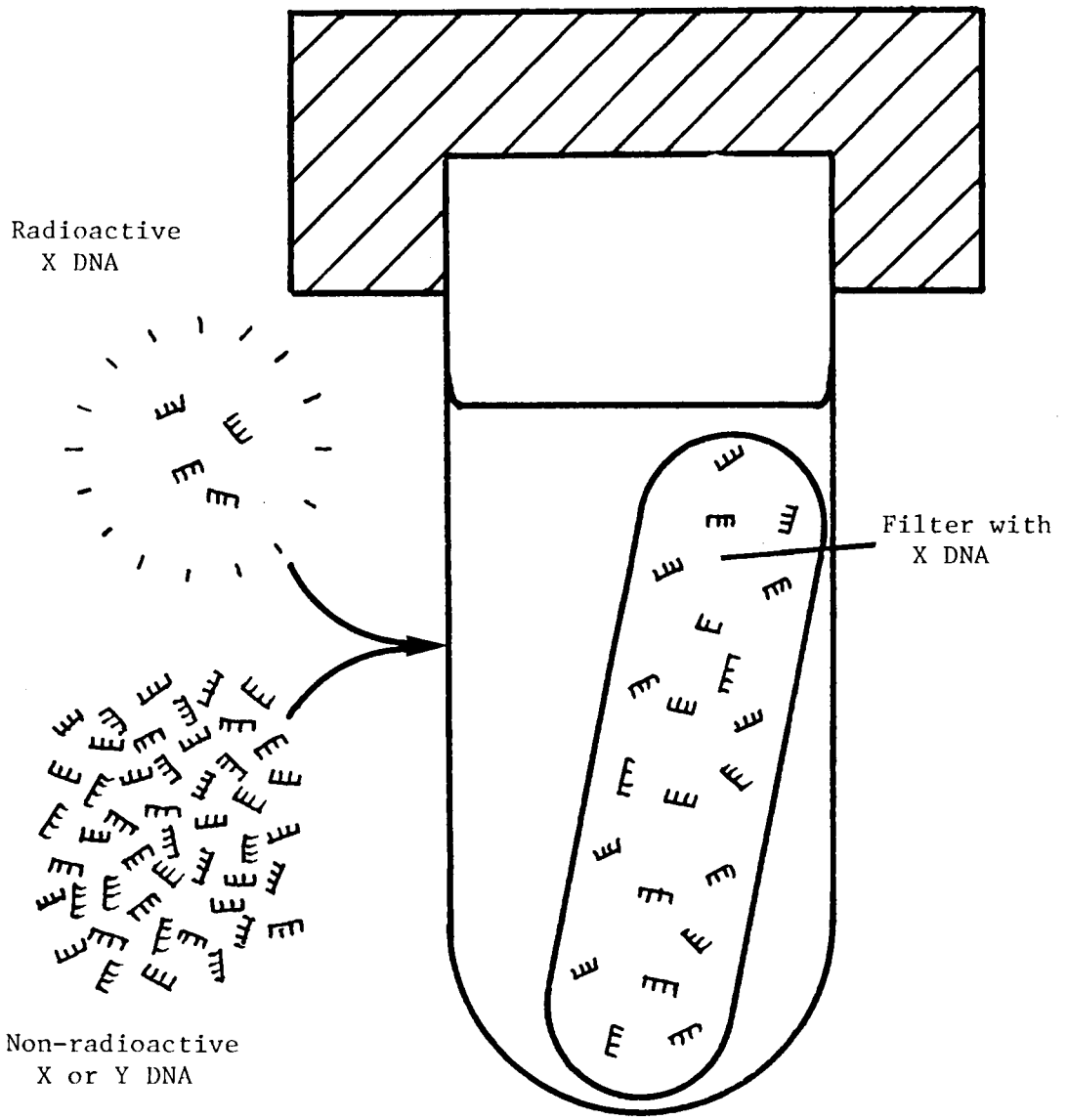

FIGURE 2. Experimental setup for DNA/DNA homology experiments, with X or Y DNA as the competitor and X DNA as the reference.

would have caused nearly 100% of the depression in binding of radioactivity that X would have caused, and the organism would be said to have nearly 100% homology with strain X. However, if strain Y were genetically quite dissimilar to strain X, the Y competitor fragments would not compete much with the radioactive X fragments for duplex formation on the filter, and the radioactivity on the filter would be nearly as high as if no competitor had been added at all. The % homology in each case would be calculated according to the following equation:

$$\% \text{ DNA homology} = \frac{\begin{array}{c}\text{Depression in binding of radioactive homologous DNA caused by non-radioactive heterologous fragments}\end{array}}{\begin{array}{c}\text{Depression in binding of radioactive homologous DNA caused by non-radioactive homologous fragments}\end{array}} \times 100$$

An example of such a calculation is presented in Table 2. It should be noted that the homology scale generated in this fashion does not represent the absolute level of genetic relatedness. I.e., strains must have at least 70% of their base sequence identical in order for any significant DNA homology to occur.

As indicated by the studies made by Ravin (45), a species based on nucleic acid homologies is termed a "genospecies" to distinguish it from those species that have been defined by phenotypic characteristics. In general, the strains within a genospecies are proposed to be related to an extent of 60% or higher by DNA homology (10,25). With regard to genus, if a significant degree of DNA homology (ca,

TABLE 2. Example of calculation of the % DNA homology for strain Y, with strain X as the reference strain

Experimental conditions	Radioactivity bound to filter, cpm
No competitor present	3400
Homologous (X) DNA used as the competitor	300
(Depression in binding = 3400 - 300 = 3100 cpm.)	
Heterologous (Y) DNA used as the competitor	600
(Depression in binding = 3400 - 600 = 2800 cpm.)	

% homology = 2800 ÷ 3100 X 100 = 90.3%, which would indicate that strain Y is genetically closely related to strain X and that the two organisms should be placed in the same species.

20 to 50%) existed between two genospecies, this would support inclusion within a single genus, but not the same species. DNA homology values less than ca. 20% are generally not considered significant (25), and thus homology data are not useful at the tribe or family level, but only for the lower taxa. They are most useful at the species level.

The techniques of DNA homology were first applied to problems of bacterial taxonomy nearly 18 years ago. One of the earliest techniques which demonstrated the formation of hybrid DNA molecules was the density gradient method. One bacterial strain was grown in a medium containing D_2O, so that its DNA was "heavy" as a result of incorporation of deuterium. Another culture of the strain was grown without D_2O, so that its DNA was "light." After the two DNA samples had been mixed, denatured, and annealed, hybrid molecules could be detected by centrifugation in a cesium chloride gradient, where they formed a band intermediate in position between those of the "light" and "heavy" duplexes. The method could also be used to compare the DNA samples from two bacterial strains, but it proved to be too cumbersome for routine use, and also had the disadvantage of detecting only reassociation between complementary strands of very high homology. An improvement occurred with the development of the agar gel method, in which long denatured strands of DNA were immobilized in agar and short fragments of labeled denatured DNA allowed to diffuse into the agar to their complementary binding sites on the immobilized DNA. This method was used by DeLey and Park (16) in 1966 in a study

of free-living nitrogen-fixing bacteria. Another development was the hydroxylapatite (HA) method, in which HA specifically adsorbs DNA duplexes, but not single-stranded DNA, under certain conditions. Reassociation between labeled and unlabeled DNA is allowed to occur in solution, after which the duplexes are adsorbed onto HA. This is then washed to eliminate any single-stranded DNA, and the adsorbed duplexes are eluted by either increasing the ionic strength of the buffer or by heating. The radioactivity of the eluate is then determined. In another method, a membrane filter is used for immobilization of single stranded DNA; this method has already been discussed in this section. The membrane filter method and the HA method are easier to carry out then the agar gel method and consequently have increased the rate at which DNA homology information can be gathered.

Some examples of the use of DNA homology experiments in bacterial taxonomy follow. In 1972 Johnson and Cummins (26) used the method to advantage in studying the taxonomy of anaerobic coryneforms, classical propionibacteria, and strains of Archnia propionica. For example, among the anaerobic coryneforms (now included in the genus Propionibacterium), three DNA homology groups occurred. The P. granulosum group showed only 15 to 20% homology to the P. avidum and P. acnes group. Within each homology group, the DNA homology was very high. The classical propionibacteria could be arranged into 4 groups, and these propionibacteria exhibited only a very low degree of DNA homology (near the level of experimental error, viz. ca. 10 - 20% homology)

with the anaerobic coryneforms. The Arachnia propionica formed still another distinct DNA homology group.

With regard to the aerobic pseudomonads, Palleroni and his colleagues (40,41,42) have performed DNA homology experiments on many strains and have divided the strains into 36 homology groups. They found that in general these groups corresponded to species which had been delineated previously on the basis of phenotypic characteristics and DNA base compositions. The P. fluorescens group, however, could be divided into several DNA homology groups, indicating that this species in fact should be divided into several species. Another example where the existence of two species rather than a single species occurred was reported by Johnson (25): certain strains of clostridia could not be distinguished from one another by phenotypic tests but could be clearly distinguished by DNA homology data, there being two homology groups having only 20 to 40% homology between each group. The two groups, named C. butyricum and C. beijerinckii, were later found to be differentiated by their growth requirements. The C. butyricum strains could grow on a glucose-mineral salts medium containing only biotin as an additional growth factor, whereas the C. beijerinckii strains were found to have more complex requirements. The distinction between the two species had not been noted previously because both species had been cultured in a complex basal medium. Another example of where DNA homology experiments have proven to be useful was in a study of phytopathogenic pseudomonads by Pecknold and Grogan (43) in 1973. Traditionally, phytopathogenic pseudomonads

have been classified into numerous species on the basis of which host plant they were isolated from. The underlying assumption was that each species was thought to be specific for a particular plant or group of plants. Pecknold and Grogan performed DNA homology experiments on a large number of such species of pseudomonads and found that the strains fell into six DNA homology groups, despite the wide diversity of their plant hosts. For example, the strain in the "morsprunorum" homology group included pseudomonads isolated from diseases of plum trees, tobacco, mulberry, cucumber leaves, soybeans, kudzu, and olives. Strains belonging to the "syringae" group were isolated from lilac, citrus plants, cowpeas, beans, cherries, diseased nasturtium leaves, diseases beet leaves, garden peas, field peas, and from the roots of ginseng. Thus, there seemed to be little justification for the traditional method of speciation among such pseudomonads.

rRNA/DNA homology. In 1965, Doi and Igarashi (20) demonstrated that ribosomal RNA (rRNA) cistrons tend to be more conserved (i.e., less likely to become altered during evolution) than the cistrons of the total bacterial genome. This finding has enabled investigators to measure and describe more distant genetic relationships than can be done by DNA/DNA homology methods. In such experiments, a low level of purified homologous labeled rRNA, and a high level of unlabeled crude competitor rRNA, are added to immobilized homologous DNA. This method probably has most value at the genus level of classification. Some recent examples of its use can be noted. Johnson and Francis (27) found 4 rRNA homology groups to occur within the

genus Clostridium, indicating considerable genetic diversity within the genus. Palleroni et al. (42) similarly found that the genus Pseudomonas could be divided into at least 5 distinct rRNA homology groups, some of which were as distantly related to each other as they were to E. coli (considered to be quite distinct from the pseudomonads). One of the most interesting findings was the occurrence of a very high degree of rRNA homology between the species Pseudomonas maltophilia and Xanthomonas campestris (95% homology). This indicated that perhaps the genus Xanthomonas should not be separated from the genus Pseudomonas, and that the phenotypic characters presently used to differentiate the two genera may be more applicable at the species level rather than the genus level.

Correlation of DNA homology data with phenotypic characters. The techniques of DNA homology experiments are relatively difficult and painstaking, and they cannot be done in ordinary bacteriology laboratories. However, once homology studies have indicated the genetic relationships among a number of strains, those biological characteristics that correlate well with the homology groupings can then be used to identify additional strains in a relatively easy manner. In other words, the phenotypic characters of an unidentified strain could be used to determine to which homology group it most likely belongs. Some phenotypic characters may be less well conserved (less well correlated with the homology data) and would have little usefulness for identification of new strains. An excellent statement of the usefulness of DNA homology data has been given by Johnson (25):

"How should DNA homology values be used in taxonomy? If they are regarded as an end in themselves, they soon become boring and lifeless sets of figures without much practical use. What I believe to be the most useful and interesting questions to ask are as follows. How do the biological properties which have traditionally been used to identify and classify bacteria correlate with DNA homology values? Do certain phenotypic characters tend to be more conserved than others, and are they thus more useful for classification and identification?"

MATERIALS AND METHODS

Strains studied and maintenance of stock cultures. Table 3 lists the various strains of the S. lipoferum group studied. Upon receipt, each strain was purified by streaking twice on peptone-succinate-salts (PSS) agar (24). Stock cultures were maintained in Döbereiner's nitrogen-deficient semisolid medium, having the following composition (g/l): KH_2PO_4, 0.4; K_2HpO_4, 0.1; $M_gSO_4.7H_2O$, 0.2; NaCl, 0.1; $Cacl_2.2H_2O$, 0.026; $FeCl_3.6H_2O$, 0.01; $NaMoO_4.2H_2O$, 0.002; L-malic acid, 3.58; brom thymol blue, 0.025; yeast extract, 0.05; agar (Difco), 1.75. The pH was adjusted to 7.0 with KOH. Strains not requiring yeast extract (as indicated by Dr. J. Döbereiner, personal communication) were transferred monthly, while strains requiring yeast extract were transferred biweekly. Stock cultures were grown and maintained at 30°C. At the beginning of the study, 11 strains were preserved in liquid nitrogen, and the remainder were similarly preserved at the end of the study. For preservation in liquid nitrogen, the cells were suspended in nutrient broth containing 10% dimethyl sulfoxide as a cryoprotective agent.

Morphology. Cellular dimensions were determined from photomicrographs taken by phase-contrast microscopy. The magnification of the photomicrographs was determined accurately from similar photomicrographs

TABLE 3. Summary of S. lipoferum strains studied

Strain No.	Country	Place	Plant roots or soil	Isolated by:
Sp 4	Brazil	Km 47, Rio de Janeiro	Digitaria	J. Döbereiner
Sp 7	"	" "	"	"
Sp 13	"	" "	"	"
Sp 13t	"	" "	"	"
Sp 34	"	" "	"	"
Sp 35	"	" "	"	"
Sp 51e	"	" "	Wheat	"
Sp 52	"	" "	Sorghum	"
Sp T60	"	" "	Wheat	"
Sp 67	"	"	Maize	"
Sp M75	"	" "	"	"
Sp 80	"	" "	"	"
Sp 81	"	" "	"	"
Sp M82	"	" "	"	"
Sp P1	Peru		Soil	"
Sp P2	"		"	"
Sp F4	U.S.A.	Gainesville, Florida	Millet	"
Sp F6	"	" "	"	"
Sp Br 8	Brazil	Brasilia	Soil	"
Sp Br 11	"	"	Maize	"
Sp Br 13	"	"	Soil	"
Sp Br 14	"	"	Wheat	"
Sp Br 21	"	"	"	"

TABLE 3. (continued)

Strain No.	Country	Place	Plant roots or soil	Isolated by:
Sp A2	Africa	Ibadan, Nigeria	Maize	J. Döbereiner
Sp A7	"	" "	Rice	"
Sp A8	"	" "	Panicum	"
Sp MT 20	Brazil	Mato Grosso	Soil	"
Sp MT 21	"	" "	"	"
Sp L69	"	Londrina	Wheat	"
Sp Col 1c	Colombia		Soil	"
Sp RG 8a	Brazil	Rio Grande do Sul	Wheat	"
Sp RG 16a	"	"	Lolium	"
Sp RG 20b	"	"	Wheat	"
JM 6A2	Ecuador		Zea mays	J. R. Milam
JM 6B2	"		" "	"
JM 24B4	"		Musa sp	"
JM 28A2	"		" "	"
JM 52B1	Venezuela		Panicum maximum	"
JM 73B3	"		Zea mays	"
JM 732B	"		" "	"
JM 73C3	"		" "	"
JM 75A1	"		Panicum maximum	"
JM 82A1	"		Zea mays	"
JM 119A4	U.S.A.	Florida	Pennisetum americanum	"
JM 125A2	"	"	" "	"
Cd	"	California	Cynodon dactylon	D. Eskew

TABLE 3. (continued)

Strain No.	Country	Place	Plant roots or soil	Isolated by:
Sp 59b	Brazil	Km 47, Rio de Janeiro	Wheat	J. Döbereiner
Sp USA 5b	U.S.A.	Pullman, Washington	Soil	"
Sp RG6xx	Brazil	Rio Grande do Sul	Wheat	"
Sp RG 8c	"	"	"	"
Sp RG 9c	"	"	"	"
Sp RG 18b	"	"	"	"
Sp RG 19a	"	"	Digitaria	"
Sp RG 20a	"	"	Wheat	"
Sp Col 2b	Colombia	Papayan	Maize	"
Sp Col 3	"	"	Brachiaria	"
Sp Col 5	"	"	Hyparrhenia rufa	"
Sp Br 10	Brazil	Brasilia	Soil	"
Sp Br 17	"	"	Maize	"
Sp A3a	Africa	Dakar, Senegal	Grass	"
Sp S1a	Sweden	Uppsala	Lawn	"

of a stage micrometer. The type of flagellation was determined by electron microscopy, using preparations shadowed with tungsten oxide.

Acidification of sugar media. In the case of glucose, a medium of the following composition (g/l) was employed: Peptone (Difco), 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 1.0; $FeCl_3.6H_2O$, 0.002; $MnSO_4.H_2O$, 0.002; brom thymol blue (aqueous), 0.025. The pH was adjusted to 7.0 - 7.1 with KOH. D-glucose, sterilized by filtration, was added aseptically to a final concentration of 1.0%. The medium was dispensed in 10 ml volumes into sterile 20 X 125 mm tubes with loose screw caps. The tubes were inoculated with a loopful from a 48-h-old PSS broth culture and incubated for 4 days at 37°C. A yellow color indicated acidification.

For other sugars, and also for glucose, a different method was used. The medium employed had the following composition (g/l): yeast extract (Difco), 0.05; K_2HPO_4, 0.25; $FeSO_4.7H_2O$, 0.01; $Na_2MoO_4.2H_2O$, 0.001; $MnSO_4.H_2O$, 0.002; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.026; $(NH_4)_2SO_4$, 1.0; biotin, 0.0001; brom thymol blue (aqueous), 0.0375; agar (Difco), 15.0. The pH was adjusted to 7.1 with KOH. Each sugar, sterilized by filtration, was added aseptically to give a final concentration of 1.0%. The sugar media were then added aseptically to sterile micro-titer plates (flat-bottomed wells, NUNC), ca. 0.16 ml per well. All the wells of a given plate contained the same kind of sugar medium. The method of Wilkins et al. (55) was used for inoculation of the wells, by use of a replicator constructed of wire brads projecting from a plastic block (see Figure 3). The

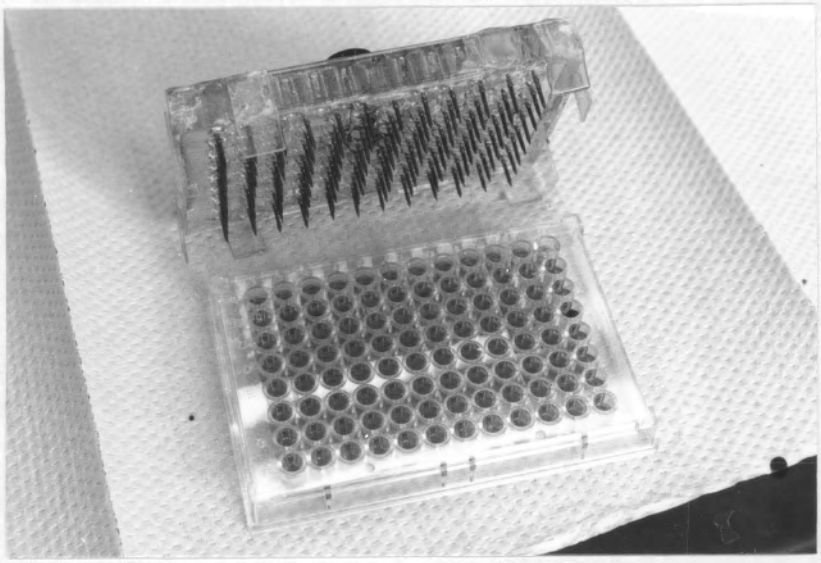

Figure 3. The replicator (top) constructed for inoculation of micro-titer plates (bottom) containing sugar media. The replicator consisted of wire brads projecting from a plastic block. Plastic guides on the border of the replicator insured precise alignment of the replicator when it was applied to a micro-titer plate.

replicator was sterilized by alcohol and by subsequent exposure to an intense source of ultraviolet light. The replicator was inoculated from a master micro-titer plate, where each well contained a different strain (0.16 ml of a 48-h-old PSS broth culture). By pressing the replicator onto the master plate, each wire brad was inoculated. The replicator was then placed over a micro-titer plate containing sugar medium and pressed onto this. Thus, each brad would inoculate a particular strain into a particular well of sugar agar. The replicator was then returned to the master plate before inoculating another plate of sugar medium. Of the 96 wells in each micro-titer plate, 35 wells located at various positions were not inoculated and served as controls for sterility of the medium and also as controls for migration of cultures from one well to another during incubation. After inoculation, the micro-titer plates were covered with their sterile lids and incubated at 37°C for 72 h. The pH of each well was then determined with a small pH electrode (Sargent No. S-30070-10) as described by Wilkins et al. (55). The pH of uninoculated wells ranged from 5.7 - 6.2, probably because of absorption of carbon dioxide. Acidification of the medium by a strain was considered positive if the pH of the well was 3.9 - 5.0.

Acidification of glucose and fructose media under anaerobic conditions was tested using the peptone-based medium described above (method 1). The medium was placed in cotton-stoppered tubes in a Gas-Pak (Bioquest) jar containing Gas-Pak catalyst (previously heated to become activated). The Jar was exhausted, filled with nitrogen,

exhausted, and subsequently filled 3 times with hydrogen. The jars were incubated at 37°C for 4 days.

The ability to grow with glucose under anaerobic conditions was determined in a manner similar to that described above, except that streaked plates of solidified medium were used. Also, in addition to the peptone-based medium, a defined solid medium (Döbereiner's medium containing $(NH_4)_2SO_4$, glucose, and biotin but lacking malate) was used.

Requirement for biotin. Preliminary evidence by an auxanographic method indicated that certain strains required biotin for growth. This method employed Döbereiner's medium lacking yeast extract but containing $(NH_4)_2SO_4$ and 1.5% agar (purified, Difco). To 100 ml of molten medium (45°C) was added 0.1 ml of a washed suspension (24 Klett units turbidity, with blue filter and 16 mm cuvettes) of the bacteria. The medium was then dispensed into Petri dishes and allowed to solidify. Crystals of various growth factors were placed on the agar, and the plates were incubated at 37°C. Very large zones of growth of yeast extract-requiring strains occurred around crystals of biotin. Small zones of growth occurred around crystals of pyridoxal; however, this compound was later found not to be a necessary requirement for growth.

A more definitive method for demonstrating the biotin requirement was later developed. The medium employed had the following composition (g/l): K_2HPO_4. 0.5; succinic acid, 5.0; $FeSO_4.7H_2O$, 0.01; $NaMoO_4.2H_2O$,

0.002; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $CaCl_2.2H_2O$, 0.026; $(NH_4)_2SO_4$, 1.0. The pH was adjusted to 7.0 with KOH pellets. Media with and without biotin (0.0001 g/l) were prepared. Cultures grown in PSS broth were inoculated into 25 ml of 1/4X nutrient broth (Difco) and incubated at 37°C for 48 h. The cells were harvested by centrifugation at 4000 X g, washed twice in 10 ml of sterile distilled water, and suspended in distilled water to a turbidity of 20 Klett units (blue filter, 16 mm cuvettes; in 1 cm flat-faced cuvettes, this turbidity gave an absorbance of 0.09 at 420 nm). One-tenth ml of this suspension was used to inoculate each 5 ml volume of medium (contained in 16 mm test tubes). Incubation was for 48 h at 37°C. In cases where 50 ml slight growth occurred in the medium lacking biotin, compared to much heavier growth in the medium with biotin, a second serial transfer was made to media with and without biotin to confirm the requirement. In cases where no growth occurred in the medium without biotin, but did occur in the medium with biotin, no further transfers were made.

Sole carbon sources. The medium used for determination of sole carbon sources had the following composition (g/l): K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.1; $FeCl_3.6H_2O$, 0.0047; $MnSO_4.H_2O$, 0.0025; $CaCO_3$, 0.001; $ZnSO_4.7H_2O$, 0.00072; $CuSO_4.5H_2O$, 0.000125; $CoSO_4.7H_2O$, 0.00014; H_3BO_3, 0.000031; $Na_2MoO_4.2H_2O$, 0.000245; and agar (purified, Difco), 15.0. Before adding the $CaCO_3$ and biotin, the medium was acidified to pH 2.5 with HCl to dissolve precipitates, and was then brought to pH 7.0 with KOH. Cells were prepared as described above for the biotin determination (second method), except

that the final suspension had a turbidity of 30 Klett units (or an absorbance of 0.125 in a 1 cm flat-faced cuvette at 420 nm). Two ml of this suspension was added to a petri dish and mixed with 20 ml of molten (45°C) medium. After the medium had solidified, sterile discs (7.0 mm) punched from Beckman electrophoresis paper (Cat. No. 319328) were dipped into 5% solutions of carbon sources which had been previously sterilized by filtration. (Organic acids such as succinic acid had been adjusted to pH 7 with KOH before use.) The paper discs were then placed near the periphery of the agar plates, 3 discs per plate. The plates were then incubated at 37°C for 72 h. Any visible zone of turbidity around a disc, as judged with the naked eye when the plates were held up to a light source against a dark background) constituted a positive growth response.

Other biochemical tests. The following characterization tests were performed according to Hylemon et al. (24): catalase, oxidase, phosphatase, urease, starch hydrolysis, esculin hydrolysis, ability to grow anaerobically with nitrate, nitrate reduction, gelatin liquefaction, and tolerance to 1% glycine or 3% NaCl. The indol test was performed by culturing cells in 1% tryptone (Difco) plus 0.1% tryptophan; after 48 h, 1 ml of ethyl ether was mixed with the culture and allowed to form a layer at the top. Ehrlich's aldehyde was allowed to run down the inside of the tube, forming a layer just beneath the ether. The occurrence of a red color in the aldehyde layer constituted a positive test. Cultures of E. coli were used as controls.

Deoxyribonucleic acid (DNA) base composition. For determination of the DNA base compositions of various strains, high molecular weight DNA was prepared according to the method of Marmur and Doty (34). Log phase cells of the bacterial strains were harvested by centrifugation and suspended in 1X saline-EDTA buffer (NaCl, 0.15 M, ethylene diamine tetra-acetic acid (EDTA), 0.01 M, pH = 8.0). Cells from 2500 ml of PSS broth cultures were suspended in 150 ml of the buffer.

(i) The cells were lysed by addition of sodium lauryl sulfate (SLS) to a final concentration of 1%. After addition of the SLS, the suspension was agitated gently in a waterbath at 60°C until lysis was complete (usually ca. 10 min).

(ii) Liquefied phenol (90% aqueous phenol) was added to the lysate (0.25 to 0.33 volumes of phenol to 1.0 volume of lysate). The mixture was shaken on a wrist action shaker for 5 - 10 min and was then cooled in an ice bath to reduce the solubility of the phenol in the aqueous phase.

(iii) The mixture was centrifuged at 10,000 to 15,000 X g for 10 min. The aqueous layer was decanted and saved, while the phenol layer with extracted protein was discarded.

(iv) The phenol extraction was repeated for the aqueous layer.

(v) Two volumes of ice-cold ethanol were added to the lysate to precipitate the DNA. The mixture was poured into a perforated polypropylene centrifuge tube, allowing the precipitated DNA to be caught in the tube and the buffer-ethanol mixture to drain off. This step removed much of the SLS and phenol. The DNA was dissolved in 0.1X SSC

(1X SSC = NaCl, 0.15 M; sodium citrate, 0.015 M; pH = 7.0) during overnight incubation in a refrigerator. The ionic strength of the solution was then increased by addition of 20X SSC, to give a final concentration of 1X SSC.

(vi) Step (v) was repeated twice. The DNA from the final alcohol precipitation was dissolved in 25 ml of 0.1X SSC, and 0.5 ml of ribonuclease ("5X recrystallized pancreatic ribonuclease," Sigma, 5 mg/ml, heated previously at 80°C for 20 min) was added. The solution was incubated at 37°C for 1 h. The ionic strength of the solution was then increased to 1X SSC.

(vii) Chloroform-octonol solution (3% 1-octonol in chloroform) was added (0.25 volume to 1.0 volume of DNA solution) to precipitate additional protein. The mixture was then shaken for 20 min on a wrist action shaker and the protein was removed by centrifugation at 10,000 X g. The aqueous layer was decanted and saved.

(viii) The DNA was again precipitated with cold ethanol. It was then spooled onto an etched glass rod and drained.

(ix) The DNA was dissolved in 5 ml of 0.1X SSC and stored in a freezer until needed.

(x) A final concentration of 50 µg/ml was prepared in 0.025M sodium phosphate buffer containing 0.01% SLS. The preparations were dialyzed overnight in this buffer together with the DNA from E. coli B (a reference DNA).

(xi) Melting point (Tm) values were determined using a Gilford thermospectrophotometer containing a temperature sensor in the

cuvette. The mol% G + C values were calculated by the formula of Marmur and Doty (34):

$$\text{mol\% G + C} = \frac{\text{Tm} - 69.3}{0.41}$$

The Tm value of E. coli B (90.5°C) was used to normalize the Tm values of the test strains.

Preparation of DNA for DNA homology experiments. In some early DNA homology experiments, DNA for binding to filters was prepared by the method of Marmur and Doty (34) as described in the previous section. However, for most experiments DNA for binding to filters was prepared by a method developed by Dr. J. L. Johnson, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg (personal communication). Competitor DNA and radioactive DNA were also prepared by this method. The details of the method are as follows.

(i) Harvested cells were suspended in 20 ml of borate buffer (NaCl, 0.1 M; EDTA, 0.01 M; sodium borate, 0.1 M; pH = 8.0) in a 250 ml flask.

(ii) One ml of 20% SLS was added and the flask swirled in a waterbath at 60°C until lysis was complete.

(iii) The preparation was subjected to sonic oscillation at 10,000 cps (Raytheon Model DF 101) for 3 15-sec bursts at 60% power.

(iv) Five-tenths ml of pancreatic RNase (see previous section) was added and the solution incubated at 37°C for 1 h.

(v) Five ml of liquefied phenol was added and the mixture shaken on a wrist action shaker for 20 min. The mixture was centrifuged at

10,000 X g for 10 min and the aqueous layer was collected. The phenol extraction was then repeated on the aqueous layer.

(vi) One ml of 1 M sodium phosphate buffer (PB), pH 6.8, was added per 20 ml of lysate to give a final concentration of 0.05 M phosphate buffer.

(vii) Two g of dry hydroxylapatite (HA, Bio-Gel DNA grade) were then added. The flask was swirled quickly to suspend the HA. The suspension was then incubated on a waterbath shaker at 55°C for 1 h. This allowed the DNA to adsorb to the particles of HA.

(viii) The HA was centrifuged at 4000 X g for 5 min. The supernatant fluid was decanted and saved for a second adsorption later. To the HA pellet was added 5 ml of 0.05 M PB and the mixture was agitated rapidly with a Vortex mixer until the HA was re-suspended. An additional 25 ml of 0.05 M PB was then added rapidly from a syringe. The mixture was centrifuged at 4000 X g for 5 min and the supernatant discarded. The HA was re-suspended and centrifuged repeatedly until the supernatant fluid no longer showed an absorbance at 270 nm, indicating removal of phenol and ribonucleotides. (Usually 7 washes of the HA were sufficient.)

(ix) To the final pellet of HA was added 5 ml of 1.0 M PB, resulting in elution of the DNA from the HA. The HA was collected by centrifugation and used for adsorption of the DNA from the supernatant fluid saved in step (viii) above. The DNA from this supernatant was later combined with that from the first adsorption.

Preparation of competitor DNA. DNA isolated by the HA method described above was sonicated for 3 bursts of 5 sec each at 60%

power in a Raytheon Model DF 101 sonic oscilator. The phosphate was then removed by dialysis with a large excess of 0.1X SSC for 24 h. The concentration of DNA was then determined by dividing its adsorbance at 260 nm by the specific extinction coefficient 20. The total amount of DNA was calculated by multiplying the concentration by the volume. The ionic strength was then raised to 1X SSC by addition of 20X SSC. Two volumes of ice-cold ethanol were added and the mixture was incubated in a freezer for 2 h. The DNA was then centrifuged and suspended in sufficient 0.001 M HEPES buffer (N-2-hydroxyethylpiperazone-N'-2-ethanesulfonic acid) containing 0.02 M NaCL to give a final DNA concentration of 3 mg/ml. NaOH was added to the DNA solution at a final concentration of 0.25 M (0.05 ml of 5.0 M NaOH per ml of DNA solution). The solution was incubated at 45°C for 15 min to allow denaturation of the DNA and also hydrolysis of any residual RNA. The solution was then cooled quickly in an ethanol-ice bath and neutralized with an equivalent volume of 5.0 M HCl. The solution was dialyzed with a large excess of cold 2.2X SSC (changed 3 times) overnight. The DNA was placed in 75 X 16 mm screw-capped test tubes and adjusted to a final concentration of 1.5 mg/ml with 2.2X SSC. The DNA was frozen until needed.

Preparation of radioactive homologous DNA. Cultures were grown with ^{32}P or ^{33}P (1 mCi per 40 ml of PSS broth). The use of isotopes of P was necessitated by the finding that a number of tritiated precursors of DNA synthesis (viz., thymine, thymidine, adenine, and deoxyadenosine) failed to become incorporated into the DNA of strain Sp 7 in sufficient amounts to yield a sufficiently high specific activity. The DNA was extracted from the cells by the HA method

described above and dialyzed with 0.1X SSC overnight. The concentration and radioactivity of the DNA were determined, and the specific activity of the DNA (cpm/μg) was calculated. The DNA was denatured just prior to an experiment by heating it in 0.1X SSC at 100°C for 20 min.

Preparation of DNA filters. The binding of long-strand (Marmur and Doty method) or short-strand (HA method) DNA to nitrocellulose membrane filters involved the slow filtration of denatured DNA through the filters. At proper buffer concentrations the single-stranded DNA will bind irreversibly to the filters. The filters used were S & S B6 nitrocellulose filters, 15 cm diameter. These were placed on the surface of distilled water, then submerged and soaked in water for 1 - 2 h. Double-stranded DNA (50 μg/ml in 0.1X SSC) was denatured by heating in a boiling water bath for 5 min. It was then colled quickly by making a 10-fold dilution in ice-cold 6X SSC. The filters were placed on a suction apparatus and distilled water, followed by 500 ml of 6X SSC, was drawn through. This was followed by 870 ml of DNA solution (containing a total of 4.37 mg DNA). The filters were washed with 500 ml of cold 6X SSC, placed on an absorbent towel, and dried overnight at 60°C. For DNA homology experiments, pieces of a filter were punched out with a Keysort punch (McBee Systems, No. 5203); the punched out pieces were lozenge shaped and were 3 X 9 mm.

As indicated previously, DNA filters prepared from DNA isolated by the HA method were used for most of the DNA homology experiments; however, filters prepared with DNA isolated by the Marmur and Doty method were used for some early experiments. The two types of

DNA filters were compared with respect to 3 strains of S. lipoferum in a DNA homology experiment. The DNA homology values obtained with DNA filters prepared with DNA isolated by the Marmur and Doty method were 93%, 79%, and 38% for strains 119A4, 73B3, and RG 9c, respectively. The corresponding DNA homology values for the same strains using filters made with DNA prepared by the HA method were 94, 82, and 34. Because these values were similar to the previous values, it was concluded that it made little difference which type of DNA filter was used in the DNA homology experiments.

DNA homology experiments. Just prior to a DNA homology experiment, the 3 X 9 jm DNA filters were pre-incubated for ½ to 2 h at 73°C in the following mixture:

Bovine serum albumin (Fraction V).....0.03%

Polyvinyl pyrrolidone (PVP).....0.02%

Ficol.....0.02%

The purpose of the pre-incubation was to prevent non-specific binding of DNA to the nitrocellulose.

(i) Direct binding experiments. Some filters were incubated for 15 h at 73°C in a mixture consisting of 10 µl of denatured radioactive homologous DNA (ca. 1 µg) and 100 µl of 2.2X SSC. This was to determine the extent of binding of homologous DNA in the absence of any competitor DNA.

(ii) Competition experiments. Filters were incubated at 73°C for 15 h in a mixture consisting of 10 µl of radioactive homologous DNA and either 50 µl of competitor DNA (ca. 75 µg) plus 50 µl of

2.2X SSC, or 100 µl of competitor DNA (150 µg) plus no additional 2.2X SSC.

(iii) At the end of the incubation period for the direct binding and competition experiments, the filters were removed from their individual reaction vessels, washed in 2X SSC at 73°C using 2 300 ml volumes of the buffer, and dried on an absorbent towel under a heat lamp. The filters were then skewered on insect pins in duplicate, placed in scintillation vials containing a toluene-soluble fluor, and counted for radioactivity in a liquid scintillation counter.

(iv) The % DNA homology values were obtained by dividing the depression in binding caused by the heterologous unlabeled DNA fragments by the depression in binding resulting from the homologous unlabeled DNA fragments and multiplying by 100.

RESULTS

DNA base composition. In our initial studies, we wished to determine the overall DNA base composition of a number of strains isolated from different regions and different plants. If significant differences occurred in DNA base composition, this alone would be strong evidence for the existence of more than one species. The melting points (Tm) and mol% G + C values for 11 strains are presented in Table 4. As indicated, the G + C values were all ca. 70%, and this similarity indicated that the strains could possibly, but not necessarily, all belong to a single species. It should be noted that in the case of strain Sp USA 5b, comparable values were obtained with DNA prepared either by the Marmur and Doty method or by the HA method.

DNA homology experiments. The results of the DNA homology experiments are presented in Table 5. It can be seen that when strain Sp 7 was used as the reference strain, the other strains fell into 2 distinct but related groups: those strains having ca. 30 - 50%. In the reciprocal situation where strain Sp 59B was used as the reference strain, those strains which had exhibited low homology with Sp 7 now exhibited high homology with Sp 59B. Conversely, the strains which had had high homology with Sp 7 now exhibited low homology with Sp 59b.

TABLE 4. Melting points (Tm values) and mol% G + C values for the DNA of 11 strains of the S. lipoferum group

Strain	Average Tm, °C	Mol% G + C (± 1%)
Sp 7	98.1	70
Sp 13	98.0	70
Sp 4	97.8	70
Sp 35	98.2	70
Sp M82	98.4	71
Sp M75	98.2	70
Sp 51e	98.2	70
Sp T60	98.4	71
Sp 59b	97.9	70
Sp RG6xx	97.8	70
Sp USA 5b	97.6, 97.8[a]	69, 70[a]

[a]In the case of the second value given, the DNA was prepared by the HA method rather than by the method of Marmur and Doty.

TABLE 5. DNA homology values for strains of the *S. lipoferum* group

Strain	DNA homology values (%)	
	Reference = Sp 7	Reference = Sp 59b
Sp 7	100	34
Sp 4	102	38
Sp 13	94	32
Sp 13t	103	47
Sp 34	81	43
Sp 35	74	42
Sp 51e	71	41
Sp 52	85	44
Sp T60	85	30
Sp 67	80	30
Sp M75	71	49
Sp 80	84	52
Sp 81	73	41
Sp M82	73	41
Sp P1	72	46
Sp P2	82	52
Sp F4	80	35
Sp F6	74	40
Sp Br 8	84	37
Sp Br 11	67	38
Sp Br 13	72	41
Sp Br 14	81	38
Sp Br 21	80	Not done
Sp A2	92	50
Sp A7	96	33
Sp A8	81	42
Sp Mt 20	82	39
Sp Mt 21	81	35
Sp L69	83	49
Sp Col 1c	80	34
Sp RG 8a	104	37
Sp RG 16a	77	35
Sp RG 20b	73	32
JM 6A2	78	49
JM 6B2	87	48
JM 24B4	83	48
JM 28A2	91	46
JM 52B1	86	40

TABLE 5. (continued)

Strain	DNA homology values (%)	
	Reference = Sp 7	Reference = Sp 59b
JM 73B3	81	45
JM 73C2B	83	49
JM 72C3	79	40
JM 75A1	77	45
JM 82A1	90	45
JM 119A4	91	47
JM 125A2	88	52
Cd	100	49
Sp 59b	31	100
Sp USA 5b	36	70
Sp RG6xx	36	73
Sp RG 8c	34	72
Sp RG 9c	35	73
Sp RG 18b	39	73
Sp RG 19a	28	72
Sp RG 20a	37	76
Sp Col 2b	43	72
Sp Col 3	34	76
Sp Col 5	46	73
Sp Br 10	38	74
Sp Br 17	30	73
Sp A3a	29	70
Sp S1a	34	73

These results provide strong evidence that two distinct but related species existed, and also that the two species belonged together in the same genus.

DNA homology experiments were also performed with a variety of organisms from other genera which had certain similarities in physiology and/or morphology. These results are presented in Table 6. If significant DNA homology were to occur between S. lipoferum and a recognized member of an established genus, this would constitute strong evidence for assigning S. lipoferum to that genus. We failed to obtain any DNA homology values greater than ca. 20% with the organisms listed. We consider such values to have little significance (25). In fact, DNA homology studies are most useful at the species level of classification, not the genus level, and lack of significant DNA homology would not preclude assignment of an organism to a particular genus.

Characteristics correlated with the two homology groups. Among the S. lipoferum strains, we found certain characteristics that were correlated with the two DNA homology groups. These characteristics are indicated in Table 7. It should be noted that especially that Group II strains appeared to possess some fermentative ability, as judged from their acidification of glucose or fructose media anaerobically, an ability to exhibit slight growth in sugar broth anaerobically and to form a small amount of gas (less than ca. 5% of the volume of the gas vials), and an ability to form minute colonies on sugar agar plates anaerobically. Yet Group II strains

TABLE 6. DNA homology values obtained for organisms of various genera when *S. lipoferum* strains were used as reference strains

Genus and species	ATCC no.	mol% G + C	DNA homology values (%)	
			reference = Sp 7	Reference = Sp 59b
Pseudomonas testosteroni	11996	61[b]	4	-2
Pseudomonas acidovorans	15668	67[c]	20	14
	15667	67[c]	1	1
Aquaspirillum metamorphum	15280	63[d]	-9	13
Aquaspirillum peregrinum[a]	15387	62[d]	22	11
Aquaspirillum fasciculus[a]	29048	65[e]	5	Not done
	27740	62[e]	16	11
	29049	64[e]	11	Not done
Vibrio percolans	8461	65[b]	-4	1
Derxia gummosa[a]	15994	70[f]	2	-6
	15995	70[f]	0	20
Azomonas insignis[a]	12523	58[g]	10	1
Azomonas macrocytogenes[a]	12335	59[g]	6	7

[a]Nitrogen-fixers.

[b-g]See references 23, 33, 24, 52, 5, and 29, respectively.

TABLE 7. Distinction between DNA homology groups on the basis of phenotypic characters

Test	% of strains positive	
	Group I	Group II
Acid from glucose	0	100
Acid from mannitol, ribose, and sorbitol	0	80[a]
Acid from glucose and fructose in anaerobic hydrogen jar (slight growth also occurs)	0	100
Biotin required for growth	0	100
In semi-solid N-free malate medium + 0.005% yeast extract, cells tend to become wider, longer (often S or spirillum-shaped), and non-motile[b]	0	100
Sole C sources (by disc test)		
α-Ketoglutarate	0	100
Mannitol	0	80, + 7% variable[c]
Sorbitol	0	67, + 13% variable[d]
Ribose	0	40, + 27% variable[e]
Glucose	9[f]	100

[a]Negative strains = Br 10, Br 17, and Col 3.

[b]See Figures 4 and 5.

[c]Negative strains = Br 10 and Br 17. Variable strains = Col 3.

[d]Negative strains = Br 10, Br 17, and Col 5. Variable strains = RG6xx and RG 8c.

[e]Negative strains = Br 10, Br 17, USA 5b, Col 5, and RG6xx. Variable strains = Rg 9c, Col 2b, Col 3, and 59b.

[f]Positive strains = T60, 67, 34, and F6.

grew far better aerobically than anaerobically, and like Group I should be considered to have mainly a respiratory type of metabolism.

Another useful difference between the two groups has been found by Döbereiner (personal communication), who inoculated strains into semi-solid N-free medium containing vitamins but with glucose substituted for malate. Here, Group II strains grew very heavily near the surface of this medium, whereas Group I strains showed only slight growth or no growth. This observation was confirmed in our laboratory.

General characteristics of the S. lipoferum group. Concerning characteristics other than those previously mentioned, many of these are summarized in Table 8. When cultured for 24 h in PSS broth, both Group I and Group II strains exhibited a similar morphology (see Figure 4), viz. short, plump, slightly-curved motile rods, averaging 1.0 μm in diameter and having a typical range in length of 2.1 to 3.8 μm. Many cells had pointed ends, and poly-β-hydroxybutyrate granules were easily visible. When cultures grown in PSS broth were transferred to semi-solid malate medium + 0.005% yeast extract, Group II strains tended to become wider and longer, and many S-shaped or spirillum-shaped cells appeared in 24 or 48 h (see Figure 5). The long cells had a diameter of 1.4 to 1.7 μm and a length of 5 to over 38 μm. The long cells seemed to eventually undergo fragmentation into large pleomorphic misshapen cells filled with enormous amounts of large granules, probably PHB. Group II strains also tended to lose their motility in the semi-solid medium. In contrast, Group I

TABLE 8. Physiological characteristics of S. lipoferum strains

Test	% of strains positive	
	Group I	Group II
Oxidase, phosphatase, urease, esculin hydrolysis, anaerobic growth with nitrate	100	100
Starch and gelatin hydrolysis, production of water-soluble fluorescent pigment, indol test	0	0
Acid from fructose	100	100
Optimum Temperature of 36-38°C	100[a]	100[a]
Acid from lactose, maltose, sucrose, rhamnose, cellobiose, erythritol, dulcitol, and melibiose	0	0
Acid from the following sugars:		
arabinose	89, + 2% var[b]	100
galactose	76, + 4% var[c]	93[c]
i-inositol	0	13[d]
xylose	0, + 9% var[e]	80, + 7% var[e]

[a]11 strains were tested, 8 from Group I and 3 from Group II.

[b]Negative strains = F6, T60, 34, and P2. Variable strains = 67.

[c]Negative strains = Br 14, T60, 82A1, RG 16a, 51e, 34, 7, 4, P2, 24B4, 28A2, Col 2b. Variable strains = 67 and F4.

[d]Positive strains = 59b and Col 2b.

[3]In Group I, variable strains = 34, Br 11, 28A2, and 82A1. In Group II, negative strains = Col 3 and Br 10; variable strains = Br 17.

TABLE 8. (continued)

Test	% of strains positive	
	Group I	Group II
Catalase:		
strong	87	27
weak	13	40[f]
negative	0	33
Growth in presence of		
1% bile	98[g]	100
1% glycine	0	0
3% NaCl[h]	51 (very weak)	0
Reduction of nitrate to nitrite only[h]	60	67[i]
Reduction of nitrate beyond nitrite stage[h]	40	33
Sole carbon sources (disc method):		
succinate, L-malate, lactate, oxaloacetate, fumarate, β-hydroxybutyrate, gluconate, pyruvate, glycerol, fructose	100	100
propionate	98	93, + 7% var
galactose	65, + 15% var	80, + 13% var
arabinose	74, + 9% var	67, + 27% var
citrate	96, + 4% var	100
malonate	0	0

[f] In Group II, negative strains = RG 20a, Col 2b, Col 3, Br 10, and Sla.

[g] Negative strains = P2.

[h] In Group I, 43 strains were tested; in Group II, 3 strains were tested.

[i] In group II, 59b reduced nitrate beyond the nitrate stage, while RG6xx and USA 5b reduced nitrate only to nitrite. However, Neyra et al. (36) have shown that nearly all Group II strains are weak denitrifiers, based on ability to form nitrous oxide from nitrate.

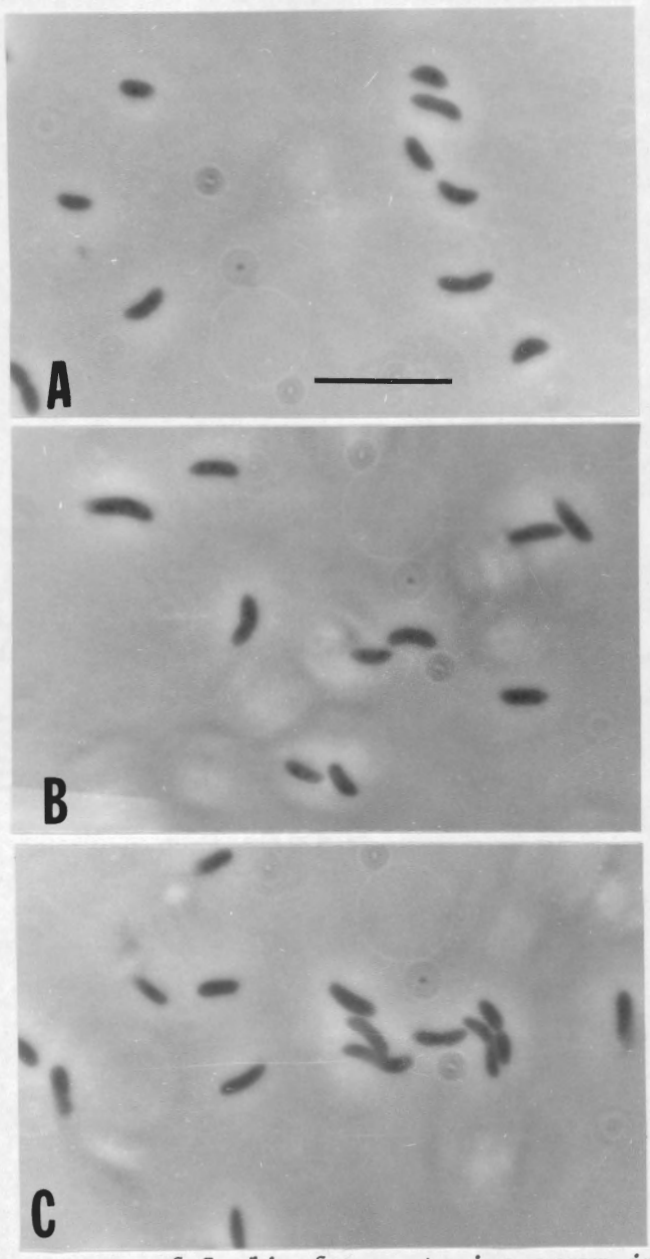

FIGURE 4. Appearance of S. lipoferum strains grown in peptone-succinate-salts broth for 24 h at 37°C. A, strain Sp 7. B, strain Sp 59b. C, strain Sp RG 20a. The bar represents 10 µm. Phase-contrast microscopy of wet mounts.

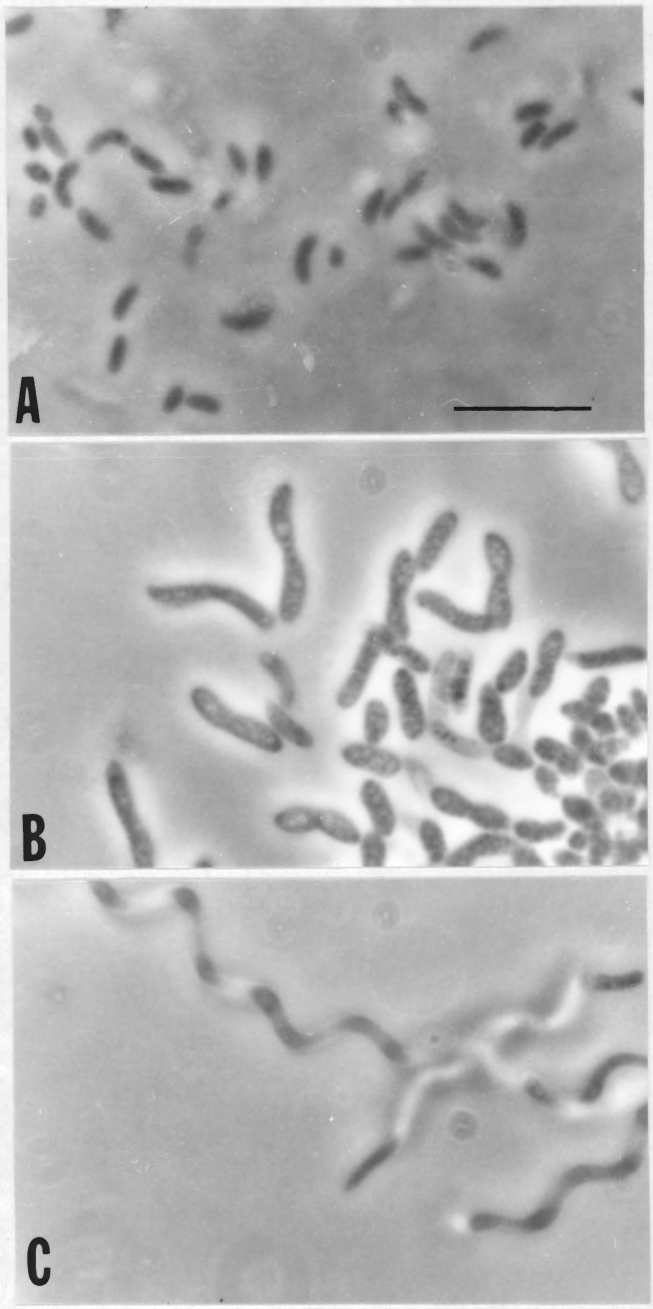

FIGURE 5. Appearance of S. lipoferum strains grown in semi-solid N-free malate medium + 0.005% yeast extract at 37°C. A, strain Sp 7 at 48 h. B, strain Sp 59B at 48 h. C, strain Sp RG 20 a at 24 h. The bar represents 10 µm. Phase-contrast microscopy of wet mounts.

strains retained motility for up to a month, and the cells tended to remain normal size. In old cultures, however, some S-shaped cells could be found (see reference 31).

One of the most interesting aspects of the strains was their type of flagellation. Until a short time ago, the cells were considered to be monotrichous, and this type of flagellation does indeed occur when the cells are cultured in liquid media such as PSS broth. However, when cultured on PSS agar at 30°C for 2 or 3 days, numerous lateral flagella of shorter wavelength occur in addition to the single polar flagellum (see Figure 6). This situation is similar to that described for Pseudomonas stutzeri (41), Vibrio parahaemolyticus (50), and Vibrio alginolyticus (8).

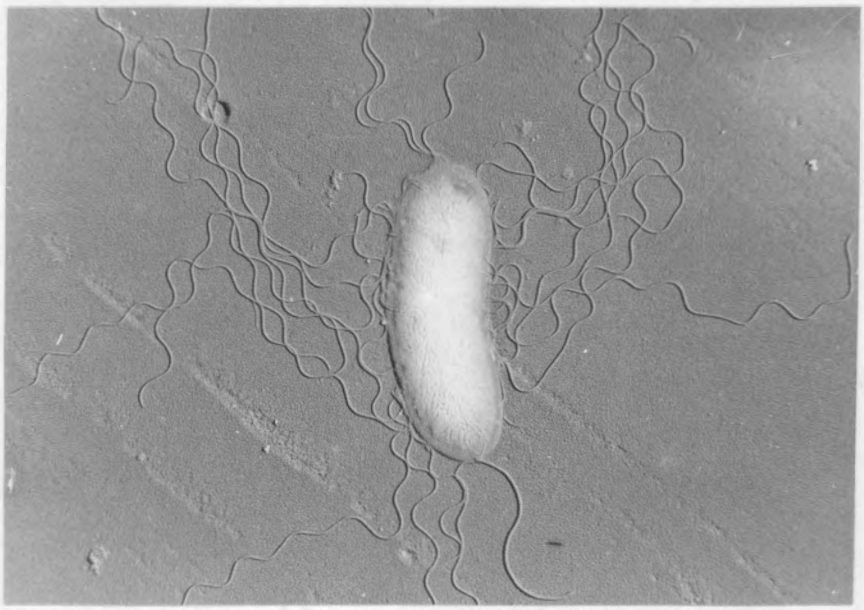

FIGURE 6a. Electron micrograph of strain Sp 7 grown on agar at 30°C, showing lateral flagella in addition to the single polar flagellum. 17,000 X.

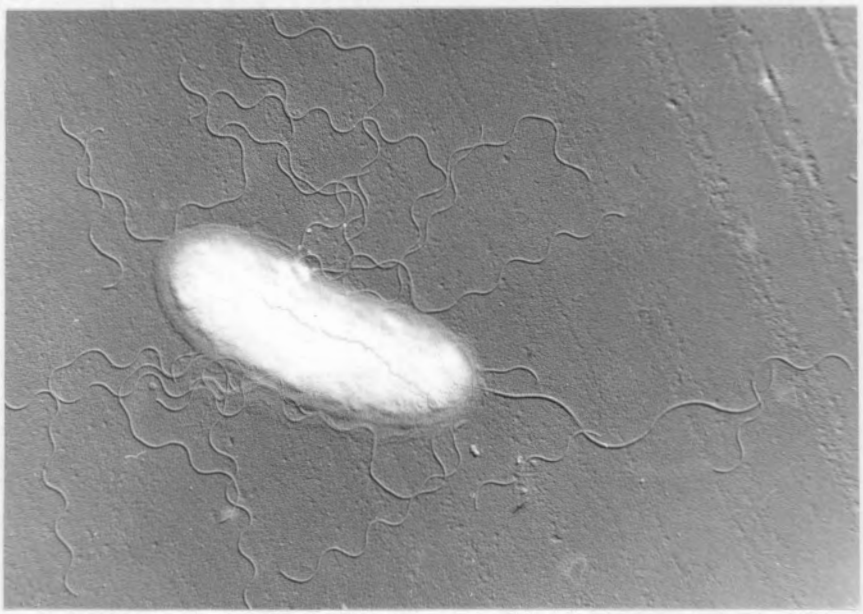

FIGURE 6b. Electron micrograph of strain Br 17 grown on agar at 30°C, showing lateral flagella in addition to the single polar flagellum. 17,000 X.

DISCUSSION

The results of the DNA homology experiments provide strong evidence that two distinct but related species exist within the S. lipoferum group, and also that the two species belong together within the same genus. Although none of the strains in Group II exhibits more than 76% homology to the reference strain Sp 59b, I consider that DNA homology values of ca. 70% or higher to be indicative of a sufficiently high degree of genetic relationship to Sp 59B as to justify considering Group II as a single species. There appear to be no apparent phenotypic differences between Sp 59b and the other Group II strains that would mitigate against this view. Within homology Group I there exists a continuum of homology values from 67 to 100%, and again no phenotypic characters appear to be uniquely correlated with higher or lower values. While there appears to be little correlation of phenotypic characteristics with homology values within Group I or Group II, there are several clear differences in phenotypic characteristics between the two homology groups, and these are listed in Table 7. These differential characteristics should enable investigators to determine to which of the two species a new isolate would belong, without the necessity of performing DNA homology experiments.

Due to the importance of denitrification for nitrogen-fixers in the soil, Neyra et al. (36) have grouped strains of S. lipoferum into 3 groups: (i) those which denitrify s=rongly, (ii) those which reduce nitrate only to nitrite, and (iii) those which denitrify weakly (with incomplete removal of the nitrite formed as an intermediate). Without DNA homology data, these groups might eventually have been considered as different species. However, the DNA homology data show this not to be the case. Whether or not the 3 denitrification groups represent subspecies is another question. One initial consideration is that the difference between weak denitrifiers and strong denitrifiers is a quantitative one rather than a qualitative one, and therefore probably of minor importance. This would leave only 2 groups, those that denitrify (either strongly or weakly) and those which reduce nitrate only to nitrite. Within DNA homology Group I, there would seem to be little correlation of these denitrification groups with homology values. For example, for those strains which reduce nitrate only to nitrite, homology values ran from 70% to 100% when the strains were tested against Sp 7. With regard to DNA homology Group II, the data in Table 8 seem to indicate that Sp 59b can denitrify, while two other strains having DNA homology values in the 70's with respect to Sp 59b did not denitrify. However, Döbereiner (personal communication) has indicated that these two strains, and also all other strains of Group II, are capable of at least weak denitrification. By the usual qualitative tests with sulfanilic acid and alpha-naphthylamine reagents this may not be apparent because some of the nitrite formed as an intermediate may

not disappear. The method used by Döbereiner was based on quantitative estimates of nitrite and also on detection of N_2O by gas chromatography. Consequently, I believe that the denitrification types described by Döbereiner and her colleagues should not be considered as subspecies. Instead, it seems preferable to refer to such groups as biotypes, as the term "biotype" implies no official taxonomic standing.

In view of the fact that a gap in homology values seems to occur within DNA homology Group II (most strains having homology values of 70 - 76% to Sp 59b, with no values intermediate between 76% and homologous 100% value), the possibility exists that two subspecies may occur within Group II: one subspecies would include Sp 59b, while the other would include the remaining strains. However, merely because a number of strains exhibit a similar DNA homology value compared to Sp 59b does not necessarily mean that these strains are all highly related to each other. This can only be determined by using one of the strains as a reference strain for additional DNA homology experiments, testing the other strains against it. Moreover, there appear to be no phenotypic characteristics uniquely associated with Sp 59b vs. the other members of Group II. Consequently, it would be best not to designate two subspecies within Group II at the present time, although it is possible that further DNA homology studies might ultimately support such a separation.

Although the DNA homology experiments indicate that the S. lipoferum group should be divided into two closely related species, the question still remains as to what genus would most appropriately contain these

species. If significant DNA homology were to occur between the S. lipoferum group and an established member of a particular genus, this would indicate that the S. lipoferum group should be assigned to that genus. As indicated previously, however, no such evidence was obtained in the present investigation (see Table 6). Nevertheless, lack of significant DNA homology cannot exclude assignment of an organism to a particular genus. DNA homology is in fact most useful at the species level, rather than the genus level, of taxonomy. It is possible that rRNA/DNA homology experiments would be more useful at the genus level, but as these have not been performed, assignment of the S. lipoferum group to an appropriate genus must presently be done on the basis of phenotypic characteristics.

The strains of the S. lipoferum group exhibit certain properties which are characteristics of a number of established genera. The following discussion indicates these genera and also certain species within the genera that resemble S. lipoferum to some extent. It will become evident from the discussion that it is very difficult to decide on the basis of phenotypic characters to which of these genera, if any, the S. lipoferum group should belong.

On the basis of their vibrioid and sometimes helical shape, and also their preference for the salts of organic acids as carbon sources, S. lipoferum was originally assigned to the genus Spirillum by Beijerinck (7). However, the genus Spirillum is presently reserved for very large, microaerophilic, helical organisms having bipolar tufts of flagella and whose mol% G + C is ca. 38 (i.e., organisms

resembling Spirillum volutans) (24). Consequently, the genus Spirillum is not appropriate for the S. lipoferum group.

Like S. lipoferum, Derxia gummosa is a nitrogen fixer and has a mol% G + C of 70. Derxia is strictly dependent on oxygen (like Group I of S. lipoferum) and has a respiratory type of metabolism. The cells have a single polar flagellum, have a cell diameter reportedly the same as that of S. lipoferum, and contain poly-β-hydroxybutyrate granules (5). Moreover, like most S. lipoferum strains, Derxia is isolated from tropical soils (South America, South Africa, and Java). These characteristics constitute a compelling reason to consider Derxia as a possible genus for the S. lipoferum group. However, a number of differences occur between S. lipoferum and Derxia. The cells of Derxia are described as straight rods rather than curved or helical. Lateral flagella have not been reported in Derxia. The cells of Derxia are said to "shrink" to a smaller diameter under nitrogen-fixing conditions in old cultures, whereas S. lipoferum does not show such shrinkage (and in fact the cells of Group II actually become larger). No association of Derxia with plants has been reported, in contrast to S. lipoferum. Unlike S. lipoferum, Derxia forms a gummy slime or capsule. Derxia is incapable of reducing nitrate even to nitrite, unlike S. lipoferum. Perhaps the most important difference is that Derxia is strictly aerobic, while Group II strains of S. lipoferum have some fermentative ability. Moreover, unlike S. lipoferum, Derxia is incapable of growing on the salts of succinic and malic acid. In view of these differences, I believe that the S. lipoferum group should not be assigned to the genus Derxia.

Because of the curvature of the cells of S. lipoferum, and their formation of S-shaped or even spirillum-shaped cells under certain conditions, it is possible that the genus Aquaspirillum (24) might be suitable for the S. lipoferum group. However, a number of considerations combine to make this unlikely. The mol% G + C for the genus ranges from 49 - 65, and the typical characteristics of the genus Aquaspirillum are as follows: (i) aerobic, possessing a strictly respiratory type of metabolism; (ii) oxidase positive; (iii) no growth in the presence of 3% NaCl; (iv) presence of bipolar tufts of flagella; (v) inability to oxidize or ferment carbohydrates; (vi) presence of intracellular poly-β-hydroxybutyrate granules; (vii) positive catalase and phosphatase reactions; (viii) no coccoid bodies formed in older cultures; (ix) inability to grow anaerobically with nitrate; and (x) a non-exacting nutrition (vitamins generally not required). Some exceptions to the above do occur in the genus, as follows (24). Three species can oxidize a very limited variety of carbohydrates, and can acidify certain sugar media under relatively anaerobic conditions (although visible growth under this condition is not evident). Three species can grow anaerobically with nitrate. Four species can form coccoid bodies in old cultures. One species has predominantly single polar flagella and is only slightly curved, and another species has predominantly bipolar single flagella. One species, although physiologically very similar to other aquaspirilla, is a straight rod. One species lacks phosphatase activity. Two species have a vitamin requirement (one of them requires biotin).

Two species exhibit nitrogenase activity. All of the above characteristics are regarded as being unusual or atypical.

S. lipoferum strains would be atypical for the genus Aquaspirillum with regard to the following: (i) a DNA base composition of 70 mol% G + C; (ii) ability to grow anaerobically with nitrate; (iii) having usually only a slightly curved shape; (iv) having only a single polar flagellum when grown in broth, or a polar flagellum plus a number of lateral flagella when grown on agar at 30°C; (v) ability to oxidize a number of carbohydrates, and, in the case of Group II, ability to acidify sugar media under anaerobic conditions and even to grow slightly; (vi) the requirement for biotin exhibited by Group II; (vii) the tolerance of some strains for 3% NaCl (although only slight growth occurs); (viii) the negative catalase reaction exhibited by some strains of Group II; (ix) an ability to fix nitrogen; and (x) an association with plant roots. None of these characteristics alone probably would be a firm basis for excluding S. lipoferum from the genus Aquaspirillum, but taken together they comprise a formidable combination of atypical characteristics. Perhaps the most important of them is the DNA base composition of S. lipoferum - 5% higher than for any species of Aquaspirillum. Because of the various considerations given above, I believe that the S. lipoferum group should not be included in the genus Aquaspirillum.

The genus Azomonas is a nitrogen-fixing genus consisting of aerobic, oxidative avoid cells (29). Two species, A. insignis and A. macrocytogenes, have polar flagella; the former has a tuft, while

the latter is monotrichous. This genus would not appear to be a suitable one for the S. lipoferum group for several reasons, but the most important reason is that the mol% G + C of the genus is only 53 - 59 (29).

Another nitrogen-fixing genus is Beijerinckia (4), which consists of straight, slightly curved, or pear-shaped rods with peritrichous flagella. The cells have intracellular poly-β-hydroxybutyrate granules. The organisms are strict aerobes and form a copious slime. They are not capable of denitrification. Like S. lipoferum, some of the species form colonies that have a light pink color. However, S. lipoferum has only polar flagella when grown in broth, can denitrify, and forms no slime. S. lipoferum cannot catabolize sucrose or any dissacharide, whereas all strains of Beijerinckia can catabolize this disaccharide. In contrast to S. lipoferum, the mol% G + C for the genus Beijerinckia ranges from 55 to 61.

The genus Azotobacter (28) consists of aerobic, ovoid cells which can fix nitrogen and which have a mol% G + C of 63 - 66. In contrast to S. lipoferum, the cells exhibit an extremely high respiratory rate which apparently functions to keep the interior of the cells anaerobic, thereby protecting the nitrogenase and allowing the cells to fix nitrogen aerobically. Also in contrast to S. lipoferum, Azotobacter forms cysts. Moreover, Azotobacter is reported to have only peritrichous flagella. Consequently, this genus would also seem to be unsuitable for inclusion of the S. lipoferum group.

The genus Rhizobium (30) consists of pleomorphic rod-shaped organisms having a mol% G + C of 59 - 66, a strictly respiratory type of metabolism, and intracellular poly-β-hydroxybutyrate granules. Some strains can reduce nitrate. Two groups of rhizobia occur: one group has peritrichous flagella, and the other has a polar or a subpolar flagellum. Although associated with the roots of plants, rhizobia are much more highly specialized than S. lipoferum for symbiotic nitrogen fixation. They invade root hairs and become intracellular, and eventually characteristic root nodules are formed. In the nodules, an oxygen-protection mechanism occurs, viz., leghaemoglobin. Also in contrast to S. lipoferum, rhizobia appear to be largely restricted to leguminous plants. Although certain strains of Rhizobium have been shown to be capable of nitrogenase activity in vitro, it is far more difficult to demonstrate this than with S. lipoferum.

On the basis of purely phenotypic characteristics, such as vibrioid shape, ability to reduce nitrate, the occurrence of peritrichous flagella in addition to a single polar flagellum, and ability to ferment as well as to oxidize sugars, the genus Vibrio (49) might seem to be most suitable for the S. lipoferum group. However, the mol% G + C for the genus Vibrio of 40 to 50 is far too low to permit inclusion of S. lipoferum in this genus.

Certain oxidative vibrioid bacteria have been included in the genus Comamonas (14, 48), represented by the type species C. terrigena

(Vibrio percolans). This organism has a mol% G + C of 64, has only polar flagella, and is incapable of catabolizing any sugar (23). It would thus appear that the genus Comamonas would not be appropriate for the S. lipoferum group.

Although the genus Pseudomonas is generally associated with straight rods, the definition of the genus indicates that curved rods (but not helically curved rods) can be included. Since S. lipoferum has mainly a vibrioid shape, it could not be excluded from the genus Pseudomonas because of its shape (although under certain conditions S-shaped or even spirillum-shaped cells can occur, especially with Group II.) The genus is defined by only a few characteristics: gram negative, motile by polar flagella, strict aerobes, and a mol% G + C of 58 - 70. Many of the species are capable of denitrification. There are presently no nitrogen fixers included in the principal list in Bergey's manual (21), but as has been indicated previously, nitrogenase activity probably carries little taxonomic importance. DNA/DNA and rRNA/DNA studies have indicated that the genus Pseudomonas is very heterogeneous and may deserve to be divided into at least 5 separate genera (42).

Of all the species of Pseudomonas, perhaps P. stutzeri resembles S. lipoferum to the greatest degree. The flagellation of P. stutzeri is similar to that of S lipoferum, with lateral flagella being formed in addition to a polar flagellum on solid media. Moreover, P. stutzeri is capable of denitrification. Although described as being straight rods, I observed many S-shaped cells in semi-solid PSS medium in

my laboratory, and the organism can apparently exhibit a vibrioid shape. Unlike S. lipoferum, however, P. stutzeri does not fix nitrogen and can catabolize disaccharides such as maltose and sucrose. The most compelling reason for not including S. lipoferum in the genus Pseudomonas, however, is that unlike P. stutzeri or any Pseudomonas species, Group II of S. lipoferum can acidify sugar media under anaerobic conditions and can even exhibit some growth. The definition of the genus Pseudomonas specifically excludes organisms having a fermentative metabolism. Even without this consideration, however, there is one other consideration which is admittedly subjective, but still compelling in a practical sense, for not including S. lipoferum in the genus Pseudomonas: all pseudomonads which are associated with plants appear to be phytopathogens. Although associated with plants, S. lipoferum is certainly not pathogenic, and indeed may be quite beneficial to the plants. To place S. lipoferum in the genus Pseudomonas could result in the implication for a government agriculture agency that the organism is a phytopathogen. The idea of inoculating fields with an organism that might be a phytopathogen would probably be met with some reservations. A further consideration is that the genus Pseudomonas is already very heterogeneous, and adding S. lipoferum to the list of Pseudomonas would make it even more so. For these reasons, I believe that Pseudomonas would not be appropriate for inclusion of the S. lipoferum group.

Although S. lipoferum appears to have no photosynthetic properties, there are some interesting similarities between it and Rhodospirillum

rubrum (44). For example, R. rubrum requires biotin, can grow aerobically in the dark, has a vibrioid to helical shape, and produces a pink pigment. Some members of the genus Rhodospirillum have a mol% G + C as high as 66. One of the most interesting similarities is that, except for S. lipoferum, R. rubrum is the only nitrogen fixer so far found to have a molybdenum-containing co-factor for the Mo-Fe protein (Component I) of its nitrogenase. Moreover, there appears to be a high degree of interchangeability in vitro between Components I and II in in vitro studies for S. lipoferum and R. rubrum. Yet, S. lipoferum strains 59b and 7 were found in the present investigation to be incapable of growth in deep, vaseline-sealed tubes of PSS agar under illumination. All species of Rhodospirillum are capable of using succinate as an electron donor, and thus if the organisms had been photosynthetic, they should have been able to grow under these conditions.

The genus Azospirillum gen. nov. It appears to me that the best course to pursue with regard to the generic placement of the S. lipoferum group would be to assign the organisms to a new genus, Azospirillum. I believe that this generic name would be suitable for the following reasons:

(i) The name Spirillum lipoferum, designated by Beijerinck in 1925, has become a familiar one since the work of Dögereiner and Day (18) in 1974, and the retention of the term "spirillum" in the new name would minimize confusion among soil microbiologists.

(ii) S. lipoferum strains, although admittedly mainly vibrioid in shape, can exhibit helical forms under certain conditions.

(iii) Certain spirilla, such as Aquaspirillum aquaticum, A. delicatum, A. metamorphum, and Oceanospirillum japonicum, generally have less than one helical turn, but are nonetheless included with other spirilla.

(iv) Like spirilla, S. lipoferum strains do have intracellular poly-β-hydroxybutyrate granules, have mainly a respiratory type of metabolism, and seem to prefer the salts of organic acids as carbon sources.

Description of the genus Azospirillum gen. nov. A·zo·spi·ril'·lum. French n. azote nitrogen; Gr. n. Spira a spiral; spirillum a small spiral; Azospirillum a small nitrogen spiral. Cells generally slightly-curved to S-shaped, ca. 1.0 μm in diameter. Motile by means of a single polar flagellum; on solid media at 30°C, numerous lateral flagella are also formed. Prominent intracellular granules of poly-β-hydroxybutyrate present. Slime not formed. Possess mainly a respiratory type of metabolism, but some fermentative ability may also be present. Oxidase positive. Grow well on salts of organic acids such as malate, succinate, pyruvate, or lactate; sugars are less satisfactory substrates. Optimum temperature, ca. 37°C. Isolated from soils and from surface-sterilized roots of various plants, especially grasses. Nitrogen fixers. The mol% G + C of the DNA is 69 - 71. The type species is Azospirillum lipoferum.

In the genus Azospirillum, two species would presently occur. Although Beijerinck's cultures are no longer in existence, it seems likely that his organisms belonged to Group II. Beijerinck referred to the development of his spirilla in solutions of glucose or mannitol inoculated with soil, although the spirilla were later displaced by overgrowth with Azotobacter and Clostridium. When malate was used as the carbon source, no such displacement occurred. Moreover, Beijerinck also provided a drawing of cells cultured in sugar medium (see Figure 1). Beijerinck's cells could also exhibit a helical shape (Figure 1). All of this strongly supports the idea that Group II organisms were involved. Consequently, I believe that the name Azospirillum lipoferum should be applied to Group II strains.

Azospirillum lipoferum. (li·po'·fe·rum. Gr. n. lipus fat; L. v. fero to carry; M. L. adj. lipoferus fat-bearing.) Capable of acidifying glucose medium. Requires biotin as a growth factor. Media containing ribose, mannitol, and sorbitol are usually acidified. Acidification of glucose or fructose medium occurs under anaerobic conditions; slight growth also occurs under this condition. In semi-solid nitrogen-deficient malate medium containing 0.005% yeast extract, cells tend to become wider (ca. 1.4 - 1.7 μm diameter), longer (often S-shaped or spirillum-shaped), and non-motile. The type strain is Sp 59b. This strain has been deposited with the American Type Culture Collection (ATCC) under the number 29707. Reference strains RG 20a and Br 17 have been deposited under the numbers 29708 and 29709, respectively.

In honor of the country of Brazil, where Döbereiner and Day isolated nitrogen-fixing organisms from the roots of tropical grasses in 1974, I believe that the species name brasilense would be suitable for the organisms belonging to DNA homology Group I.

Azospirillum brasilense. (bra·si·len' se pertaining to the country of Brazil, South America.) Acidification of glucose medium does not occur. Vitamins not required for growth. Media containing ribose, mannitol, and sorbitol are not acidified. No fermentative capacity present. In semi-solid nitrogen-deficient malate medium containing 0.005% yeast extract, cells do not become wider; they retain their motility. In old cultures, S-shaped cells may occur. The type strain is Sp 7 (ATCC 29145). Reference strains Cd and Sp 35 have been deposited with the ATCC under the numbers 29710 and 29711, respectively.

LITERATURE CITED

1. Albrecht, S., Y. Okon, J. Lunnquist, and R. H. Burris. 1976. Corn roots fix nitrogen. Crops and Soils Mag. 28: 16.

2. Barber, L. E., J. D. Tjepkema, S. A. Russell, and H. J. Evans. 1976. Acetylene reduction (nitrogen fixation) associated with corn inoculated with Spirillum. Appl. Environ. Microbiol. 32: 108 - 113.

3. Becking, J. H. 1963. Fixation of molecular nitrogen by an aerobic Vibrio or Spirillum. Antonie van Leeuwenhoek J. Microbiol. Serol. 29: 326.

4. Becking, J. H. 1974. The genus Beijerinckia, p. 256 - 260. In R. E. Buchanan and N. E. Gibbons (ed), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

5. Becking, J. H. 1974. The genus Derxia, p. 260 - 261. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

6. Beijerinck, M. W. 1922. Azotobacter chroococcum als indikator van de vruchtbarrheid van den grond. K. Ned. Akad. Wet. Versl. Gewone Vergad. Afd. Natuurkd. 30: 431 - 438.

7. Beijerinck, M. W. 1925. Uber ein Spirillum, welches freien Stickstoff binden kann? Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 63: 353 -359.

8. Boer, W. E., C. Golten, and W. A. Sheffers. 1975. Effects of some chemical factors on flagellation and swarming of Vibrio alginolyticus. Antonie va. Leeuwenhoek 41: 385 - 403.

9. Breed, R. S. 1957. The genus Spirillum, p. 253 - 257. In R. S. Breed, E. G. D. Murray, and N. R. Smith (ed.), Bergey's manual of determinative bacteriology, 7th ed. The Williams and Wilkins Co., Baltimore.

10. Brenner, D. J. 1973. Deoxyribonucleic acid reassociation in the taxonomy of enteric bacteria. Int. J. Syst. Bacteriol. 23: 298 - 307.

11. Brill, W. J. 1977. Biological nitrogen fixation. Sci. Amer. 236: 68 - 81.

12. Cato, E. P., and J. L. Johnson. 1976. Reinstatement of species rank for Bacteroides fragilis, B. ovatus, B. distasonis, B. thetaiotaomicron, and B. vulgatus: designation of neotype strains for Bacteroides fragilis (Veillon and Zuber) Castellani and Chalmers and Bacteroides thetaiotaomicron (Distaso) Castellani and Chalmers. Int. J. Syst. Bacteriol. 26: 230 - 237.

13. Dalton, H. 1974. Fixation of dinitrogen by free-living microorganisms. CRC Crit. Rev. Microbiol. 3: 183 -220.

14. Davis, G. H. G., and R. W. A. Park. 1962. A taxonomic study of certain bacteria currently classified as Vibrio species. J. Gen. Microbiol. 27: 101 - 119.

15. Day, J. M., and J. Döbereiner. 1976. Physiological aspects of N_2-fixation by a Spirillum from Digitaria roots. Soil Biol. Biochem. 8: 45 - 50.

16. DeLey, J., and I. W. Park. 1966. Molecular biological taxonomy of some free-living nitrogen-fixing bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. 32: 6 - 16.

17. De-Polli, H., E. Matsui, J. Döbereiner, and E. Salati. 1977. Confirmation of nitrogen fixation in two tropical grasses by $^{15}N_2$ incorporation. Soil Biol. Biochem. 9: 119 - 123.

18. Döbereiner, J., and J. M. Day. 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In W. E. Newton and C. J. Nymans (ed.), Symposium on nitrogen fixation, Washington State University Press, Pullman 2: 518 - 538.

19. Döbereiner, J., I. E. Marriel, and M. Nery. 1976. Ecological distribution of Spirillum lipoferum Beijerinck. Can. J. Microbiol. 22: 1464 - 1473.

20. Doi, R. H., and R. T. Igarashi. 1965. Conservation of ribosomal and messenger ribonucleic acid cistrons in Bacillus species. J. Bacteriol. 90: 384 - 390.

21. Doudoroff, M., and N. J. Palleroni. 1974. The genus Pseudomonas, p. 217 - 243. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

22. Evans, H. J., and L. E. Barber. 1977. Biological nitrogen fixation for food and fiber production. Science 197: 332 - 339.

23. Hugh, R. 1965. A comparison of Pseudomonas testosteroni and Comamonas terrigena. Int. Bull. Bacteriol. Nomencl. Taxon. 15: 125 - 132.

24. Hylemon, P. B., J. S. Wells, Jr., N. R. Krieg, and H. W. Jannasch. 1973. The genus Spirillum: a taxonomic study. Int. J. Syst. Bacteriol. 23: 340 - 380.

25. Johnson, J. L. 1973. Use of nucleic acid homologies in the taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. 23: 308 - 315.

26. Johnson, J. L., and C. S. Cummins. 1972. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and strains of Archnia propionica. J. Bacteriol. 109: 1047 - 1066.

27. Johnson, J. L., and B. S. Francis. 1975. Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. J. Gen. Microbiol. 88: 229 - 244.

28. Johnstone, D. B. 1974. The genus Azotobacter, p. 254 - 255. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

29. Johnstone, D. B. 1974. The genus Azomonas, p. 255 - 256. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

30. Jordan, D. C., and O. N. Allen. 1974. The genus Rhizobium, p. 262 - 264. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

31. Krieg, N. R. 1976. Biology of the chemoheterotrophic spirilla. Bacteriol. Rev. 40: 55 - 115.

32. Kumari, L. M., S. K. Kavimandan, and N. S. Subba Rao. 1976. Occurrence of nitrogen-fixing Spirillum in roots of rice, sorghum, maize, and other plants. Ind. J. Exptl. Biol. 19: 638 - 639.

33. Mandel, M. 1966. Deoxyribonucleic acid base composition in the genus Pseudomonas. J. Gen. Microbiol. 43: 273 - 292.

34. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. 5: 109 - 118.

35. Neyra, C. A., and P. van Berkum. 1977. Nitrate reduction and nitrogenase activity in Spirillum lipoferum. Can. J. Microbiol. 23: 306 - 310.

36. Neyra, C. A., J. Döbereiner, R. Lalande, and R. Knowles. 1977. Denitrification by N_2-fixing Spirillum lipoferum. Can. J. Microbiol. 23: 300 - 305.

37. Normore, W. M. 1973. Guanine-plus-cytosine (GC) composition of the DNA of bacteria, fungi, algae, and protozoa. In A. I. Laskin and H. A. Lechevalier (ed.), CRC Handbook of microbiology, Vol. II. CRC Press, Cleveland.

38. Okon, Y., S. L. Albrecht, and R. H. Burris. 1976. Factors affecting growth and nitrogen fixation of Spirillum lipoferum. J. Bacteriol. 127: 1248 - 1254.

39. Okon, Y., S. L. Albrecht, and R. H. Burris. 1976. Carbon and ammonia metabolism of Spirillum lipoferum. J. Bacteriol. 128: 592 - 597.

40. Palleroni, N. J., R. W. Ballard, E. Ralston, and M. Doudoroff. 1972. Deoxyribonucelic acid homologies among some Pseudomonas species. J. Bacteriol. 110: 1 - 11.

41. Palleroni, N. J., M. Doudoroff, and R. Y. Stanier. 1970. Taxonomy of the aerobic pseudomonads: the properties of the Pseudomonas stutzeri group. J. Gen. Microbiol. 60: 215 - 231.

42. Palleroni, N. J., R. Kunisawa, R. Contopoulou, and M. Doudoroff. 1973. Nucleic acid homologies in the genus Pseudomonas. Int. J. Syst. Bacteriol. 23: 333 - 339.

43. Pecknold, P. C., and R. G. Grogan. 1973. Deoxyribonucleic acid homologies among phytopathogenic Pseudomonas species. Int. J. Syst. Bacteriol. 23: 111 - 212.

44. Pfennig, N., and H. G. Trüper. 1974. The genus Rhodospirillum, p. 26 - 29. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

45. Ravin, A. W. 1963. Experimental approaches to the study of bacterial phylogeny. Amer. Natur. 97: 307 - 318.

46. Rogerson, A. C. 1977. Nitrogen fixation in grass-Spirillum systems. Science 195: 1362.

47. Schröder, M. 1932. Die Assimilation des Luftstickstoff durch einige Bakterien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 85: 177 - 212.

48. Sebald, M., and M. Véron. 1963. Teneur en bases de l'ADN et classification des vibrions. Ann. Inst. Pasteur Paris 105: 897 - 910.

49. Shewan, J. M., and M. Véron. 1974. The genus Vibrio, p. 340 - 345. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

50. Shinoda, S., and K. Okamoto. 1977. Formation and fun-tion of Vibrio parahaemolyticus lateral flagella. J. Bacteriol. 129: 1266 - 1271.

51. Smith, R. L., J. H. Bouton, S. C. Schank, K. H. Quesenberry, M. E. Tyler, M. H. Gaskins, and R. C. Littell. 1976. Nitrogen fixation in grasses inoculated with Spirillum lipoferum. Science 193: 1003 - 1005.

52. Strength, W. J., B. Isani, D. M. Linn, F. D. Williams, G. E. Vandermolen, B. E. Laughon, and N. R. Krieg. 1976. Isolation and characterization of Aquaspirillum fasciculus sp. nov., a rod-shaped nitrogen-fixing bacterium having unusual flagella. Int. J. Syst. Bacteriol. 26: 253 - 268.

53. Von Bülow, J. F. W., and J. Döbereiner. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. 72: 2389 - 2393.

54. Watson, J. D. 1976. Molecular biology of the gene, 3rd ed. W. A. Benjamin, Inc., Menlo Park, Calif.

55. Wilkins, T. D., and C. B. Walker. 1975. Development of a micromethod for identification of anaerobic bacteria. Appl. Microbiol. 30: 825 - 830.

56. Winter, H. C., and R. H. Burrus. 1976. Nitrogenase. Annu. Rev. Biochem. 45: 409 - 426.

The vita has been removed from the scanned document

A TAXONOMIC STUDY OF THE SPIRILLUM LIPOFERUM GROUP, WITH DESCRIPTIONS OF A NEW GENUS, AZOSPIRILLUM GEN. NOV., AND TWO SPECIES, AZOSPIRILLUM LIPOFERUM (BEIJERINCK) COMB. NOV. AND AZOSPIRILLUM BRASILENSE SP. NOV.

by

Jeffrey James Tarrand

ABSTRACT

Sixty-one strains of the root-associated nitrogen fixer Spirillum lipoferum exhibited a similar morphology (slightly-curved to S-shaped cells having a cell diameter of 1.0 μm) and had a DNA base composition of 69 - 71 mol% G + C (Tm). When grown in broth the cells had a single polar flagellum, but when grown on agar at 30°C they also formed numerous lateral flagella of shorter wavelength. DNA homology experiments using a membrane filter competition method indicated the occurrence of two distinct but related homology groups: 46 strains were in Group I and 15 strains were in Group II. Group I strains gave DNA homology values of 67% when tested against reference strain Sp 7, whereas Group II strains gave 28 - 46%. Group II strains gave homology values of 70% when tested against reference strain 59b, whereas Group I strains gave 30 - 52%. Group II strains were distinguished by their acid reaction with glucose, a weak fermentative ability, a requirement for biotin, and formation of wider, longer, S-shaped to spirillum-shaped cells when transferred to nitrogen-deficient malate

medium. Pigmentation, catalase activity, and denitrifying ability varied considerably. All strains could grow anaerobically in peptone media with nitrate as the electron acceptor. The results indicate that two species exist within the S. lipoferum group, and the characteristics of the organisms suggest that they should be assigned to a new genus, Azospirillum. Strains belonging to Group II are named A. lipoferum, while those belonging to Group I are named A. brasilense.