A NUCLEIC ACID HOMOLOGY STUDY OF THE GENUS *AZOSPIRILLUM*

by

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(ABSTRACT)

The results of deoxyribonucleic acid homology experiments with the type strains of *Azospirillum lipoferum*, *Azospirillum brasilense*, and *Azospirillum amazonense*, and 19 additional strains of *A. amazonense*, confirmed that *A. amazonense* is a distinct new species. The description of the genus *Azospirillum* is emended to accommodate *A. amazonense*.

Ribosomal RNA (rRNA) homology studies indicated 90 to 96% homology between *Azospirillum lipoferum* and *Azospirillum brasilense*, and 64 to 70% homology between these species and *Azospirillum amazonense*. This supports the inclusion of these three species in the genus *Azospirillum*. In contrast, "*Azospirillum seropedicae*" had very little homology with the other *Azospirillum* species (<22% RNA homology) and should not be considered a member of the genus. The taxonomic placement of "*A. seropedicae*" is uncertain. The nearest relatives of the genus *Azospirillum* were *Aquaspirillum itersonii* and *Rhodospirillum rubrum* (>65% RNA homology); *Gluconobacter oxydans* and *Beijerinckia indica* exhibited 30 to 60% RNA homology with *Azospirillum* species. Deoxyribonucleic acid (DNA) studies indicated that *Conglomeromonas largomobilis* subsp. *largomobilis* was related to *Azospirillum lipoferum* by >45% DNA homology and by 99% RNA homology; moreover, it was found to be a microaerophilic nitrogen fixer. Thus *C. largomobilis* subsp. *largomobilis* is a

subjective synonym of *A. lipoferum*. In contrast, DNA homology studies indicated that *Conglomeromonas largomobilis* subspecies *parooensis* was unrelated to *C. largomobilis* or *A. lipoferum*, or to any other species tested, and its taxonomic position is uncertain. Several strains of azospirilla which form unique star-shaped colonies were identified as *A. lipoferum* by DNA homology.

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Introduction

"Spirillum lipoferum" was isolated by Döbereiner and Day in 1976 from the roots of certain tropical grasses in Brazil. The organisms were described as being gram-negative, motile, generally vibrioid in shape and containing lipid (poly-ß-hydroxybutyrate) granules. "S. lipoferum" exhibited nitrogenase activity as indicated by acetylene reduction, but nitrogen fixation occured only under microaerobic conditions. Since 1976, these organisms have been isolated from the roots of a variety of grasses, cereal crops and tuber plants.

Because these organisms have the ability to fix nitrogen in the roots of cereal crops such as maize and wheat, it was thought that "S. lipoferum" might be useful in helping to solve the problem of world hunger: larger quantities of cereal crops might be produced without the expense of a nitrogen fertilizer. Thus there was an explosion in research on these organisms.

Although many strains were isolated from different plants and different geographic regions, all these strains were initially called "S. lipoferum". Lack of taxonomic research led to various opinions as to how many species there really were. It was important to determine the number of species before the name "S. lipoferum" became too firmly entrenched in the literature. If this were to occur, it would then become increasingly difficult to make taxonomic revisions and accompanying nomenclatural changes.

Some investigators found that certain strains of "S. lipoferum" required low levels of yeast extract in their media for growth. These same strains could also grow on glucose as the sole carbon source, whereas the strains which did not require the yeast extract failed to use glucose as a sole carbon source for growth. Thus the concept of two major groups began to arise.

In 1978 Tarrand, Krieg and Döbereiner performed DNA homology experiments to determine how many species could be distinguished in the "S. lipoferum" group. The results indicated the occurrence of two distinct but related homology groups. These homology groups were found to correlate well with certain physiological characteristics, such as the ability to use glucose. The two groups were interpreted as representing two species, and since these species were related, they belonged together in a single genus. However, they could not be assigned to any established genus. Therefore Tarrand et al. decided that these organisms would be better assigned to a new genus, Azospirillum. This name was devised because Tarrand et al. thought the term "spirillum" should be retained in the new generic name to minimize confusion, even though the organisms were actually vibrioid in shape. In some respects they did resemble spirilla: they could form helical cells under certain conditions and, like spirilla, they had a mainly respiratory type of metabolism and grew well on salts of organic acids as carbon sources.

Organisms belonging to homology group II were named Azospirillum lipoferum because they seemed similiar to the "S. lipoferum" described by Beijerinck in 1925. These strains were capable of using glucose as a sole carbon source for growth in nitrogen-free semisolid medium, and they required the vitamin biotin for growth. On the other hand, the organisms in homology group I, which were named Azospirillum brasilense, could not use glucose as a sole carbon source and did not require biotin.

A new sucrose-utilizing group of strains of Azospirillum-like organisms was isolated by Magalhães and Baldani in Rio di Janeiro in 1983. These strains resembled A. brasilense and A. lipoferum in size, shape, characteristic spinning

motility, and microaerophilic nitrogen-fixing ability, but differed in several other phenotypic characteristics, including the ability of these new strains to use sucrose as their sole carbon source, unlike the two known species of Azospirillum. The name A. amazonense was assigned to these strains. However, whether the strains formed a single DNA homology group, and whether they were related to A. lipoferum and A. brasilense was not known.

In 1984, Baldani et al. reported a fourth species isolated from cereal roots, and proposed the name "Azospirillum seropedicae" for it. That this was a new species distinct from the others was determined on the basis of phenotypic tests. For instance, the cells were smaller in diameter and possessed multiple polar flagella. However, as with A. amazonense, whether this new species was genetically related to A. lipoferum and A. brasilense was not known.

Döbereiner also isolated another new group of Azospirillum strains which formed unique star-shaped colonies on potato agar after one to two weeks. Although these "star-like" strains seemed similiar in other respects to A. lipoferum, their inclusion in this species was debatable.

In 1983, Skerman, Sly and Williamson had proposed a new genus, Conglomeromonas, consisting of one species, C. largomobilis, and two new subspecies, C. largomobilis subspecies largomobilis and C. largomobilis subspecies parooensis. The strains of C. largomobilis had been isolated from freshwater sources rather than from plant roots or soil. Nevertheless, many of the phenotypic traits described for Conglomeromonas largomobilis seemed similiar to those of A. lipoferum, and the possibility existed that C. largomobilis might actually belong to the genus Azospirillum.

The degree of relatedness between bacterial groups can be determined by nucleic acid hybridization studies, in which the entire genome, or a certain portion of the genome, of one organism can be compared with that of another organism. Such studies could be useful in clarifying the taxonomic relationships between the species of *Azospirillum* and the relationships between this genus and other genera. Therefore, the specific objectives of this research were to use DNA and/or RNA homology experiments to determine:

1. The relatedness of *A. amazonense* to *A. lipoferum* and *A. brasilense*.
2. The relatedness of the proposed fourth species of *Azospirillum*, "*A. seropedicae*" to the other species.
3. The relatedness of the "star-like" *Azospirillum* strains to *A. lipoferum* *A. brasilense* and *A. amazonese*.
4. The relatedness of *Conglomeromonas largomobilis* to the genus *Azospirillum*.
5. Whether all the species included in the genus *Azospirillum* are genetically related at a high enough level to be included in the same genus, and to determine their relatedness to representatives of other nitrogen-fixing genera, including *Aquaspirillum itersonii*, *Beijerinckia indica*, *Gluconobacter oxydans*, *Rhodospirillum rubrum* and *Rhodospirillum tenue*.

Literature Review

Spirillum lipoferum. Up to 1976 nitrogen fixing bacteria were considered in three main physiological categories: (a) aerobic organisms such as Azotobacter and Derxia, (b) facultative organisms, which fixed nitrogen only under anaerobic conditions, such as Klebsiella and (c) anaerobes, such as Clostridium pasteurianum. The possibility that microaerophilic nitrogen fixers might exist was given scant attention, although Beijerinck in 1921-1925 (4,5) had suggested that a spirillum he had observed in cultures from soil might be a microaerophilic nitrogen fixer. Beijerinck found that mixed cultures containing this spirillum could fix nitrogen but that pure cultures could not. He noted that the spirillum formed microaerotactic bands in wet mounts, and thus he suggested that the spirillum might depend on other bacteria for providing microaerobic conditions suitable for growth. Beijerinck originally named the spirillum "Azotobacter spirillum" but later changed the name to "Spirillum lipoferum" (the specific epithet being based on the presence of lipid granules in the cells). However, except for a few scattered reports (2,3,14,32) "S. lipoferum" was largely forgotten by soil microbiologists.

In 1976 Döbereiner and Day (11) reported the isolation of a plump, vibrioid gram-negative rod from the roots of the forage grass Digitaria decumbens in Brazil. Döbereiner and Day used a semisolid nitrogen-deficient medium to test the ability of the organism to fix nitrogen, and it is doubtful that recognition of the organism as a microaerophilic nitrogen fixer would have been achieved unless such a semisolid medium had been used. Döbereiner and Day attempted to classify the organism and after examining the literature on nitrogen fixers they believed that it resembled the "S. lipoferum" that had been described by Beijerinck. The occurence of a nitrogen fixer within the roots of grasses

suggested that the organisms might have agricultural potential, and the report by Döbereiner and Day stimulated microbiologists to examine other plants for the organism. The organism began to be found in a great variety of plant roots (including those of maize, wheat, and rice), in various soils and rhizospheres, and in various geographic regions (12,25,38). However, it was not clear just how many species were represented by all these isolates. Okon et al. (30) recognized two physiological groups based on a requirement for yeast extract and the ability to use glucose, but whether these two groups really represented separate species was not certain. In 1978 Tarrand et al. (36) performed a taxonomic study of 64 strains isolated from various plants and geographic regions. Through the use of DNA homology experiments two distinct but related homology groups were recognized; these were interpreted as being separate species. By the membrane-filter competition method for determining DNA homology, the two groups were related by 30 to 50% homology and thus belonged together in the same genus. However, which genus they both should be assigned to was unknown. Although the organisms had been assigned to the genus *Spirillum* by Beijerinck, they obviously did not belong to this genus, since it was restricted to very large microaerophilic spirilla having large bipolar tufts of flagella and whose mol% G + C of the DNA was much lower (38% versus 70%) (18). The genus *Aquaspirillum* was also considered but the overall combination of characteristics was not typical of this genus (see reference 36 for a discussion). Similarly, the organisms could not be placed in established genera of nitrogen-fixing organisms, such as *Derxia*, *Azotobacter*, *Azomonas*, or *Beijerinckia* (36). Consequently, Tarrand et al. assigned the species to a new

genus, Azospirillum. Based on these two species, the description of the genus as published in Bergey's Manual of Systematic Bacteriology (24) was as follows:

Strains of A. lipoferum and A. brasilense are characterized by gram-negative, vibrioid cells, about 1 μm in diameter and 3.5 μm in length. They show motility in liquid media by a single polar flagellum and exhibit a characteristic spinning movement. When grown on solid media, the cells form lateral flagella as well. Intracellular PHB granules are present. Azospirillum possess mainly a respiratory type of metabolism with O_2 and, with some strains, NO_3^- as terminal electron acceptors, however, some fermentative ability may be present. Pellicle formation occurs in semi-solid media. The organisms are microaerophilic nitrogen-fixers with optimum growth for most strains being from 0.35 to 4% O_2. When O_2 is limiting, some strains dissimilate nitrate to nitrite, nitrous oxide, or nitrogen gas. Azospirillum species grow well on salts of organic acids, such as malate, succinate, pyruvate and lactate. Certain carbohydrates may also serve as carbon sources. Some strains require biotin. The optimum growth temperature for Azospirillum is 34 to 37°C. The organisms are isolated from soils and from the roots of cereal crops, grasses, and tuber plants. The mol% G + C of the DNA is 67 to 71 by thermal denaturation methods.

Azospirillum lipoferum. Characteristics are cited in Table 1.

Azospirillum brasilense. Characteristics are cited in Table 1.

Azospirillum amazonense. A sucrose utilizing Azospirillum-like group of organisms were isolated by F. M. Magalhães and J. I. Baldani in Rio de Janeiro in 1983 (27). These microaerophilic nitrogen-fixing organisms resembled A. lipoferum and A. brasilense in size, shape, and characteristic spinning motility.

Table 1. Comparison of A. lipoferum and A. brasilense based on phenotypic characteristics[a]

Characteristics	A. lipoferum	A. brasilense
Biotin required for growth	+[b]	-
Glucose, 2-Oxoglutarate and mannitol used as sole carbon sources for growth in nitrogen-free semisolid medium	+	-
Acidification of peptone-based glucose medium, 96 h	+	-
In semisolid N-free malate medium nearly all cells become wider (1.4 - 1.7 µm), longer (5 µm to over 30 µm) and non-motile in 24-48 h. Eventually many cells become ovoid or pleomorphic and filled with highly refractile granules. Multicellular forms may also occur.	+	-
Acid from glucose or fructose anaerobically; slight growth also occurs; a small amount of gas is sometimes formed.	+ or v	-
Oxidase	+	+
Catalase	d	+
Esculin hydrolysis	+	+
Phosphatase	+	+
Pink pigment on BMS agar	+	+
Anaerobic growth with nitrate in peptone-based medium	+	+
NO_3^- to NO_2^-	+	+
NO_2^- to N_2O	d	d
N_2O to N_2	+	+
Starch hydrolysis	-	-
Urease	+	+
Gelatin hydrolysis	-	-
Indole	-	-
Growth in presence of 1% oxgall	+	+
Growth in presence of 3% NaCl	d	-
Voges-Proskauer, 2% glucose, 5 days	d	-
Acid produced aerobically from carbohyrates:		
Fructose	+	+
Galactose, arabinose	+	d
Mannitol, sorbitol, ribose, i-inositol, xylose	d	-
Rhamnose, dulcitol, erythritol, maltose, sucrose, cellobiose, melibiose lactose	-	-

Table 1. (continued)

Characteristics	A. lipoferum	A. brasilense
Sole carbon sources (auxanographic method)[c] with $(NH_4)_2SO_4$ as nitrogen source:		
Succinate, malate, lactate, pyruvate, oxaloacetate, fumarate, gluconate ß-hydroxybutyrate, glycerol, fructose, propionate,citrate	+	+
2-Oxoglutarate	+	-
Glucose	+	d[d]
Galactose, arabinose	d	d
Mannitol, sorbitol, ribose	d	-
Malonate	-	-

[a] All tests done with incubation at 37°C by the methods of Tarrand et al., 1978 (see reference 24, Procedures for Testing Special Characters).

[b] +, Positive in more than 90% of the strains; d, positive in 11 to 89% of the strains; -, negative in 90% or more of the strains; v, strain instability (NOT equivalent to "d").

[c] See Procedures for Testing Special Characters, reference 24.

[d] Only a few strains show this character under the conditions of the test (see reference 24, Procedure for Testing Special Characters) and the growth response is weak. The strains are unable to use glucose as a sole carbon source for N_2-dependent growth in nitrogen-free semisolid medium.

These strains grow on organic acids such as malate, lactate, pyruvate, fumarate and succinate, as both A. lipoferum and A. brasilense. These strains do not use citrate, glycerol or fructose, as the known species of azospirilla do, but use sucrose as a preferential carbon source. Unlike the known species of azospirilla, the majority of these strains do not dissimilate nitrate to nitrite and nitrite is not dissimilated to nitrous oxide. These strains also differ from A. lipoferum and A. brasilense in that there is no anaerobic growth on nutrient agar in the presence of nitrate. These strains were shown to have a slight fermentative ability. In 1984 the name Azospirillum amazonense was validly published for this new species (19). See table 2 for comparison of phenotypic traits with the other Azospirillum species.

"Azospirillum seropedicae". In 1984 Baldani et al. (1) reported a fourth Azospirillum species, "A. seropedicae", isolated from cereal roots, resembling the other Azospirillum species in shape, microaerophilic nitrogen-fixing ability and its ability to de-nitrify. "A. seropedicae" was reported to have a mol% G + C of the DNA of 66.8, lower than that of the other Azospirillum species. "A. seropedicae" also differs from the other Azospirillum species in that it has a smaller cell width (0.6 to 0.7 μm), a pair of polar flagella instead of one, its capacity to use glucose without a biotin requirement, a distinct colony form and color, and a strong smell on complex media. See table 2 for a comparison of phenotypic traits with the other Azospirillum species.

DNA homology experiments in bacterial taxonomy. DNA homology experiments have become of major importance since their development 20 years ago. This procedure measures similarity in which the entire genome of one organism is compared to that of another organism. Because of this broad

Table 2. Differential characteristics among Azospirillum species[a]

	A. lipoferum	A. brasilense	A. amazonense	"A. seropedicae"
Cell width (μm)	1.0 - 1.5	1.0 - 1.2	0.8 - 1.0	0.6 - 0.7
Flagellation:				
One flagellum, monopolar	+[b,c]	+[c]	+	-
>1 flagellum, mono or bipolar	-	-	-	+
Pleomorphic cells in alkaline media	+	-	-	-
Colony type on potato agar:				
pink, raised, curled	+	+	-	-
white, flat, raised margin	-	-	+	-
brown, raised, smooth, small	-	-	-	+
Dissimilation of:				
$NO_3^- > NO_2^-$	+	+	d[d]	+
$NO_2^- > N_2O$	d	d	-	d
Anaerobic growth on nutrient agar in the presence of NO_3-	+	+	-	-
Biotin requirement	+	-	-	-
Use of glucose	+	-	+	+
Use of sucrose	-	-	+	-
Use of fructose	+	+	-	+
Use of glycerol	+	+	-	+
Use of 2-oxoglutarate	+	-	-	+
Use of citrate	+	+	-	+
pH range for good growth	5.7 - 6.8	6.0 - 7.3	5.7 - 6.5	5.3 - 8.0
mol% G + C of DNA[e]	69 - 70	70 - 71	67 - 68	66 - 67

[a] The characteristics of "A. seropedicae" are as described by J. I. Baldani, V. L. D. Baldani, and J. Döbereiner, personal communication; the characteristics of A. amazonense are as described in reference 27; the characteristics of A. lipoferum and A. brasilense are as described in reference 24.

[b] +, Positive in more than 90% of the strains; d, positive in 11 to 89% of the strains; -, negative in all strains.

Table 2. (continued)

[c] In liquid media the cells possess only a single polar flagellum; on nutrient agar at 30°C numerous lateral flagella of shorter wavelength occur in addition to the polar flagellum.

[d] Only 4 of the 35 strains described by Magalhães et al. (reference 27) produced NO_2^-, which was afterward assimilated.

[e] As determined by the thermal denaturation method.

comparison, only bacteria with a high degree of base sequence similarity (ca. 90% or more) show any significant level of DNA homology. Proposed taxonomic groupings based upon DNA homology data are most useful at the species and subspecies level (21).

To determine the extent of similarity of the base sequences in the DNA of the different organisms, the DNA is denatured into single strands and combined with single-stranded DNA from various organisms. The strands are then allowed to re-anneal. The amount of heteroduplex formation occurring as compared to the amount of homoduplex formation is a measure of the DNA homology of the different organisms. Renaturation of heterologous DNA (single strands from different organisms) is compared to renaturation of homologous DNA (single strands from the same organism). In the membrane-filter competition method or in the hydroxylapatite procedure, an 80% or greater DNA homology between two organisms indicates that they are two members of the same species, while a 65 to 80% homology is considered borderline.

There are several different methods for determining DNA homology. One method is the direct binding method, in which denatured single-stranded DNA from an organism is bound to a nitrocellulose membrane, which is treated afterwards to prevent any further DNA from nonspecifically binding onto the membrane. Radioactively-labeled single-stranded DNA is then allowed to hybridize with the DNA on the membrane under standardized conditions of temperature, pH, salt concentration and time. Formamide may be added to lower the reaction temperature of the mixtures in all methods. After incubation, unbound labeled DNA is washed off the membrane and the remaining radioactivity is measured. The percent homology is calculated by comparing the

amount of heterologous DNA which was bound on the membrane to the amount of homologous DNA which was capable of binding to the membrane (20).

In the membrane-filter competition method (20), single-stranded labeled DNA is allowed to hybridize with single-stranded unlabeled DNA bound to a membrane in the presence of a large amount of a single stranded unlabeled competitor DNA. The degree to which the unlabeled DNA is able to compete with labeled DNA is used as a measure of DNA homology.

In free solution reassociation all of the component nucleic acids are in solution rather than being immobilized in some manner. Reassociation of DNA may be monitored optically by ultraviolet spectophotometry or by means of a labeled probe. In the optical method the rates of reassociation are determined. The general procedure for comparing DNAs from two organisms is to measure the rate of reassociation of equivalent concentrations from each of the organisms separately and compare those rates with that of an equal mixture of the two DNA preparations. This method is based on the fact that mixtures containing single-stranded DNA from two organisms which are very similiar in their base pair sequence reassociate at a rate very similiar to the rate of the DNA from one of the strains alone. The rate will be proportionately slower if the base pair similarity is lower (31). The second major type of free solution homology experiment employs the use of a labeled DNA probe. In this type of reassociation a small amount of single-stranded labeled DNA probe is incubated at a standard temperature, pH and salt concentration with a large amount of single-stranded unlabeled (driver) DNA. This method is based on the fact that, under these conditions, the DNA will reassociate into duplexes at a rate directly proportional to the concentration of DNA present in the reaction mixture.

Because the labeled DNA probe is used at a very low concentration, very little of it will reassociate, compared to the unlabeled test DNA with which the probe is incubated, which is used at a very high concentration. Because of this, most of the unlabeled test DNA will reassociate faster than the labeled DNA, unless the labeled DNA is identical to the unlabeled test DNA, in which case, the labeled DNA will reassociate with the unlabeled DNA at the same rate at which the unlabeled DNA is reassociating. Therefore if the two DNAs are unrelated, the unlabeled DNA will reassociate at a faster rate, leaving most of the labeled DNA in its single-stranded state. After incubating for 24 hours, if the two DNAs share a high degree of base pair similarity, most of the labeled DNA will form duplexes with the unlabeled driver DNA. The level of heteroduplex formation is compared to the amount of homoduplex formation to determine the level of DNA homology. There are two ways to determine exactly how much of the labeled DNA has formed heteroduplexes with the unlabeled DNA, which are the hydroxylapatite method and the S1 nuclease method. In the hydroxylapatite (HA) procedure, the incubation mixture is placed on an HA column or mixed with HA in a batch procedure. The DNA duplexes are adsorbed onto the HA particles at a phosphate concentration of 0.14 M. The single-stranded DNA is then removed by centrifuging and washing the batch, or washing the column. Double-stranded DNA is then eluted from the HA with 0.28 M phosphate, and precipitated with trichloroacetic acid (TCA). The amount of reassociated DNA can be calculated using the radioactivity of the precipitate, as described by Johnson (20). In the S1 nuclease method the resulting duplexes from the reassociation mixture are subjected to S1 nuclease digestion, a hydrolytic enzyme isolated from *Aspergillus oryzae*. This enzyme is specific for single-stranded DNA and gets rid

of unpaired portions of the duplexes. Then the remaining double-stranded DNA is precipitated with TCA, collected on nitrocellulose membranes and the radioactivity is measured. This gives the % homology which is equal to cpm of heterologous S1-resistant DNA divided by the cpm of homologous S1-resistant DNA multiplied by 100. Sheared native salmon sperm DNA, which has no homology with bacterial DNA is used as a control.

Labeling DNA for homology experiments. The type of radioisotope used for labeling DNA for homology experiments varies among investigators. Tritiated nucleotides may be used to label DNA *in vitro* using a "nick-translation" procedure, whereby DNA is nicked by deoxyribonuclease (DNase) and a DNA polymerase is used to reconstruct part of the DNA using the added radioactive nucleotides (20). Radioactive iodine (^{125}I) can be used in an *in vitro* procedure, where the radioactive iodine bonds covalently to the cytosine residues of the DNA in the presence of a thallium catalyst. This procedure does not involve the use of enzymes (7,33,37). A third method that is sometimes used is to grow the organisms from which the reference DNA is to be isolated in the presence of ^{32}P, ^{3}H, or ^{14}C (16).

Comparison of homology results obtained using different methods. Results from DNA homology studies employing various methods cannot be directly compared. For example, for homology values up to 60 to 70%, results with the S1 nuclease method are about 20% lower than those obtained by either the hydroxylapatite or the membrane competition procedures (23). Differences in these three methods are less pronounced with homology values ranging from 80 to 100%. This is due to the fact that only true hybrids remain intact after S1 nuclease treatment, whereas in the other two methods both hybrids and tails

resulting from unmatched single-stranded labeled DNA are detected as radioactive counts.

One of the major experimental parameters that affects reassociation rates is the sodium ion concentration (21). The most commonly used sodium ion concentration is about 0.4 M although concentrations up to 1 M do not alter the results significantly.

The other major experimental parameter that can affect homology results is reassociation temperature. A standardized temperature of about 25°C below the "T_m" (melting temperature) is used most commonly (28) which is considered the "optimal temperature", although formamide may be included to the reaction mixture to lower the reaction temperature if the DNA has an unusually high guanine-plus-cytosine content (mol% G + C). This reduces the amount of thermal degradation of the nucleic acids. The T_m of DNA is lowered by 0.60°C per percent formamide in buffers containing from 0.035 to 0.88 M NaCl (17). Johnson and Ordal (22) showed that at the optimal temperature the heteroduplexes formed from single-stranded DNA from highly related organisms exhibited a thermal stability very similar to that of native DNA. It is important that this standardized optimal temperature be employed during homology experiments because at temperatures below the optimal temperature considerable renaturation would occur between single-stranded DNAs from organisms which were not highly related, although the thermal stability of the resulting heteroduplexes would be found to be much lower than that of native DNA. The reassociation temperature effect is approximately linear for the membrane competition and the hydroxylapatite procedures; for organisms having less than 50% homology, the homology values will increase by about 20% at 10°C

below the T_m - 25°C temperature and decrease by about 20% at 10°C above the T_m - 25°C temperature (21). Reassociation temperature differences do not have as great an effect on the optical (9) or the S1 nuclease methods (15). Schleifer and Stackenbrandt (31) suggested using an incubation temperature of 15°C below the T_m of the DNA, which is sometimes termed "stringent" conditions. These conditions can be used in determining the level of base-pair mismatching between DNA hybrids. Thermal stability profiles may also be used to determine the amount of base-pair mismatching. Estimates range from 1% to 2.2% base-pair mismatching for each °C that the T_m of the hybrid DNA is below the T_m of the native DNA.

DNA homology studies of azospirilla. Limited DNA homology studies have been performed on species within the genus *Azospirillum*. In 1978 Tarrand and Krieg (36) were the first to perform a taxonomic study on the bacterium, then known as *Sprillum lipoferum*. Using the membrane-filter competition method, Tarrand and Krieg reassigned the *Spirillum lipoferum* group to a new genus, *Azospirillum*. This genus was found to contain two homology groups, which correlated well with differences in phenotypic characteristics. Homology group II, which is capable of using glucose as a sole carbon source for growth in nitrogen-free semisolid medium containing biotin was classified as *Azospirillum lipoferum*. Homology group I, which was not capable of using glucose as a sole carbon source for growth under nitrogen-fixing conditions, and did not require biotin for growth, was classified as *Azospirillum brasilense*.

rRNA hybridization experiments in bacterial taxonomy. Ribosomal RNA (rRNA) hybridization experiments differ from DNA homology experiments because rRNA is coded for by only 0.1 to 0.3%, a small fraction, of the

bacterial genome. Because rRNA is complementary to only one of the DNA strands, there is no association between the rRNA molecules. Due to their essential role in determining both structural and functional aspects of the ribosome, rRNA cistrons of the DNA appear to have evolved less rapidly than the bulk of the cistrons in the DNA (40). rRNA homology experiments are therefore used to detect similarities between more distantly related organisms. Consequently, these techniques are useful for determining genetic relationships at the genus, family and superfamily levels.

Homology of rRNA samples can be determined by immobilizing denatured DNA on a nitrocellulose membrane and incubating this with denatured labeled rRNA at standard conditions of temperature, pH and salt concentration. This is known as the direct binding procedure. Hybridization is measured as filter-bound radioactivity after treatment with RNase and washing to remove unreacted rRNA (20). Because of the variability of DNA membrane filters, direct experiments are not very quantitative. Another method of determining RNA homology is through the membrane competition procedure. This procedure is based on the fact that when an excess of unlabeled rRNA (competitor RNA) is added to the reaction vial, the hybridization of the labeled rRNA to the immobilized DNA on the membrane will be depressed. This reduction in binding can be used to determine percent homology between the bound DNA and the rRNA competitor. Direct binding is used to give total possible radioactive counts, because no competitor is present to compete with the labeled RNA probe for binding sites on the DNA immobilized on the membrane. Homologous competition is used to determine the background number of radioactive counts, because the competitor RNA is homologous to the DNA immobilized on the

membrane. Heterologous competition involves varying the competitor RNA, while keeping the probe RNA and immobilized DNA constant.

RNA thermal stability experiments. Another method for measuring nucleotide sequence similarity between DNA and rRNA involves comparing the thermal stability differences between the heterologous and homologous hybrids. The direct binding procedure is used and the labeled RNAs are varied across a matrix with various DNAs immobilized on membranes. The greater the amount of base mismatching between the strands, the less stable the hybrid will be. In order to remove any unhybridized RNA, RNase can be employed. This should attribute any remaining counts to labeled RNA bound to the homologous DNA on the membrane. The integral elution profile curves are obtained by summing the radioactivity up to each temperature and dividing by the total radioactivity. The temperature at which 50% of the duplexes have dissociated is called $T_{m(e)}$ (elution melting temperature). The difference between the homologous and heterologous $T_{m(e)}$ values is the $\Delta T_{m(e)}$ value. The $\Delta T_{m(e)}$ values for heterologous duplexes range from 0 (no mismatching) to 18°C (considerable mismatching).

Labeling of rRNA for hybridization experiments. Methods for labeling rRNA are similiar to those for labeling DNA. 16S and 23S fractions are usually isolated by polyacrylamide gel electrophoresis (6,13,26,34) or by sucrose gradient centrifugation (29). Either 23S or 16S, or both pooled fractions may be labeled _in vitro_ with ^{125}I (33). RNA may also be labeled _in vivo_ using ^{14}C or ^{3}H uracil or uridine, or $H_3{}^{32}PO_4$ and during the last 0.5 hour of incubation, the cultures are often incubated with a large excess of unlabeled precursor to decrease the specific activity of messenger RNA.

rRNA hybridization studies of azospirilla. Limited rRNA hybridization studies have been performed on members of the genus Azospirillum. In 1980 De Smedt et al. (10) performed hybridizations with reference rRNA from Azospirillum brasilense ATCC 29145^{T}. The study included 10 other strains of this species obtained from Vlassak, and LMD 50.39. Two strains of A. lipoferum were included in this study on the rRNA cistrons of nitrogen-fixing bacteria. These were A. lipoferum Sp Br17 and RG 18c. De Smedt et al. reported that the rRNA cistrons of all Azospirillum strains studied were very similar ($T_{m(e)}$ values between 80 and 82.5°C using 2X SSC, pH 7.0, 20% formamide at the stringent temperature of 50°C using the method of De Ley and De Smedt, 1975 and Takahashi et al., 1967). De Smedt et al. also reported Spirillum polymorphum NCIB 9072 and S. itersonii subsp. vulgatum NCIB 9071 had $T_{m(e)}$ values of 71.5 and 72.5, respectively. De Smedt concluded from these results that although these organisms belong in one rRNA group, the inclusion of the nitrogen-fixing azospirilla in a separate genus seemed justified. With respect to their rRNA cistrons, they differ as much from the "authentic" spirilla as Azotobacter differs from Alteromonas communis and Alteromonas vaga. The Azospirillum strains and Spirillum polymorphum ATCC 11332 and Spirillum itersonii subspecies vulgatum ATCC 11331 strains investigated by De Smedt constituted a separate branch in their fourth rRNA superfamily. De Smedt et al. concluded that because representatives of Paracoccus, Agrobacterium, Acetobacter, Gluconobacter, Beijerinckia, Xanthobacter autotrophicus, and "Mycobacterium" flavum had $T_{m(e)}$ values between 65 and 69°C, that these taxa all belong in the same rRNA superfamily as Azospirillum.

In 1982 Woese et al. (39) reported a phylogenetic analysis of certain helical bacteria- the genera Spirillum, Aquaspirillum, Oceanospirillum, and Azospirillum -based on the method of oligonucleotide cataloging. This involves a two dimensional T_1 ribonuclease digest which cleaves between the 3'-guanylic acid and the 5'-hydroxyl group of the adjacent nucleotide. This results in a guanine residue at the 3' end of each oligonucleotide. Then a 2 dimensional electrophoresis is used to separate the oligonucleotides, which can then be picked out and sequenced. The unique oligonucleotides (usually only one per rRNA molecule) of each organism are catalogued into computer storage. Then the nucleotides that any two organisms have in common are compared by a similarity coefficient, or S_{AB}, between organism A and organism B. This is calculated by dividing the number of unique oligonucleotides in each of their RNA molecules that they both share by the average total number of unique nucleotides. S_{AB} values can range between 0.28 and 1.00, with values below 0.28 indicating no RNA homology within bacterial groups. The study by Woese et al. included only one strain of A. brasilense ATCC 29145^T. This strain was found to be related to Aquaspirillum itersonii ATCC 12639 with an S_{AB} value of 0.48 and Rhodospirillum rubrum with an S_{AB} value of 0.51.

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Chapter 1

Deoxyribonucleic Acid Homology of *Azospirillum amazonense* Magalhães et al. 1984 and Emendation of the Description of the Genus *Azospirillum*

ABSTRACT

The results of deoxyribonucleic acid homology experiments with the type strains of *Azospirillum lipoferum*, *Azospirillum brasilense*, and *Azospirillum amazonense* and 19 additional strains of *A. amazonense* confirmed that *A. amazonense* is a distinct new species. The description of the genus *Azospirillum* is emended to accommodate *A. amazonense*.

In 1983 Magalhães et al. (6) reported the isolation of 35 strains of a microaerophilic, nitrogen-fixing Azospirillum-like organism from soils and roots of various Gramineae and other plants in the Amazon region and Rio de Janeiro state, Brazil. In shape, size, characteristic spinning motility, and microaerophilic nitrogen-fixing ability, this organism resembled the previously described species of the genus Azospirillum (viz., Azospirillum brasilense and Azospirillum lipoferum) (5, 8); moreover, the guanine-plus-cytosine content of the deoxyribonucleic acid (DNA) was 67 to 68 mol%, only slightly lower than the value of 69 to 70 mol% reported for azospirilla (8). However, several phenotypic differences were described that differentiated the new organism from previously described azospirilla (Table 1). On the basis of these differences, it was proposed that the new organism be assigned to a new species, Azospirillum amazonense, and this name has since been validly published (2). Strain ATCC 35119 (= strain Am14 of Magalhães et al.) was designated as the type strain. In this paper we describe the results of DNA homology experiments in which we used the type strains of A. brasilense, A. lipoferum, and A. amazonense and 19 additional strains of A. amazonense described by Magalhães et al. (6). We also provide an emended description of the genus Azospirillum to accommodate the new species.

DNA was extracted and purified by the hydroxylapatite procedure described by Johnson (3). Portions of the DNA preparations from the type strains of A. brasilense, A. lipoferum, and A. amazonense were labeled by in vitro iodination. The $TlCl_3$ iodination procedure of Selin et al. (7) was modified by the addition of sodium perchlorate to a final concentration of

TABLE 1. Characteristics differentiating Azospirillum amazonense from A. lipoferum and A. brasilense[a].

Characteristic	A. amazonense	A. lipoferum	A. brasilense
Cell width (µm)	0.9-1.0	1.0-1.5	1.0-1.2
Flagellar arrangement:			
Monotrichous	+[b]	+	+
Peritrichous	-	+[c]	+[c]
Enlarged, pleomorphic cells develop in alkaline media	-	+	-
Biotin required	-	+	-
Growth at pH:			
>6.8	W	+	+
6.0	+	+	+ or W
Pigmentation on potato agar			
White	+	-	-
Pink	-	+	+
Dissimilation of:			
$NO_3^- \rightarrow NO_2^-$	d[d]	+	+
$NO_2^- \rightarrow N_2O$	-	d	d
Anaerobic growth on nutrient agar in the presence of NO_3^-	-	+	+
Sole carbon sources for N_2 fixation			
Sucrose	+	-	-
Fructose	-	+	+
Glucose	+	+	-
Guanine-plus-cytosine content of DNA (mol%)[e]	67-68	69-70	70-71

[a] The characteristics of A. amazonense are described in reference 6; characteristics of A. lipoferum and A. brasilense are described in references 5 and 6.

[b] +, Positive in more than 90% of the strains; d, positive in 11 to 89% of the strains; -, negative in all strains; W, scant growth.

[c] In liquid media the cells possess only a single polar flagellum; on nutrient agar at 30°c numerous lateral flagella of shorter wavelength occur in addition to the polar flagellum.

[d] Only 4 of the 35 strains described by Magalhães et al. (6) produced NO_2^-, which was afterward assimilated (strains Am 17, Am 22, Am 27, and Am 31).

[e] As determined by the thermal denaturation method.

5.0 M in the reaction mixture to ensure single-stranded DNA (1). The S1 nuclease procedure described by Johnson (4) was used for the DNA homology experiments. The reassociation mixtures consisted of 10 µl of labeled DNA (0.03 µg), 50 µl of unlabeled DNA (20 µg) or 0.1 X SSC (1X SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 25 µl of 5.28 M NaCl-1.0 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and 25 µl of formamide. The mixtures were reassociated at 65°C (25°C below the thermal melting point in this buffer system) for 24 h. The S1-resistant duplexes and 60 µg of sheared salmon sperm DNA were coprecipitated by adding 0.25 volume of a mixture containing 1 N HCl, 1% sodium pyrophosphate, and 1% NaH_2PO_4. After refrigeration for 1 h at 4°C the precipitates were collected on glass fiber filters (Whatman GF/F). Radioactivity was measured with a Beckman model 5500 gamma scintillation counter.

The results of the DNA homology experiments are shown in Table 2. All of the new strains tested had high levels of homology (>56%) to the type strain of A. amazonense but only very low levels of homology (2 to 6%) to the type strains of A. brasilense and A. lipoferum. These data confirm that A. amazonense is a distinct species. A. lipoferum ATCC 29707^T (T = type strain) and A. brasilense ATCC 29145^T exhibited a lower degree of homology (14 to 17%) toward each other than the previous values of 31 to 34% reported by Tarrand et al. (8); this is due to use of the S1 nuclease method rather than the membrane-filter competition method used previously.

TABLE 2. DNA homology among *Azospirillum* strains

Unlabeled DNA from strain:	% Homology to DNA from reference strain:		
	A. lipoferum ATCC 29707^T	*A. brasilense* ATCC 29145^T	*A. amazonense* ATCC 35119^T
A. lipoferum ATCC 29707^T	100.0	16.9	2.9
A. brasilense ATCC 29145^T	14.1	100.0	7.8
A. amazonense ATCC 35119^T	3.3	2.5	100.0
Am 15	2.5	2.5	67.6
Am 16	3.9	4.1	56.2
Am 17	3.4	3.5	66.8
ATCC 35120 (=Am 18)	3.0	2.1	88.0
Am 19	2.5	3.0	87.3
Am 20	3.7	4.0	66.5
Am 21	2.5	2.9	70.1
Am 22	4.0	6.0	62.8
Am 24	2.6	2.3	57.3
Am 25	2.3	3.7	60.4
Am 26	3.2	4.8	58.4
Am 27	5.8	5.2	67.4
Am 28	3.5	4.2	65.7
Am 29	3.6	4.8	68.6
Am 31	2.0	5.0	67.1
Am 32	2.9	4.4	64.5
Am 33	2.6	4.8	59.0
Am 34	3.0	3.3	67.4
Am 35	5.3	5.3	72.4

Inclusion of A. amazonense in the genus Azospirillum requires a modification of the genus description, as given below.

Emended description of the genus Azospirillum Tarrand et al. 1978. Plump, vibrioid, or straight rods, often with pointed ends. Gram-negative to gram-variable. Intracellular granules of poly-ß-hydroxybutyrate are present. Motile with a characteristic corkscrew-like or vibratory motion in liquid media by means of polar flagella. Lateral flagella may also be formed by some strains when cells are cultured on solid media at 30°C. The colonies of some strains are pigmented (light or dark pink) on potato agar. Optimum growth temperature, 34 to 37°C. Some strains grow well at pH 7; others prefer more acidic conditions. Nitrogen-fixers, exhibiting N_2-dependent growth under microaerobic conditions. Grow well under an air atmosphere in the presence of a source of fixed nitrogen, such as an ammonium salt. Possess mainly a respiratory type of metabolism with O_2 and, with some strains, NO_3^- as terminal electron acceptors. Weak fermentative ability may also occur. Under severe O_2 limitation some strains may dissimilate nitrate to NO_2^- or to N_2O and N_2. Oxidase positive. Chemoorganotrophic; some strains are facultative hydrogen autotrophs. Grow well on the salts of organic acids such as malate, succinate, lactate, and pyruvate; certain carbohydrates may also serve as carbon sources. Some strains require biotin. Occur free-living in soil or in association with the roots of cereal crops, grasses, and tuber plants. Root nodules are not induced. The guanine-plus-cytosine content of the DNA is 67 to 71 mol% (thermal denaturation method).

Type species: Azospirillum lipoferum (Beijerinck) Tarrand et al. 1978.

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Chapter 2

Deoxyribonucleic and Ribonucleic Acid Homology Studies of the Genera *Azospirillum* and *Conglomeromonas*

ABSTRACT

Ribosomal RNA (rRNA) homology studies indicated 90 to 96% homology between *Azospirillum lipoferum* and *Azospirillum brasilense*, and 64 to 70% homology between these species and *Azospirillum amazonense*. This supports the inclusion of these three species in the genus *Azospirillum*. In contrast, "*Azospirillum serodedicae*" had very little homology with the other *Azospirillum* species (<22% RNA homology) and should not be considered a member of the genus. The taxonomic placement of "*A. seropedicae*" is uncertain. The nearest relatives of the genus *Azospirillum* were *Aquaspirillum itersonii* and *Rhodospirillum rubrum* (>65% RNA homology); *Gluconobacter oxydans* and *Beijerinckia indica* exhibited 30 to 60% RNA homology with *Azospirillum* species. Deoxyribonucleic acid (DNA) studies indicated that *Conglomeromonas largomobilis* subsp. *largomobilis* was related to *Azospirillum lipoferum* by >45% DNA homology and by 99% RNA homology; moreover, it was found to be a microaerophilic nitrogen fixer. Thus *C. largomobilis* subsp. *largomobilis* is a subjective synonym of *A. lipoferum*. In contrast, DNA homology studies indicated that *Conglomeromonas largomobilis* subspecies *parooensis* was unrelated to *C. largomobilis* or *A. lipoferum*, or to any other species tested, and its taxonomic position is uncertain. Several strains of azospirilla which form unique star-shaped colonies were identified as *A. lipoferum* by DNA homology.

Introduction

In 1976 Döbereiner and Day reported the isolation of a microaerophilic nitrogen-fixing vibrioid bacterium from the roots of forage grasses in Brazil (5). The organism was identified as "Spirillum lipoferum" Beijerinck 1925 (2). Many strains were isolated subsequently from various kinds of plants and soils and from various geographic regions of the world and, although all these strains were called "S. lipoferum", it was not clear whether they all belonged to a single species. Subsequent taxonomic studies indicated the occurrence of two deoxyribonucleic acid (DNA) homology groups, which were interpreted as representing two species (16). Based on membrane-filter competition DNA homology experiments there was 30-50% interspecies DNA homology, which was interpreted to mean that the two species belonged to the same genus (21). However, they could not be assigned to any established genus and thus a new genus, Azospirillum, was created for them (16). The species were named Azospirillum lipoferum (type species) and Azospirillum brasilense. The former could be differentiated from the latter by, among other characteristics, its ability to use glucose as a sole carbon source for nitrogen fixation and a requirement for biotin. Neither species could use disaccharides such as sucrose. Detailed descriptions of the two species have recently been published in Bergey's Manual of Systematic Bacteriology (10).

In 1983 a new species, Azospirillum amazonense, was described (11). The strains were related to one another by >57% DNA homology as measured by the S1 nuclease method (6); moreover, they had no significant homology with the

other two species, indicating that they represented a distinct species. Because of lack of DNA homology with the other two species, assignment to the genus Azospirillum was made solely on the basis of phenotypic characteristics. Among the differences between A. amazonense and the other two species were the formation of white rather than pink colonies on potato agar (BMS agar; see reference 10), the absence of lateral flagella, and the ability to use sucrose as a sole carbon source for nitrogen fixation (6, 11).

In 1984 a fourth species, "Azospirillum seropedicae", was reported (1; Baldani, J. I., V. L. D. Baldani, and J. Döbereiner, "Azospirillum seropedicae", a fourth species associated with cereal roots, poster presentation at Third Int. Symp. Nitrogen Fixation with Non-Legumes, Helsinki 2-8 September 1984). DNA hybridization experiments indicate that the strains are related to one another but are distinct from other Azospirillum species (J. I. Baldani, V. L. D. Baldani, and J. Döbereiner, personal communication). Baldani et al. (1) assigned the species provisionally to the genus Azospirillum on the basis of a vibrioid cell shape, an ability to fix nitrogen under microaerobic conditions with malate as the carbon and energy source, and an association with plant roots. However, the organisms differed from other azospirilla by having a smaller cell diameter; by having one to three flagella at one or both poles rather than a single polar flagellum; by forming small, moist, green-centered colonies on nitrogen-free basal medium (Nfb medium; see reference 10) plus yeast extract, rather than the dry white colonies formed by other azospirilla; and by having a slightly lower G + C content of the DNA (66-67 mol%).

In 1983 Skerman et al. (15) proposed a new genus, Conglomeromonas, which contained the species Conglomeromonas largomobilis with two subspecies,

Conglomeromonas largomobilis subsp. largomobilis and Conglomeromonas largomobilis subsp. parooensis. Although the organisms were isolated from freshwater sources rather than from soil or plant roots, and although they were unable to fix nitrogen under aerobic conditions, other characteristics reported for these organisms have suggested that they might be related to the genus Azospirillum. These characteristics include the high G + C content of their DNA (67-70 mol%), a straight or slightly curved shape, development of multicellular elongated forms, and occurrence of lateral flagella in addition to a single polar flagellum. On the basis of these characteristics a further comparison of the organisms with members of the genus Azospirillum seems to be warranted.

Recently, one of us (VLBD) isolated several new strains of azospirilla which differed from other azospirilla by forming colonies shaped like stars on BMS agar. In other respects they resembled strains of A. lipoferum but their identity was uncertain.

The purpose of the present research was to determine the relatedness of A. amazonense, "A. seropedicae", the new "starlike" strains, and the genus Conglomeromonas to the genus Azospirillum by use of DNA and ribosomal ribonucleic acid (rRNA) homology experiments.

MATERIALS AND METHODS

Organisms and growth conditions. The strains used and their sources are listed in Table 1.

For isolation of nucleic acids, *Azospirillum* species were cultured in nutrient broth at 37°C or, in the case of "*A. seropedicae*", at 30°C. *Conglomeromonas largomobilis* subsp. *largomobilis* was cultured in a peptone-succinate-salts (MPSS) broth (3) at 37°C whereas *C. largomobilis* subsp. *parooensis*, which did not grow well at 37°C, was cultured in nutrient broth at 32°C. *Beijerinckia indica* strains were cultured at 30°C in Nfb medium containing 0.1% ammonium sulfate. *Gluconobacter oxydans* strains were cultured at 23°C in sorbitol-yeast extract-peptone medium (13). *Rhodospirillum tenue* was grown aerobically in the dark at 30°C in a medium containing 0.1% K_2HPO_4, 0.05% $MgSO_4$, and 1% yeast extract (pH 7.0), whereas *Rhodospirillum rubrum* was grown aerobically in trypticase soy broth in the dark at 30°C. *Aquaspirillum itersonii* was cultured in MPSS broth at 30°C.

Isolation of nucleic acids. Low molecular weight DNA was extracted and purified by the hydroxylapatite procedure described by Johnson (8). High molecular weight DNA was extracted and purified by the procedure described by Marmur (12). DNA preparations were stored at -20°C in 0.1X standard saline citrate (1X SSC is 0.15 M plus NaCl, 0.015 M trisodium citrate, pH 7). Low molecular weight DNA preparations were further fragmented by passage (two times) through a French pressure cell at 16,000 psi; they were then denatured by heating in a boiling water bath for 5 min and adjusted to 0.4 mg/ml for use

TABLE 1. Bacterial strains used in this study

Taxon	Strain	Source[a]	Origin
Aquaspirillum itersonii	ATCC 11331	N.R. Krieg	Freshwater
	ATCC 12639[T]	N.R. Krieg	Freshwater
Azospirillum amazonense	ATCC 35119[T]	J. Döbereiner	Roots of *Digitaria decumbens*
	ATCC 35121	J. Döbereiner	Roots of *Pennisetum purpureum*
	ATCC 35120	J. Döbereiner	Roots of *Hyparrhenia rufa*
Azospirillum brasilense	ATCC 29145[T]	J. Döbereiner	Roots of *Digitaria decumbens*
	ATCC 29710	J. Döbereiner	Roots of millet
Azospirillum lipoferum	ATCC 29707[T]	J. Döbereiner	Roots of wheat
	ATCC 29731	J. Döbereiner	Roots of maize
	Sp RG9c	J. Döbereiner	Roots of wheat
"*Azospirillum seropedicae*"	Sp Z67 (= ATCC 35892)[b]	J. Döbereiner	Roots of rice
	Sp Z78 (= ATCC 35893)	J. Döbereiner	Roots of sorghum.
	Sp Z152 (= ATCC 35894)	J. Döbereiner	Roots of maize
Beijerinckia indica	ATCC 9039[T]	ATCC	Soil
	ATCC 9038	ATCC	Soil
Conglomeromonas largomobilis subsp. *largomobilis*	UQM 2041[T]	V.B.D. Skerman	Freshwater
	UQM 2043	V.B.D. Skerman	Freshwater
Conglomeromonas largomobilis subsp. *parooensis*	UQM 2042[T]	V.B.D. Skerman	Freshwater
Gluconobacter oxydans	ATCC 19357[T]	G.W. Claus	Beer
	ATCC 621	G.W. Claus	Unknown
Rhodospirillum rubrum	ATCC 11170[T]	S. Herbein	Unknown
Rhodospirillum tenue	ATCC 25093[T]	ATCC	Pond

TABLE 1, continued . . .

Taxon	Strain	Source[a]	Origin
"Starlike" strains	MA-1	J. Döbereiner	Roots of maize
	MA-3	J. Döbereiner	Roots of maize
	TSE-2	J. Döbereiner	Roots of wheat
	TSE-7	J. Döbereiner	Roots of wheat
	IIISa-4	J. Döbereiner	Rhizosphere of sorghum
	IIISb-6	J. Döbereiner	Roots of sorghum
	260d	J. Döbereiner	Rhizosphere of sorghum

[a]N. R. Krieg, G. W. Claus, or S. Herbein, Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Va.; J. Döbereiner, EMBRAPA-PNPBS, Km 47, 23460 Seropédica, Rio de Janeiro, Brazil; ATCC, American Type Culture Collection, Rockville, Md.; V. B. D. Skerman, Department of Microbiology, University of Queensland, St. Lucia, Queensland, Australia.

[b]Suggested type strain (J. Döbereiner, personal communication).

in the DNA homology experiments. The high molecular weight DNA preparations were used in RNA homology experiments.

Ribosomal RNA (rRNA) was isolated by a variation of the Kirby procedure described by Johnson (9). The rRNA preparations were stored at -20°C in 1X SSC (pH 7) containing 1% sodium dodecylsulfate. Competitor rRNA concentrations were adjusted to 2 mg/ml. Ribosomal RNA to be labeled (see below) was fractionated into 16S and 23S components by centrifugation for 15 h at 8°C (Sorval AH628 rotor) through 5 to 20% sucrose gradients prepared in 1X SSC - 0.5% Sarkosyl. The 16S and 23S components were collected and used for labeling.

Nucleic acid labeling. Portions of the 0.4 mg/ml DNA preparations from the type strains of *A. brasilense*, *A. lipoferum*, *A. amazonense*, "*A. seropedicae*", and *C. largomobilis*, and also strains UQM 2042 and UQM 2043 of *C. largomobilis*, were labeled by in vitro iodination. The $TlCl_3$ iodination procedure of Selin et al. (14) was modified by the addition of sodium perchlorate to a final concentration of 5.0 M as described previously (6).

The 16S and 23S rRNA components of the type strains of the four *Azospirillum* species were labeled with ^{125}I by the procedure described by Selin et al. (14, 17). The RNA was dissolved in 1X SSC-1 mM HEPES (*N*-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid, pH 7)-0.5% SDS to a concentration of 30 µg/ml and stored at -20°C.

DNA homology experiments. The S1 nuclease procedure described by Johnson (9) was used. The reassociation mixtures consisted of 10 µl of labeled DNA (0.03 µg), 50 µl of unlabeled DNA (20 µg) or 0.1X SSC, 25 µl of 5.28 M NaCl-1.0 mM HEPES, and 25 µl of formamide. The mixtures were reassociated at

65°C (25°C below the thermal melting point in this buffer system) for 24 h. The S-1 resistant duplexes and 60 μg of sheared salmon sperm DNA were coprecipitated by adding 0.25 volume of a mixture containing 1 N HCl, 1% sodium pyrophosphate, and 1% NaH_2PO_4. After refrigeration for 1 h at 4°C, the precipitates were collected on glass fiber filters (Whatman GF/F). Radioactivity was measured with a Beckman Model 5500 gamma scintillation counter.

rRNA hybridization experiments. The general procedures and equipment used for the rRNA hybridization experiments were as described by Johnson (9). Denatured high molecular weight DNA (4.35 mg) from the type strains of the four Azospirillum species, was immobilized on 15 cm BA 85 nitrocellulose membranes (Schleicher and Schuell, Inc.) at a level of 25 $\mu g/cm^2$ by using an adaption fo the Gillespie and Spieglman (7) procedure described by Johnson (9). A paper punch was used to cut the 3 X 9 mm filters (about 4 μg DNA/filter) used in the hybridization experiments. The membranes were incubated in reassociation mixtures which consisted of 10 μl of labeled rRNA (0.3 μg), 25 μl of competitor rRNA (50 μg), 25 μl of 17.6X SSC-1mM HEPES (pH 7), and 50 μl of deionized formamide. For direct hybridization, the competitor rRNA was replaced with 25 μl of 1X SSC-0.5% SDS. Each competition experiment contained a series of eight direct-binding-reaction vials, eight homologous-binding reaction vials and four competitor-binding-reaction vials per competitor RNA. The hybridization vials were incubated for 17 h at 50°C and the membranes were washed in two 300-ml volumes of 2X SSC at 50°C for 5 min each. The membranes were then washed in 2X SSC containing 10 μg/ml of RNase A and 0.25 units of RNase T1 for 1 h at 37°C; they were then washed in a 300-ml volume of 2X SSC at room temperature for 5 min followed by drying under a

heat lamp for 15 min. Radioactivity of the membranes was measured with a Beckman model 5500 gamma scintillation counter.

RESULTS

DNA homology results. As indicated in Table 2, the type strains of A. lipoferum and A. brasilense were related by about 15% homology, and both were related to the type strain of A. amazonense by <7% homology; these results were consistent with results reported previously (6) but were lower than the value of 30 to 50% reported by Tarrand et al. (16) for DNA homology between A. brasilense and A. lipoferum, due to use of the S1 nuclease method. Reference strain ATCC 35120 of A. amazonense had high homology (83%) with the type strain of this species, similar to the value of 88% previously reported (6). Reference strains ATCC 29731 and Sp RG9c of A. lipoferum exhibited 52 to 54% homology to the type strain of this species; these values were lower than the previous values of 73% obtained by Tarrand et al., who used the membrane-filter competition method.

The "starlike" strains (MA-1, MA-3, TSE-2, TSE-7, IIISa-4, IIISb-6, and 260d) were related to A. lipoferum by 45 to 60% homology and to the type strain of C. largomobilis subsp. largomobilis by 36 to 54% homology. They showed only 20 to 24% homology with the type strain of A. brasilense and only very low homology values (<8%) with A. amazonense and the type strain of C. largomobilis subsp. parooensis.

"A. seropedicae" strains showed only very low levels of homology with other species of Azospirillum and with both subspecies of C. largomobilis.

The type strain of C. largomobilis subsp. largomobilis exhibited 67% homology with reference strain UQM 2043 of this species, and also exhibited 47% homology with the type strain of A. lipoferum. It showed only low levels of

TABLE 2. Levels of DNA homology among *Azospirillum* and *Conglomeromonas* strains

Species and strain	% DNA homology with reference DNA from strains:					
	A. lipoferum ATCC 29707[T]	*A. brasilense* ATCC 29145[T]	*A. amazonense* ATCC 35119[T]	*C. largomobilis* subsp. *largomobilis* UQM 2041[T]	*C. largomobilis* subsp. *largomobilis* UQM 2043	*C. largomobilis* subsp. *parooensis* UQM 2042[T]
A. lipoferum						
ATCC 29707[T]	100	14	7	47	47	4
ATCC 29731	52	12	4	45	50	4
Sp RG9c	54	15	7	45	42	8
A. brasilense						
ATCC 19145[T]	16	100	7	11	19	5
A. amazonense						
ATCC 35119[T]	4	4	100	5	5	4
ATCC 35120	3	2	83	1	1	1
"*A. seropedicae*"						
ATCC 35892[T]	13	3	0	4	10	0
ATCC 35893	1	2	0	3	0	0
ATCC 35894	7	2	1	0	3	0
C. largomobilis						
subsp. *largomobilis*						
UQM 2401	47	13	5	100	65	5
UQM 2043	40	14	7	67	100	12

TABLE 2, continued . . .

Species and strain	% DNA homology with reference DNA from strains:					
	A. lipoferum ATCC 29707[T]	A. brasilense ATCC 29145[T]	A. amazonense ATCC 35119[T]	C. largomobilis subsp. largomobilis UQM 2041[T]	C. largomobilis subsp. largomobilis UQM 2043	C. largomobilis subsp. parooensis UQM 2042[T]
C. largomobilis subsp. parooensis						
UQM 2042	8	7	4	8	8	100
"Starlike" strains						
MA-1	49	21	3	41	40	4
MA-3	47	21	4	36	39	4
TSE-2	60	21	3	ND[a]	ND	ND
TSE-7	46	22	2	37	44	0
IIISa-4	45	20	4	42	28	4
IIISb-6	50	24	8	54	46	3
260d	53	21	8	37	42	4

[a] ND = not done.

homology with A. brasilense, A. amazonense, "A. seropedicae", and C. largomobilis subsp. parooensis.

RNA homology results. Results from rRNA competition experiments are shown in Table 3. The rRNA homology values ranged from 8 to 100%. Reciprocal homology values among the various type and reference strains were for the most part very similar; the greatest differences in reciprocal homology values, 10 to 12%, occurred with A. amazonense vs. A. lipoferum strains.

A. lipoferum and A. brasilense were highly related, yielding RNA homology values of 91 to 96%. Lower degrees of relationship occurred between A. lipoferum and A. brasilense vs. A. amazonense (64 to 76%). These three species of azospirilla exhibited only very low homology values with "A. seropedicae"; indeed, the latter species was most closely related to R. tenue and exhibited only low homology values with the other species tested.

Both A. lipoferum and A. brasilense were highly related to C. largomobilis subsp. largomobilis (90 to 99% homology) but not to C. largomobilis subsp. parooensis. Lower degree of relatedness were observed between A. lipoferum and species of other genera, the highest being with A. itersonii (52-73%).

TABLE 3. Levels of RNA homology

Species and strain	% RNA homology with reference RNA from strain:			
	A. lipoferum ATCC 29707^T	A. brasilense ATCC 29145^T	A. amazonense ATCC 35119^T	"A. seropedicae" ATCC 35892
A. lipoferum				
ATCC 29707^T	100	91	64	13
ATCC 29731	99	91	65	13
A. brasilense				
ATCC 29145^T	95	100	66	14
ATCC 29710	96	100	70	15
A. amazonense				
ATCC 35119^T	76	67	100	22
ATCC 35121	75	65	100	21
"A. seropedicae"				
ATCC 35892	22	18	9	100
ATCC 35893	20	20	18	100
C. largomobilis				
subsp. largomobilis				
UQM 2041^T	99	92	66	14
UQM 2043	99	90	67	13
A. itersonii				
ATCC 12639^T	73	58	47	16
ATCC 11331	72	52	47	15
R. rubrum				
ATCC 11170^T	65	41	44	15
R. tenue				
ATCC 25093^T	40	21	19	47
G. oxydans				
ATCC 19357^T	53	38	39	14
ATCC 621	60	47	41	16
B. indica				
ATCC 9039^T	55	32	40	17
ATCC 9038	32	30	11	8

DISCUSSION

The results of the DNA homology experiments indicate that A. brasilense, A. lipoferum, and A. amazonense are genetically distinct species. However, whether these three species should be classified in a single genus is not entirely clear from the DNA homology data. By using the membrane-filter competition method, Tarrand et al. (16) found a sufficient level of DNA homology (30 to 50%) between A. brasilense and A. lipoferum to justify inclusion of these two species in a single genus. In the present study, however, the level of interspecies homology was much less (about 15%), due to use of the more rigorous S1 nuclease method. A. amazonense has even less homology with the other two species.

Because of the conservation of rRNA cistrons, rRNA homology experiments can provide evidence regarding relationships above the species level of classification. The rRNA cistrons of A. brasilense and A. lipoferum show a very high degree of similarity (91 to 96% homology), in agreement with RNA hybridization data reported by De Smedt et al. (4). Moreover, A. amazonense was related to these two species by 65 to 76% RNA homology. This provides strong evidence that the three species do belong in a single genus.

By both DNA and RNA homology, "A. seropedicae" was found to be genetically distinct from the other three species of Azospirilllum, the RNA homology values being only 9 to 22% with the other three species. This indicates that "A. seropedicae" should not be included in the genus Azospirillum. Indeed, "A. seropedicae" is much more highly related to R. tenue (47% RNA

homology) than to species of Azospirillum. To what genus "A. seropedicae" belongs is not yet certain; possibly a new genus should be created for it.

With regard to the group of unidentified strains that formed star-shaped colonies, our DNA homology results indicate that these strains are closely related to the type strain of A. lipoferum, with homology values ranging from 45 to 60%. These values are similar to those of 52 and 54% homology obtained between the type strain of A. lipoferum and strains ATCC 29731 and Sp RG9c, which are reference strains of A. lipoferum established by Tarrand et al. (16). Consequently, we regard these strains as belonging to A. lipoferum.

C. largomobilis subsp. largomobilis UQM 2041^{T} and UQM 2043 showed significant DNA homology with A. lipoferum ATCC 29707^{T}, with the other A. lipoferum strains tested, and with the "starlike" strains. Moreover, strain UQM 2041^{T} exhibited 99% RNA homology with the type strain of A. lipoferum. These results prompted us to examine the two C. largomobilis subsp. largomobilis strains for their ability to fix nitrogen — a key characteristic of azospirilla. Skerman et al. (15) reported that these strains failed to fix nitrogen under aerobic conditions; however, in our laboratory both strains grew in semisolid nitrogen-free malate medium provided that biotin was present, and they gave a positive acetylene reduction test (from 31 to 80 nmoles ethylene per culture per hour). They did not reduce acetylene when grown in a similar medium containing 0.1% $(NH_4)_2SO_4$, and they failed to grow in liquid nitrogen-free medium. Thus they did appear to be microaerophilic nitrogen fixers. Other phenotypic traits described by Skerman et al. regarding cell morphology, flagellar arrangement, mol% G + C of the DNA, and various physiological and nutritional characteristics, were compatible with the characteristics of A. lipoferum, with

the exception of acid production from melibiose and hydrolysis of starch. When we performed these two tests using media and conditions that have been employed for azospirilla (10, 16), both strains were negative. Consequently we believe that, despite its isolation from freshwater sources, C. largomobilis subsp. largomobilis should be considered as a subjective synonym of A. lipoferum.

Surprisingly, C. largomobilis subspecies parooensis strain UQM 2402^T showed negligible DNA homology with the type strain of C. largomobilis subsp. largomobilis and with A. lipoferum. Thus this strain neither represents a subspecies of C. largomobilis nor a strain of A. lipoferum. Unlike the strains of C. largomobilis subsp. largomobilis, it failed to grow in semi-solid nitrogen-deficient malate medium and appeared to be incapable of nitrogen fixation. To what genus this strain should be assigned is uncertain.

On the basis of our RNA homology experiments, the nearest relatives of the genus Azospirillum, of those species tested, are the freshwater spirillum A. itersonii and the phototroph R. rubrum. A. itersonii had 72 to 73% RNA homology with A. lipoferum, and R. rubrum had 65% homology. Indeed, these homology values were of the same magnitude as those between A. amazonense vs. A. lipoferum and A. brasilense. These findings are in agreement with the report by Woese et al. (18) who found that these organisms are related on the basis of rRNA oligonucleotide cataloging with an S_{AB} value of 0.5. Our findings are also in agreement with the results of De Smedt et al. (4), who found that the rRNA cistrons of Azospirillum species were more similar to those of A. itersonii (and also Aquaspirillum polymorphum) than to those of a variety of other bacteria tested. De Smedt et al. have defined an rRNA superfamily which

contains, among other organisms, *Azospirillum*, *A. itersonii*, *Gluconobacter*, and *Beijerinckia*. With regard to *Gluconobacter*, the strains of *G. oxydans* used in our study showed RNA homology values ranging from 38 to 60% with *A. lipoferum*, *A. brasilense*, and *A. amazonense*. Similarly, the type strain of *B. indica* was related to these three *Azospirillum* species by 32 to 55% RNA homology; however, reference strain ATCC 9038 showed a lower degree of relatedness (11 to 32%).

In summary, our results indicate that (a) *A. brasilense*, *A. lipoferum*, *A. amazonense* comprise a genus having high interspecies RNA homology; (b) the genus *Azospirillum* belongs to an RNA homology group which also contains *A. itersonii*, *R. rubrum*, *R. tenue*, *G. oxydans*, and *B. indica*; (c) "*A. seropedicae*" is not a member of the genus *Azospirillum*; (d) *C. largomobilis* subsp. *largomobilis* should be considered as a subjective synonym of *A. lipoferum*; (e) *C. largomobilis* subsp. *parooensis* has no close relationship to *C. largomobilis* subsp. *largomobilis* or *A. lipoferum*, and (e) the previously unidentified "starlike" *Azospirillum* are strains of *A. lipoferum*.

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Summary and Conclusions

My DNA homology experiments showed that the group of 35 microaerophilic, nitrogen-fixing, sucrose-utilizing strains isolated by Magalhães et al. in 1983 is a distinct new species, *Azospirillum amazonense*. This correlates well with differences in phenotypic traits, which include formation of white rather than pink colonies on potato agar, the absence of lateral flagella, and the ability to use sucrose as a sole carbon source for nitrogen fixation.

My DNA and rRNA homology experiments indicated that the taxon proposed by Skerman et al. (1983), *Conglomeromonas largomobilis* subspecies *largomobilis* was genetically related at a level high enough to be included in the species *Azospirillum lipoferum*. Although these organisms were isolated from freshwater sources rather than from soil or plant roots, their phenotypic characteristics corresponded to those of *A. lipoferum*. *C. largomobilis* subspecies *parooensis* was found to have no close relationship to *C. largomobilis* subspecies *largomobilis* or *A. lipoferum*.

Other DNA homology experiments indicated that the *Azospirillum* strains which formed unique star-shaped colonies are strains of *A. lipoferum*.

Further DNA homology experiments indicated that "*A. seropedicae*" strains had no homology with strains of *A. lipoferum*, *A. brasilense*, or *A. amazonense*. Strains of "*A. seropedicae*" had previously been assigned to the genus *Azospirillum* based on their source (isolated from cereal roots), vibriod shape, microaerophilic nitrogen-fixing ability and denitrifying ability. However, my rRNA hybridization studies indicated that these organisms do not belong in the genus *Azospirillum*. This group of strains did not show significantly high homology values to any other genus tested including *Aquaspirillum itersonii*, *Beijerinckia indica*, *Gluconobacter oxydans*, *Rhodospirillum rubrum* and

Rhodospirillum tenue. Thus I propose that "A. seropedicae" be considered as a species incertae sedis.

My rRNA homology studies indicated that the genus Azospirillum shows enough genetic relatedness to Aquaspirillum itersonii, Beijerinckia indica, Gluconobacter oxydans, Rhodospirillum rubrum and Rhodospirillum tenue to be placed in the same rRNA superfamily. Practical considerations suggest that azospirilla (A. lipoferum, A. brasilense and A. amazonense) should be retained in a separate genus from these other organisms.

Appendix A

Unpublished Data

Thermal Stability Profiles. Another method besides rRNA hybridization experiments for comparing rRNA cistrons involves measuring the $\Delta T_{m(e)}$ values of DNA-rRNA hybrids. The difference between the $T_{m(e)}$ of a heterologous hybrid and that of a homologous hybrid is referred to as the $\Delta T_{m(e)}$, and is used as a measure of the degree of base-pair mismatching in the heterologous hybrid. The greater the amount of base mismatching between the DNA and rRNA of the heterologous hybrid, the less stable the hybrid will be. Thus the degree of RNA homology and heterologous hybrid stability are proportional. Estimates of base pair mismatching have indicated a range from 1 to 2.2% mismatching per degree of $\Delta T_{m(e)}$.

Thermal stability profiles of the homologous and heterologous rRNA hybrids of the type strains of Azospirillum species were determined. Direct binding reactions were carried out using a modification of the rRNA hybridization procedure. The washing buffers used were 4X SSC, and the membranes were not dried but skewered onto stainless steel insect pins which were stuck into rubber-stopper cored glass tubing. Membranes were transferred into 84-mm polypropylene tubes containing 1.0 ml of 4X SSC-1mM HEPES-50% formamide. As the temperature was increased in 5°C increments from 35°C to 90°C, the membranes were transferred through a series of tubes containing the same elution buffer which had been preheated to the existing waterbath temperature for 10 minutes. The radioactivity eluted at each temperature was measured by placing these tubes directly in a Beckman model 5500 gamma scintillation counter.

The thermal stability results are reported as midpoint temperatures ($T_{m(e)}$) and differences in midpoint temperatures ($\Delta T_{m(e)}$) (Table A1). The rRNA

TABLE A1. $\Delta T_{m(e)}$ values of DNA-rRNA thermal stability profiles

Species and strain	$\Delta T_{m(e)}$ (°C) with reference RNA from strain:			
	A. lipoferum ATCC 29707[T]	*A. brasilense* ATCC 29145[T]	*A. amazonense* ATCC 35119[T]	"*A. seropedicae*" ATCC 35892
A. lipoferum				
ATCC 29707[T]	68.8[a]	2.1	8.3	18.7
A. brasilense				
ATCC 29145[T]	6.7	66.5	8.9	19.3
A. amazonense				
ATCC 35119[T]	10.6	8.7	66.5	19.0
"*A. seropedicae*"				
ATCC 35892	19.4	16.0	16.2	67.6

[a] Homologous values are reported as midpoint temperatures.

homology values obtained by the competition technique were compared with the $\Delta T_{m(e)}$ values (correlation coefficient, -0.958) (Fig. A1). The data indicated that rRNA cistrons of *A. lipoferum*, *A. brasilense* and *A. amazonense* showed relatively little base pair mismatching ($\Delta T_{m(e)}$ values of 2.1 to 8.9°C), which we consider to be sufficiently low to warrant inclusion of these organisms in the same genus. The $T_{m(e)}$ values for *A. brasilense* and *A. amazonense* were identical (66.5°C) whereas the value for *A. lipoferum* was slightly higher (68.8°C). This suggests possible contamination with RNase, which would account for a decrease in thermal stability of the DNA-rRNA hybrid.

"*A. seropedicae*" however, exhibited a high level of base-pair mismatching with the rRNA cistrons of other *Azospirillum* species ($\Delta T_{m(e)}$ values of 16.0 to 19.4°C). These values were near the limit at which hybrids can be formed. These data indicate that "*A. seropedicae*" should not be included in the genus *Azospirillum*.

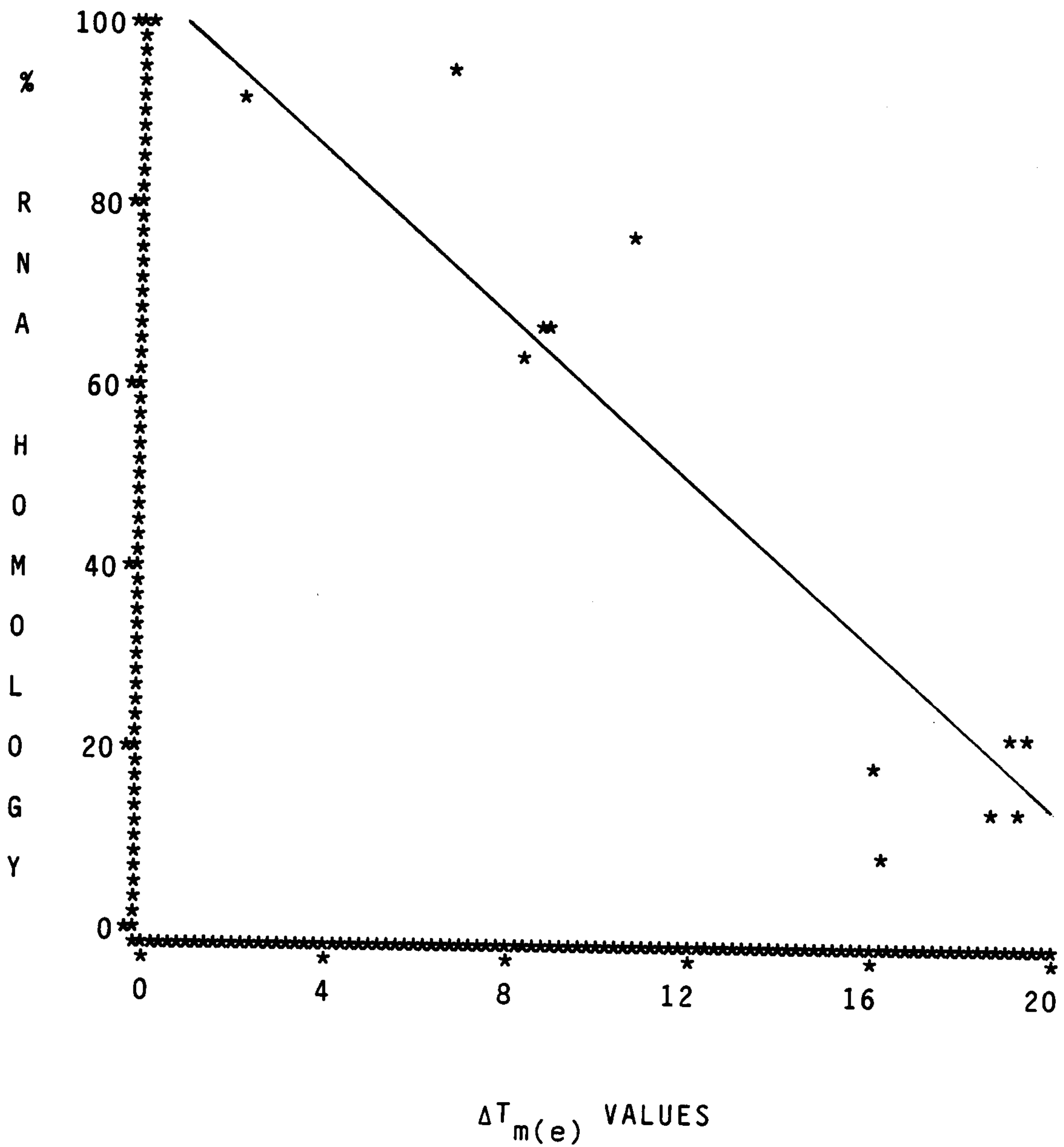

Figure A1. % RNA homology values compared to $\Delta T_{m(e)}$ values.

Appendix B

Materials and Methods

Culture media

The following culture media were used during the course of this study:

1. Nutrient broth and agar.
2. T-soy broth.
3. MPSS broth:

Ingredient	Grams per liter
Bacto-Peptone (Difco)	5.0
$(NH_4)_2SO_4$	1.0
$MgSO_4.7H_2O$	1.0
Succinic acid (free acid)	1.0

Add 1,000 ml of distilled water. Add 1.0 ml of 0.2% $FeCl_3.6H_2O$. Add 1.0 ml of 0.2% $MnSO_4$. Adjust pH to 7.0 with 2N KOH.

4. MPSS semisolid: MPSS broth supplemented with 1.5 g agar per liter.
5. MPSS agar: MPSS broth supplemented with 15.0 g agar per liter.
6. Döbereiner Semisolid Nitrogen-free Malate medium:

Ingredient	Grams per liter
KH_2PO_4	0.4
K_2HPO_4	0.1
$MgSO_4.7H_2O$	0.2
NaCl	0.1
L-Malic acid	3.58
Bromothymol blue	0.025

(Dissolve the bromothymol blue in a little water + a few drops of KOH before adding.) Add 2.0 ml of 1.0% $CaCl_2$ solution. Add 2.0 ml of 0.85% $FeCl_3.6H_2O$ solution. Add 1.0 ml of 0.2% Na_2MoO_4 solution. Adjust pH to

7.0 with KOH. Add 1.5 g agar; boil. When testing for biotin requirement, all glassware must be ashed, and caps boiled. Sterilize by autoclaving.

7. Döbereiner Semisolid Nitrogen-free Malate medium + Biotin: Döbereiner semisolid nitrogen-free malate medium supplemented with 1.0 ml of a 0.01% solution of biotin (prepared by adding 10 mg of biotin in 100 ml of distilled water - dissolve by heating).

8. Döbereiner Semisolid Nitrogen-free Glucose medium: Döbereiner semisolid nitrogen-free malate medium + biotin, replacing the malic acid with 100 ml of a 10% solution of glucose which had been sterilized by filtration (0.2 µm pore size), and replacing 1,000 ml of distilled water with 900 ml of distilled water. The sterile glucose solution was warmed to 45°C in a waterbath, and added aseptically to the 900 ml of basal medium, which had been boiled and autoclaved and placed in the 45°C waterbath. and mixed. The medium was then dispensed by pipette into sterile empty screw-capped tubes (20 x 125 mm).

9. Döbereiner Semisolid Malate medium: Döbereiner semisolid nitrogen-free malate medium + biotin is supplemented with 0.1% $(NH_4)_2SO_4$.

10. Döbereiner Liquid Malate medium: Agar is ommited from Döbereiner semisolid nitrogen-free malate medium + biotin.

11. Döbereiner Semisolid Nitrogen-free Sucrose medium: Malic acid is ommited from Döbereiner Semisolid Nitrogen-free Malate medium, and replaced with 50 ml of 10% sucrose (w/v), and 950 ml of distilled water is used in place of 1,000 ml. The sucrose solution is sterilized by filtration, and aseptically added to the 950 ml of basal medium, and mixed. The

medium is then dispensed aseptically into sterile screw-capped tubes, 10 ml per tube.

12. Peptone-based glucose medium:

Ingredient	Grams per liter
Peptone (Difco)	2.0
$MgSO_4.7H_2O$	1.0
$(NH_4)_2SO_4$	1.0
$FeCl_3.6H_2O$	0.002
$MnSO_4.H_2O$	0.002
Bromothymol blue	0.025

The bromothymol blue is dissolved in a little water and KOH before adding. The medium is made up to a volume of 950 ml, adjusted to pH 7.0 and sterilized by autoclaving. After it has cooled, 50 ml of a 20% (w/v) solution of glucose (sterilized by filtration) is added aseptically.

13. Peptone-based melibiose medium: Glucose is replaced by melibiose in the peptone-based glucose medium.

14. Starch agar:

Ingredient	Grams per liter
Bactopeptone (Difco)	10.0
Succinic acid (free acid)	1.0
$(NH_4)_2SO_4$	1.0
$MgSO_4.7h_2O$	1.0
$FeCl_3.6H_2O$	0.002
$MnSO_4.H_2O$	0.002
Starch	100.0
Agar	15.0

pH to 6.8 with KOH, boil, sterilize by autoclaving, temper to 45°C, Pour onto plates.

15. Van Niel's Yeast broth:

Ingredient	Grams per liter
K_2HPO_4	1.0
$MgSO_4$	0.5
Yeast Extract	10.0

Add to 1,000 ml of tap water, pH to 7.0, sterilize by autoclaving.

16. Sorbitol-Yeast Extract-Peptone Broth:

Ingredients	Grams per liter
Yeast extract	1.0
Sorbitol	5.0
Peptone	1.0

Add to 1,000 ml of distilled water, pH to 6.0, sterilize by autoclaving.

17. Variation of Döbereiner semisolid Malate medium:

Ingredient	Grams per liter
Malic acid	3.58
K_2HPO_4	0.8
KH_2PO_4	0.2
$MgSO_4.7H_2O$	0.5
$FeCl_3.6H_2O$	0.025
Na_2MoO_4	0.005
$CaCl_2$	0.05
$(NH_4)_2SO_4$	1.0

Add to 1,000 ml of distilled water, pH to 6.9, sterilize by autoclaving.

18. Soil Extract:

Ingredient	Grams per liter
African Violet Soil	385.0
Na_2CO_3	1.0

Add to 1,000 ml of distilled water, autoclave for 1 hour, filter through paper before using in media.

19. Azotobacter Basal Medium:

Ingredient	Gram per liter
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$FeSO_4$	0.005

Add 100 ml of soil extract, 900 ml of tap water, 15 g of agar and boil. Sterilize by autoclaving.

20. Azotobacter Supplement: Add 2% glucose to Azotobacter Basal medium. pH to 6.0.

Maintenance and preservation of cultures

All cultures were maintained in 10 ml volumes of media in 20 x 125 mm screw-capped tubes, and transferred weekly with sterile Pasteur pipets unless otherwise stated. *Azospirillum amazonense* cultures were maintained in Döbereiner semisolid nitrogen-free sucrose medium at 30°C, and transferred monthly with sterile Pasteur pipets. MPSS Semisolid medium was used for the maintenance of *A. lipoferum* and *A. brasilense*, which were kept at 30°C. "*A. seropedicae*" cultures, "star-like" *Azospirillum* strains and *Conglomeromonas largomobilis* subspecies *largomobilis* strains were maintained in Döbereiner semisolid nitrogen-free malate + biotin medium at 30°C. *Conglomeromonas largomobilis* subspecies *parooensis* was maintained in peptone semi-solid medium at 30°C. *Aquaspirillum* cultures were maintained in MPSS semisolid medium at 30°C. *Rhodospirillum rubrum* cultures were maintained in T-soy broth at 30°C. *Rhodospirillum tenue* cultures were maintained in Van Niel's yeast agar at 30°C. *Beijerinckia* cultures were maintained in Azotobacter Supplement medium at 30°C. *Gluconobacter* cultures were maintained at room temperature in sorbitol-peptone-yeast extract medium.

Type strains, reference strains and strains having unusual characteristics were stored in liquid nitrogen. Strains to be stored in liquid nitrogen were grown for 24-48 h on nutrient agar slants, or in the case of

Beijerinckia,Rhodospirillum or Gluconobacter the medium used was that stated for maintenance of the culture. The growth from the slant, or in the case of Beijerinckia, Rhodospirillum or Gluconobacter the top 1-2 ml of growth from this culture was resuspended in 2.0 ml of nutrient broth containing 10% sterile dimethyl sulfoxide (DMSO). Approximately 0.5 ml of this mixture was pipetted into a sterile, cotton-stoppered glass cryule (Wheaton), sealed, and placed in liquid N_2.

Growth of cultures for nucleic acid extraction

All Azospirillum amazonense strains used in this study were grown in nutrient broth, pH 6. Two liters of nutrient broth was poured into a four liter flask and the flask stoppered with a cotton plug, and covered with a layer of paper towels. The flasks were autoclaved for 40 min. Each flask was inoculated with 24-48 h old cultures grown in 10 ml of nutrient broth pH 6. Inoculations were performed with sterile 10 ml pipets. After inoculation, the flasks were incubated at 37°C in a shaking water bath at a setting of 90 rpm or on a stir-plate until the broth in the flasks was fairly turbid, usually 24 hours, before harvesting.

All other cultures were grown as above, with the exception that all other Azospirillum strains and Conglomeromonas largomobilis subspecies largomobilis strains were grown in MPSS broth, otherwise the same medium used for maintenance was used throughout. All incubations were at 37°C for Azospirillum strains, otherwise, maintenance temperatures were used. Both Rhodospirillum strains were grown in the dark, aerobically. Two flasks usually yielded enough

DNA or rRNA for the homology experiments. The contents of each flask were checked by phase microscopy before pooling prior to nucleic acid extraction.

Nucleic Acid Isolation

Buffers and reagents

The following reagents and buffers were used in the isolation procedures for DNA and rRNA. Glassware used for rRNA isolation was baked in a dry-heat sterilizing oven. Aqueous buffers and solutions used for rRNA isolation were autoclaved first, and then treated by adding 0.2% diethyl pyrocarbonate.

The following reagents and buffers are stored under refrigeration:

Chloroform: phenol mixture: 490 ml saline-EDTA saturated liquified phenol, 490 ml chloroform and 20 ml octanol. 8-hydroxyquinoline is added to a final concentration of 0.1%. The saline-EDTA saturated phenol is prepared by adding approximately 800 ml of liquified phenol to a glass beaker containing a magnetic stirring bar. The beaker is placed on a magnetic stirring plate under a hood and saline-EDTA buffer is added slowly until no more will go into solution and a phase separation occurs. This mixture is kept in a 2 liter ground glass stoppered reagent bottle under refrigeration.

Diethyl pyrocarbonate: Sigma Chemical Company No. D-5758; Lot 53F-3666, 100 ml, Molecular weight - 162.1. This reagent is very unstable in water; add it just prior to lysing the cells. This reagent is kept refrigerated in its original container.

Distilled water: Kept in a 1,000 ml screw-capped flask under refrigeration.

Phenol-cresol mixture: 550 ml liquefied phenol, 70 ml 10% M-cresol and 0.5 µg 8-hydroxyquinoline. This mixture is kept in a 2 liter ground glass stoppered reagent bottle jar under refrigeration.

Protease: "Pronase" (Ex Strep. griseus), Calbiochem-Behring Corp., La Jolla, CA; 53702, 5 g., Lot 903524, B grade, Activity : 56,300 PUK/gm. This was kept in the original bottle under refrigeration.

Saline-EDTA buffer: 0.15 M NaCl, 0.01 M Na EDTA, pH 8.0. This buffer is kept in a 1,000 ml screw-capped flask under referigeration.

3 M Na acetate, pH 6.0: This is kept in a 1,000 ml screw-capped flask under refrigeration.

Sodium naphthalene 1,5-disulfonate: 10% (w/v) and 0.5% (0.5 ml 10% + 9.5 ml distilled water). This is kept in a 250 ml screw-capped flask under refrigeration.

Standard Saline Citrate (SSC) buffer: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0. This buffer is used in several different concentrations, i. e. 1X SSC, 0.1X SSC. A stock solution of 20X SSC was prepared and adjusted to pH 7.0. Other working concentrations were prepared from this stock solution. After dilution, the pH of the working buffers was checked and adjusted to pH 7.0 if necessary. SSC containing 1% SDS was also prepared for rRNA isolation.

The following reagents are stored in a -20°C freezer:

Ethanol: 95% and 80%. Stored at -20°C in 1 liter screw-capped bottles.

Ribonuclease A, bovine pancreatic (Sigma): 100 mg was dissolved in 100 ml of 0.1X SSC and heated in a hot water bath at 80°C for 20 min to inactivate any residual DNase activity. 100 ml of cold 0.1X SSC was added and the mixture

chilled on ice. The mixture was then placed into 16 x 125 mm screw-capped tube in 10 ml amounts and stored until needed at -20°C.

Ribonuclease T_1, from Aspergillus oryzae (Sigma): 5000 units of RNase T_1 was dissolved in 10 ml of 0.1X SSC-10^{-2}M MES buffer. This mixture was heated as above to remove any DNase activity and 10 ml of 0.1X SSC-10^{-2} MES was added and the mixture chilled on ice. Ten ml amounts of RNase T_1 were stored at -20°C in 16 x 125 mm screw-capped test tubes.

The following reagents are stored at room temperature:

1 M phosphate buffer: 1 M NaH_2PO_4 and Na_2HPO_4. Prepared by mixing equal volumes of 1 M NaH_2PO_4 and 1 M Na_2HPO_4. The pH of a 1:10 dilution of this buffer is approximately 6.8, but the concentrated stock solution will be somewhat higher. Stored in 1 liter screw-capped bottles at room temperature. A few drops of chloroform were added periodically to prevent fungal growth. **DO NOT REFRIGERATE!.** Refrigeration causes the phosphate to precipitate.

20% (w/v) Sodium dodecyl sulfate (SDS): standard reagent grade SDS was used for the DNA isolations and electrophoretic grade SDS was used for rRNA isolations. Stored in a screw-capped bottle at room temperature. In cold weather, the bottle was stored in a 37°C incubator.

Low Molecular Weight DNA Isolation Procedures

The cells were harvested by centrifugation in 250 ml polypropylene centrifuge tubes in a refrigerated centrifuge at 10,000 rpm (15300 x g) for 10 min at 0-to-4°C. The supernatant was carefully poured off. The cell pellets were

resuspended in saline-EDTA buffer. The EDTA in this buffer deactivates any DNase activity that might be present when the cells are lysed by binding up Mg^{++}, which is required for DNase activity. It may also make lysing of the cells easier by binding divalent cations that lend strength to the outer membrane of Gram-negative bacteria. The final volume of the cell mixture was adjusted to 50 ml or 100 ml depending on the volume of cells being harvested or the procedure being used for isolating the DNA. The hydroxyapatite (HA) procedure was used to isolate all of the DNA (low molecular weight) used in the DNA homology experiments. The final volume of the lysing mixture was usually adjusted to 50 ml for the DNA isolations, or 25 ml if the yield of cells was very bad.

One-half ml of RNase A (bovine pancreatic, Sigma) and 0.5 ml of RNase T_1 was added to the cell suspension and mixed well. RNase is a good general purpose ribonuclease and RNase T_1 is effective against rRNA from organisms having a high mol% G + C content. Next, 20% sodium dodecyl sulfate was added to a final concentration of 1% (2.5 ml to 50 ml, 1.2 ml to 25 ml) and the mixture incubated overnight in a 60°C shaking water bath. This provided a much higher DNA yield than merely swirling the mixture under hot running tap water. The SDS disrupts the integrity of the cell membrane because of its detergent action. Lysis of the cells was indicated by an increase in the viscosity of the lysate and a clearing of the mixture. The amount of viscosity is a good estimate of the amount of DNA present- the higher the viscosity, the more DNA is present. Next, the viscosity of the lysate was reduced by brief sonication. This allows the phenol:chloroform used for protein removal to become mixed in more easily and results in shorter strands of double stranded DNA which are more easily adsorbed onto the HA particles. A probe sonicator was used (Lab Line

Ultratip Labsonic System, Lab Line Instruments, Inc., Melrose Park, Illinois) and the lysate was subjected to 2 to 3,and 3 to 5 sec intervals of sonication with an intermediate sized tip. After sonication, a pinch of Pronase was added and the mixture was incubated at 50°C in a shaking water bath for 1 hour. The proteins were then extracted from the lysate with the chloroform:phenol mixture. Fifteen ml of this mixture was added for every 50 ml of lysate and the mixture shaken on a wrist-action shaker for 20 min. The mixture was placed in a 50 ml polypropylene centrifuge tube and centrifuged at 12,000 rpm (17,400 x g) for 10 min. The upper (aqueous) layer was carefully removed, using an inverted 10 ml pipet in a propipette and returned to the lysing flask for an second chloroform-phenol extraction. This extraction and centrifugation were performed as before. Care must be taken to avoid pulling up the white layer of denatured protein that forms at the interface of the aqueous and the chloroform-phenol phases. This is especially true after the second phenol extraction. After the second protein extraction, The supernatant was dialyzed to remove RNase inhibitors. Spectrapor (Spectrum Medical Industries, Inc., Los Angeles) membrane tubing, 45mm, 12,000 to 14,000 m. w. cutoff was used as dialysis tubing and was boiled for a few minutes in 2% Na_2CO_3 solution, rinsed under running tap water and washed in distilled water. The supernatant was dialyzed in a 4 liter Nalgene bucket against 4 liters of cold saline-EDTA on a stir-plate in a refrigerated cold room for a few hours, then the saline-EDTA was changed for fresh saline-EDTA, and the sample was allowed to dialyze overnight. The material was then removed from the dialysis bag to a flask. One-half ml of RNase T1 and 0.5 ml of RNase A were added and the mixture was incubated at 37°C on a waterbath shaker (gentle shaking) for an hour. Then a chloroform extraction was

performed using 5 to 10 ml of chloroform, shaking as for the phenol extraction technique, centrifuged, and the upper layer removed just as with the phenol extraction technique. Then enough 1 M phosphate buffer is added to make the final concentration of the lysate 0.1 M phosphate. Then 2g (1 tbsp.) of DNA grade hydroxyapatite (Biogel, Biorad) was added to each. This mixture was then shaken on a wrist action shaker for 1 h. The HA-lysate suspension was placed in a 50 ml polypropylene centrifuge tube and centrifuged at top speed in a IEC Model CL clinical centrifuge for 1-2 min to pellet the HA. The supernatant was then poured back into the flask for a second HA elution. The pellet was then washed with 0.1 M PO_4 in the following manner. Eight ml of 0.1 M PO_4 was added to the tube with a Cornwall syringe and the tube was vortexed at a setting of 7 on a Vortex-Genie. Twenty-four ml of 0.1 M PO_4 was added in aliquots of 8 ml each at the interface of the liquid and the tube wall with sufficient force to vigorously mix the HA and the PO_4 buffer. This mixture was centrifuged as before and the supernatant discarded. This washing procedure was repeated 6 times. After the sixth wash, 5 ml of 0.5 M PO_4 was added to the HA pellet and the tube vortexed at the maximum speed on the Vortex-Genie. The tube was placed in the clinical centrifuge and spun as before. The supernatant, which contained the eluted DNA was poured into a 10 ml B-D Multifit glass syringe and filtered through a Whatman glass fiber filter (934-AH, 2.4 cm dia.) in a Gelman 1 in dia. Easy Pressure filter holder. The filtered DNA solution was stored in a 18 x 125 mm screw-capped tube at -20°C until needed. The second HA elution was performed in the same manner described above.

High Molecular Weight DNA Isolation

The cells were harvested by centrifugation in 250 ml polypropylene centrifuge bottles in a refrigerated centrifuge at 10,000 rpm (15300 x *g*) for 10 min at 0-to-4°C. The supernatant was carefully poured off. The cell pellets were resuspended in 100 ml of saline-EDTA buffer. One-half ml of RNase A and 0.5 ml of T1 RNase was added to the cell suspension and mixed well. Next, 5 ml of 20% SDS was added, and the mixture was placed in a 60°C shaking water bath overnight. After this, a pinch of Pronase was added, and the mixture was incubated for 1 hour in a 50°C shaking waterbath. Sodium perchlorate was added to a final concentration of 1 M. Then 0.5 volume of chloroform:phenol:octanol was added and the mixture was shaken on a wrist-action shaker in a 500 ml Erlenmeyer flask covered with foil for 20 minutes, using a shaking speed just sufficient to produce an emulsion. Very vigorous shaking is not desirable. The emulsion was centrifuged at 12,000 rpm (17,400 x *g*) for 15 minutes in a refrigerated centrifuge at 0-to-4°C. The upper layer was removed with an inverted 10 ml serological pipette, with the tip inserted into a Propipette (Fisher Scientific Co., Pittsburg, Pa.), being careful not to collect any of the white protein precipitate at the interface between the two phases. The aqueous layer contains the DNA and is therefore very viscous. It is helpful to move the pipette continually back and forth in the tube to avoid collecting any of the protein at the interface. The lysate was extracted again with 0.5 volume of chloroform:phenol:octanol. After this the aqueous phase was placed in a beaker and slowly overlayed with cold 95% ethanol in an amount equal to 2 volumes of the aqueous phase. The precipitated DNA was collected with a glass stirring rod by gently stirring the two phases while spinning the rod. The DNA adhered or

"spooled" onto the rod. The excess ethanol was removed by pressing the rod against the side of the beaker. The spooled DNA was washed in a test tube containing 10 to 15 ml 80% ethanol. The DNA was washed twice in separate tubes. The excess ethanol was allowed to drain off by placing the rod vertically with the DNA end up for a few minutes. The spooled DNA was dissolved in 30 ml of 0.1X SSC. The glass rod was allowed to stand in the SSC until the DNA loosened and could be slipped off the rod. It was sometimes necessary to add 3 to 5 drops of chloroform in the tube and leave overnight in the refrigerator. After the DNA was completely dissolved, the SSC concentration was adjusted to 1X SSC by adding a suitable volume of 20X SSC. RNase A and RNase T1 were added at 0.5 ml volumes to the DNA preparations and the mixture was incubated in a 37°C waterbath for 30 minutes to 1 hour. Then the chloroform:phenol:octanol extraction was repeated until very little protein was observed between the two phases after centrifugation (This was done two times at the most). The DNA was precipitated again using the same procedure with ethanol. This was done to remove ribonucleotides. The SSC concentration was adjusted to 1X SSC before each ethanol precipitation. Finally the DNA was dissolved in 0.1X SSC in a 29 x 120 mm screw-capped tube and stored in a freezer at -20°C until needed.

Ribosomal RNA Isolation Procedure

The cells were harvested by centrifugation in sterile 250 ml polypropylene centrifuge bottles in a refrigerated centrifuge at 10,000 rpm (15300 x *g*) for 10 min at 0-to-4°C. The supernatant was poured off and the cell pellets combined and washed with sterile distilled water. The cells were suspended in 15 ml of

cold distilled water. The volume was checked with a graduated cylinder. One-half ml of 10% naphthalene disulfonate was added for each 1.0 ml of the suspension. Diethyl pyrocarbonate was added to a final concentration of 0.2% just before the cells were lysed. The cells were disrupted immediately by passage through a French pressure cell at 10,000 to 12,000 psi into a mixture containing 10 ml of phenol-cresol, 10 ml of 0.5% napthalene disulfonate, and 20 µl of diethyl pyrocarbonate. The flask was then shaken for 20 minutes on a wrist action shaker. The mixture was then placed in a sterile polypropylene centrifuge tube and centrifuged at 12,000 rpm (17,400 x g) for 10 minutes in a refrigerated centrifuge at 0-to-4°C. The upper aqueous layer was carefully drawn off with a sterile inverted 10 ml pipette, with the tip placed in a Propipette. Twenty-times SSC was then added in a ratio of 1 part to 20 parts of the aqueous phase, and 20% SDS was added to a final concentration of 1%. The mixture was re-extracted with 15 ml of phenol-cresol until the aqueous layer was clean. The rRNA was precipitated by adding a double volume of ice-cold 95% ethanol. This was mixed and placed in a -20°C freezer for 1 hour or overnight in a sterile 250 ml polypropylene centrifuge bottle. The rRNA was then centrifuged at 8,000 rpm (9820 x g) for 10 minutes. The supernatant was decanted and the rRNA pellet in the centrifuge bottle was allowed to drain well. The rRNA pellet was then extracted with 25 ml of cold 3 M sodium acetate. This was mixed until the pellet was re-suspended. The mixture was then centrifuged at 8,000 rpm (9820 x g) for 10 minutes. The acetate buffer was then decanted and the rRNA pellet was allowed to drain well. The rRNA pellet was then dissolved in 30 ml of 1X SSC. The ethanol precipitation, centrifugation, and dissolution was repeated.

The final rRNA pellet was dissolved in 10 ml of 1X SSC containing 1% SDS and stored in a 16 x 150 mm sterile screw-capped tube at -20°C.

Determination of concentration and purity of DNA preparations

DNA concentration and purity was determined on a Gilford recording spectrophotometer using the following procedure. A 1:20 dilution of the DNA preparation was made by placing 50 µl of the DNA preparation in 0.95 ml of 0.1X SSC in a 12 x 75 mm serum tube . This mixture was vortexed and 0.25 ml of this mixture placed into a quartz microcuvette. The OD at 260 nm was determined. The OD of a 1:20 dilution will give the concentration of DNA in mg/ml if the DNA preparation is 100% pure. The purity was determined from the increase in absorbance that occured when the preparation was heated until it became single-stranded. The microcuvette was heated in a thermal cuvette holder and the temperature was raised from an original temperature of 50°C at a rate of 1°C per min. The rate was controlled by a thermal progammer (Gilford) and the absorbance was followed on a chart recorder. The purity could be determined by dividing the change in OD by the original OD and dividing this value by 0.4. One hundred percent pure DNA should have an increase in OD of 40% when it becomes single stranded. The concentration was then determined by multiplying the OD at 260 nm by the purity.

Determination of concentration of rRNA preparations

Ribosomal RNA concentration was determined on a Gilford recording spectrophotometer using the following procedure. A 1:50 dilution of the rRNA preparation was made directly in a quartz microcuvette (Gilford 1242x103) by

placing 5 µl of the sample in with 245 µl of 1X SSC. The OD at 260 nm was determined. The OD was then multiplied by the dilution, and this adjusted OD was divided by 23 to yield the milligrams of rRNA per ml.

Preparation of Low Molecular Weight DNA for homology experiments

The first step was to remove the phosphate from the DNA preparations. This was accomplished by dialyzing the preparations against 0.1X SSC. Five to 6 inch strips of dialysis tubing (Spectrapor 2, Fisher, 25 mm dia. 12,000 to 14,000 M.W. cutoff) were cut and boiled in a solution of 2 to 5% $NaCO_3$ for several min. The tubing was then washed first in tap water and then with distilled water. The DNA preparations were placed in the dialysis bags and 15 to 20 bags were placed in 4 liters of 0.1X SSC, pH 7.0 in a 4 liter Nalgene beaker with a magnetic stir bar. The beaker was placed on a magnetic stirrer in the cold room and the solution stirred at an intermediate speed for 4 to 6 hours, after which the bags were placed in another 4 liter Nalgene beaker containing fresh dialysis buffer and the dialysis continued with stirring overnight. The next day, the bags were opened with scissors over small glass beakers and the DNA preparations placed into 18 x 125 mm screw-capped tubes and stored at -20°C until needed. Most of the DNA preparations used in the DNA homology experiments were at a concentration of 0.4 mg/ml. If the yield of DNA from certain strains was lower than this, several preparations had to be pooled and concentrated by ethanol precipitation and dissolving in a smaller volume of 0.1X SSC. This was accomplished in the following manner. Several DNA preparations from the same strain, in 0.1X SSC buffer, were combined in one or more 50 ml polypropylene centrifuge tubes and 1/10 volume of 3M sodium acetate added to each tube. The

maximum volume in each tube was 13 ml, because 2 volumes of ice-cold ethanol was then added. The tubes were then placed at -20°C for at least 1 hour. The tubes were then centrifuged at 17,000 x g for 10 min, the supernatant carefully poured off, so that the precipitated DNA was not lost, and the tube inverted and drained. After most of the ethanol was gone, the DNA pellet was resuspended in a small volume of 0.1X SSC, the exact volume depending on the amount of DNA present in the pellet, which could be estimated from the concentrations and purities of the preparations used. DNA preparations for use in homology experiments were then sheared by two passages through a French pressure cell at 15,000 psi. Prior to shearing, a small amount of the DNA prep was removed, diluted to a concentration of 50 μg/ml with 0.5X SSC (final volume 2 or 3 ml) and stored at -20°C in a 15 x 75 mm screw-capped tube for use in mol% G + C determinations. The preparations were then denatured by boiling for 10 min and placed directly in an ice bath. After cooling, they were centrifuged in 25 ml polypropylene centrifuge tubes at 15,000 rpm for 15 min. at 0-4°C. The DNA preparation was carefully drawn off with a Pasteur pipette, being careful not to draw up any of the unwanted material that had pelleted in the tube. The concentration of these preps was then determined by measuring the OD at 260 nm of a 1:20 dilution of the prep in 0.1X SSC. The concentration (mg/ml) was then obtained by multiplying this value by 0.87, which takes into account the increased absorbance of single-stranded DNA. All preparations were then adjusted to 0.4 mg/ml with 0.1X SSC and stored at -20°C in 15 x 75 mm screw-capped tubes.

Preparation of competitor rRNA for hybridization experiments

Competitor rRNA samples were adjusted to a concentration of 2 mg/ml with 1X SSC. If the concentration of the sample was below 2 mg/ml, the sample was precipitated with two and one-half volumes of cold ethanol in a 250 ml polypropylene centrifuge bottle by placing the mixture at -20°C for one hour, centrifuging in a refrigerated centrifuge at 8,000 rpm (9820 x g) for 15 minutes, and decanting the ethanol. The pellet was drained and re-dissolved in 1X SSC, adjusting the concentration to 2 mg/ml. The preparations were then heat denatured for 5 minutes in a boiling water bath and after rapid cooling in an ice bath, stored at -20°C. One percent SDS (electrophoretic grade) was added to all samples.

Preparation of low molecular weight DNA for I-125 labeling

The low molecular weight DNA from reference strains that was to be labeled was prepared in the following way. Thirty µl of the DNA preparation (0.4 mg/ml) was placed into a Microfuge tube (Brinkman 22 36 430-8) along with 10 µl of 3 M Na acetate, pH 6.0, 60 µl sterile distilled water and 200 µl of ethanol. The tube was then placed in a freezer at -20°C for 1 h. The tube was then spun in a Eppendorf Model 5413 Microfuge for 8 to 10 min and the supernatant was drawn off with a Pasteur pipet. Care was taken to follow the meniscus and not dislodge the DNA pellet. One ml of an ice-cold mixture of 80% ethanol, 20% 0.2 M Na acetate, pH 4.6 was added and the tube placed at -20°C for 15 to 30 min. The tube was then spun and the supernatant drawn off as before. Another brief spin (10 to 15 sec) was performed to collect any residual fluid. The DNA pellet was then dissolved in 50 µl of a mixture of $NaClO_4$ and 0.05 M Na acetate.

Preparation of rRNA for labeling with I-125

There are several classes of RNA, therefore the RNA that one wishes to label for use in a hybridization experiment in usually separated from the rest. Although the ribosomes may be isolated and their subunits separated, and rRNA isolated from them, the total RNA is usually fractionated. In separating the RNA components by sucrose gradient centrifugation, the first step was to obtain a 2 ml sample of the isolated rRNA sample, adjusted to a final concentration of 1.5 mg/ml with 1X SSC. These samples were gently layered using a sterile pasteur pipette onto a sucrose gradient prepared with 5 to 20% sucrose in 1X SSC. The sucrose gradients were then centrifuged in a Sorval OTD-75 Ultra Centrifuge-Oil Turbine Drive using a AH627 rotor at 8°C, 25 K for 15 hours. Twenty-three S and 16 S fractions were then collected together using a Gilford 2400 recording spectrophotometer to monitor the optical densities of progressive layers of the sucrose gradient. The samples were then precipitated with two and one-half volumes of cold ethanol in 250 ml polypropylene centrifuge bottles at -20°C for 2 hours. The samples were then centrifuged in a refrigerated centrifuge at 8,000 rpm (9820 x *g*) for 15 minutes. The ethanol was decanted, and the samples were re-dissolved in sterile 1X SSC. DNA concentration was determined on a Guilford 2400 recording spectrophotometer by preparing a 1 to 20 dilution with 1X SSC directly in a quartz microcuvette (Gilford 1242x103).

Preparation of High Molecular Weight DNA for homology experiments

The first step was to adjust the concentration of the 4.35 mg of DNA to 50 μg per ml with 0.1X SSC. Then the DNA was denatured by heating in a 125

ml flask in a boiling water bath for 10 minutes. The DNA was cooled quickly by pouring it into 800 ml of cold 6X SSC, which should give 5 µg per ml of DNA. A 15-cm diameter BA 85 nitrocellulose membrane filter (Schleicher and Schuell, Inc., Keene, N. H.) was floated on distilled water so that the pores were filled with water. If the membranes were to be submerged immediately, air pockets would form and cause uneven filtration. The wet membrane filter was placed on a filtration device constructed mainly from Plexiglass and having a porous polyethylene filtration surface. The base of the apparatus is a short Plexiglass cylinder with the outside diameter the same as that of the membrane. The porous polyethylene is mounted on the inside and flush with the top. The base has a single exit tube, the flow rate of which can be controlled with an adjustable pinch clamp. The top part of the device consists of a Plexiglass cylinder with a Neoprene (1/8 inch thick) gasket glued to the bottom rim. The top part is placed on top of the membrane and is held on by spring tension. The membrane on the filtration device was washed with 500 ml of cold 6X SSC, using a flow rate of approximately 30 ml/min. The denatured DNA was then passed through the filter, and washed again with 500 ml of 6X SSC. The membrane was then dried at room temperature, and then overnight at 60°C. Then the membrane was labeled on the edge with a pencil, the filter was cut in half, and the halves were seperated with half a sheet of the paper that is used to separate the membranes in the shipping box, and placed in an envelope. The membranes were handled only by the outside edges to avoid touching the surface on which the DNA was bound.

<u>Labeling of reference low molecular weight DNA with I-125</u>

The procedure used for labeling low molecular weight DNA from reference strains is a modification of the procedure of Commerford (1971) and Tereba and McCarthy (1973) as described by Selin et al. (1983). Twelve µl of each DNA (30 µl of 0.40 mg/ml) plus 3 µl of 3M Sodium acetate buffer, pH 6.0 was added to 1 ml serum vial (Wheaton 400) and mixed and 66 µl of cold ethanol was added, mixed and left in at -20°C for 30 minutes. The mixture was then centrifuged for 10 minutes, and the pellet was washed with 0.5 ml of 0.2 M sodium acetate. The pellet of DNA was then dissolved in 35 µl of $NaClO_4$ (7.2 M)-Na acetate (0.08 M)-KI (2.8×10^{-5}) buffer. The $NaClO_4$ was added to insure single-stranded DNA (Chan et al., 1976). The vial was then capped and sealed with an teflon-lined aluminum seal (Wheaton) which was crimped on. One ml of air was removed with a 1 ml syringe and 2 µl of ^{125}I and 12.5 µl of $TlCl_3$ (1 mg/ml) was injected into the reaction vial. The $TlCl_3$ is the catalyst and initiates the reaction when added. The vial was mixed and incubated in a water bath at 70°C for 15 min. During this time the ^{125}I forms covalent bonds with the cytosine moieties of the DNA. The reaction mixture was then cooled on ice and 150 µl of 0.5 M phosphate buffer (pH 6.8) containing 0.01% mercaptoethanol was added to stop the reaction. A second incubation at 70°C was performed to disrupt any weak bonds between the iodine and the DNA. The contents of the vial were then removed with a 1 ml disposable syringe and placed on a Pharmacia PD-10 column which had been equilibriated with 1X SSC containing 0.4% electrophoretic grade SDS. The DNA was eluted from the column in a 2 ml volume into a collection tube which also contained 40 µg of sheared, denatured

salmon sperm DNA. The column was discarded as radioactive waste after use. The DNA preparation was then heated in a boiling water bath for 5 min and then passed through a Pasteur pipet packed with glass wool and a column of activated hydroxyapatite (DNA grade, Biogel, Bio-Rad) 2.5 cm in length which was equilibriated with 0.14 M phosphate buffer (pH 6.8) containing 0.4% SDS. The Pasteur pipet columns were placed inside of 16 x 125 mm screw-capped test tubes in a 70°C water bath and the elutes collected in the test tubes. After the first gravity elution, the columns were transfered to clean, preheated test tubes. The purpose of the HA column was to remove any double stranded DNA along with any weakly bound ^{125}I. The labeled DNA preparations were then passed through another Pharmacia PD-10 column equilibriated with 0.1X SSC and eluted with 2 ml of 0.1X SSC into a 50 ml centrifuge tube. This step removed the phosphate. The DNA samples were then precipitated by adding 0.2 ml of 3 M Na acetate, pH 6 and 5 ml of ice-cold ethanol. After at least 2 h in a freezer at -20°C, the sample was centrifuged at 12,000 x g for 10 min and the DNA pellet redissolved in 3.3 ml of 0.1X SSC and stored at -20°C until needed. The final concentration of the ^{125}I DNA was 3 µg/ml.

Labeling of reference 23S and 16S rRNA with I-125

The procedure used for labeling rRNA from reference strains is a modification of the procedures of Tereba and McCarthy (1973) as described by Selin et al. (1983). The rRNA samples (500 - 600µg) were precipitated with 0.1 M sodium acetate (pH 6.0) and two volumes of ethanol. After centrifugation the rRNA pellet was extracted with 1 ml of 80% ethanol-20% 0.2 M sodium acetate buffer (pH 4.8) and then dissolved in 0.05 M sodium acetate buffer (pH 4.8). The

iodination reaction components (except for the $TlCl_3$) were added to a one ml serum vial. The components included 10 µl 0.2 M Na acetate buffer, pH 4.8, 2 µl 7×10^{-4} M KI, 2 µl ^{125}I and 20 µl rRNA. The vial was then capped, one ml of air was removed with a syringe and 12.5 µl $TlCl_3$ was then added by injection. The vial was mixed and incubated in a water bath at 70°C for 20 minutes. The vial was then cooled on ice and two volumes of 0.5 M phosphate buffer (pH 6.8) containing 0.01% mercaptoethanol was added to stop the reaction. After incubating at 70°C for a second period of 20 minutes to disrupt weak iodine associations, the contents of the vial were removed using a disposable syringe and placed on a PD-10 column (Pharmacia) equilibrated with 1X SSC. The front peak of labeled rRNA was collected in a 2.0 ml volume and the column was capped and discarded. The labeled rRNA was again incubated at 70°C for 20 minutes to ensure removal of all of the weakly bound ^{125}I and, after cooling placed on a second PD-10 column (equilibrated with 0.1X SSC). The rRNA was eluted in the front peak (3.5 ml) and precipiated by adding 0.3 ml of 3 M sodium acetate (pH 6.0) and two volumes of ethanol. After at least 2 hours at -20°C the rRNA was collected by centrifugation. Labeled rRNA was centrifuged two times in sucrose gradients. Labeled rRNA was dissolved in 3.3 ml in 1X SSC - 0.5% SDS (30 µg/ml) and stored at -20°C.

DNA homology experiments

Buffers and reagents

The following buffers and reagents were used in the DNA homology experiments:

Buffer A: 0.05 M sodium acetate-0.3M NaCl-0.5 mM $ZnCl_2$, pH 4.6. Stored in a screw-capped 1 liter bottle in the refrigerator.

5.28 M NaCl-10^{-2} HEPES: Stored in the refrigerator in 50 ml screw-capped tubes.

1 N HCl mixture for DNA precipitation: 1N HCl, 1% NaH_2PO_4, 1% $Na_4P_2O_7$. Stored at room temperature in a ground glass stoppered Erlenmeyer flask or a dispenser bottle. A 1:4 dilution of this mixture was used as the HCl wash. This wash mixture was stored in the refrigerator in ground-glass stoppered Erlenmeyer flasks.

S-1 nuclease: An enzyme from Aspergillus oryzae that, under proper conditions is specific for single-stranded DNA. The enzyme is supplied in glycerol and must be diluted before use. The proper dilution to use is determined by "titrating" the S-1 nuclease. In this procedure, two-fold dilutions of the nuclease are prepared and each of these dilutions is incubated with radioactively-labeled, single-stranded bacterial DNA under the same conditions that are to be employed in the homology experiments (50°C, 1 h). The contents of each tube is then acid precipitated, the DNA collected on nitrocellulose membranes and the radioactivity measured in a scintillation or gamma counter. The highest dilution that "chews up" all of the single-stranded DNA, so that no radioactivity can be precipitated is determined. The S-1 nuclease is then used at one-half of this dilution. For example, if a 1:50 dilution will get rid of all of the single-stranded DNA during the incubation period, the S-1 nuclease is used at a dilution of 1:25 in the homology experiments. The activity of the nuclease should be checked from time to time, to ensure that activity is not lost in storage. A 1:25 dilution of the S-1 nuclease was used for most of the experiments performed during the course of this project. Buffer A (described above) is used to dilute the S-1 nuclease.

Sheared, denaturated salmon sperm DNA : high molecular weight DNA, 0.5 mg/ml, sheared by 2 passages through a French pressure cell at 10,000 psi and denaturated by boiling for 10 min. Stored in 15 x 75 screw-capped tubes at -20°C. Added to the tubes prior to S-1 digestion to ensure that the substrate concentration of the enzyme, in this case, single-stranded DNA, is high enough to for the enzyme to be operating at maximum efficiency.

Sheared, native salmon sperm DNA: high molecular weight DNA 0.4 mg/ml and 1.2 mg/ml. Sheared and stored as described above. Used in the vials containing only labeled reference DNA, so that the DNA concentration will be high enough to be effectively precipitated by the HCl mixture after S-1 treatment.

Procedure for DNA homology experiments

The DNA homology experiments were performed in the following manner. Six x 22 mm vials were used for the DNA hybridization. Two tubes were used for each heterologous strain (heterologous re-associations, "driver" DNA) and four tubes for the reference strain (homologous re-associations). In addition, 4 vials were included in which the driver DNA was replaced by sheared native salmon sperm DNA (0.4 mg/ml), to measure the amount of self-renaturation of the labeled DNA over the hybridization period. This represented the background hybridization. Two tubes were included which contained sheared native salmon sperm DNA (0.4 mg/ml) in addition to the labeled reference DNA, but no S1 nuclease was added, and no denatured DNA was added. This set of tubes served as a control for total radioactive counts. The reassociation mixtures were composed of 10 µl of labeled DNA, 25 µl 5.28 M NaCl-10^{-2} HEPES, 25 µl formamide and either 50 µl sheared, native salmon sperm DNA or 50 µl of driver

DNA. The vials were capped with trimmed serum bottle stoppers, vortexed, and placed in a metal rack and completely immersed in a 65°C water bath for 24 hours. The contents of the vials were removed with a 100-µl Eppendorf pipettor and placed into 15 x 100 mm polypropylene tubes containing 1 ml of Buffer A. Each vial was rinsed out with an additional 100 µl of Buffer A and this also placed into the polypropylene tubes. The same pipet tip was used throughout the procedure, only changing tips between different labeled DNAs. The empty vials and caps were placed into separate beakers of Isoclean. It is important to rinse the Isoclean out of these very shortly after use, especially the caps, which the Isoclean will corrode. Next 50 µl of diluted S-1 nuclease was added to each tube. The tubes were vortexed and placed in a covered water bath at 50°C for 1 h. After the 1 h incubation, 50 µl of 1.2 mg/ml sheared native salmon sperm DNA and 0.5 ml of 1N HCl mixture was added to each tube, the tubes vortexed and placed in a refrigerator for 1 h. The acid-precipitated duplex DNA was then collected on glass fiber filters in the following manner. A strip of glass fiber filter paper (Whatman GF/F, 1" x 15") was placed in a collection device designed to hold 15 tubes and wet with the HCl wash mixture. This device was connected to an aspirator so that a vacuum would draw the liquid down through the filter and the device divided the filter strip into 15 individual circles. The device also had a reservoir to hold the radioactive waste. The contents from each of fifteen tubes was poured into each of the 15 holes in the device, each tube rinsed with the HCl wash and allowed to drain in the holes. The tubes were removed and the holes rinsed with the HCl wash. The filter paper was then removed and placed on a tray for drying under a heat lamp for 1 hour. The dried filter papers were separated with tweezers and placed into separate

polypropylene tubes (< 50 cpm) and placed to count in a Beckman model 5500 gamma scintillation counter for five minutes per tube, and no background was subtracted. Radioactivity of the HCl wash from the filtering trough was measured by taking a 2 ml sample of the fluid from the trough, counting it for 1 minute with 60 cpm subtracted for background. Total μCi were calculated by the following formula:

$$\text{cpm/ml} \times 1.33 = \text{dpm/ml}$$

$$\text{dpm/ml} \times \text{total ml in trough} = \text{total dpm}$$

$$\text{total dpm}/(2.2 \times 10^{6}\,\text{dpm}/\mu\text{Ci}) = \text{total } \mu\text{Ci}$$

If the total μCi are less than 1 μCi, it can be discarded down the drain, after entering in the "swipe test" book. If not the waste must be cut to 1 μCi and discarded over a period of days.

The filter papers from the experiment must be discarded in a radioactive waste can, and the polypropylene tubes which were used must be re-counted at 1 minute per tube, and 60 cpm subtracted for background. Tubes with more than 50 cpm should be placed in a separate labeled radioactive waste bucket.

The ticker-tape from the experiment is run through a program to calculate DNA homology values using a Wang computer.

Ribosomal RNA hybridization experiments

Buffers and reagents

The following buffers and reagents were used in the rRNA hybridization experiments:

17.6X SSC-10^{-3} HEPES: For description of SSC see DNA isolation buffers and reagents. 0.5 ml of 0.1 M, pH 7 HEPES was added to 50 ml of 17.6X SSC.

1X SSC-0.5% SDS: For description of SSC and SDS see DNA isolation buffers and reagents.

2X SSC: For description of SSC see DNA isolation buffers and reagents.

Ribonuclease A, bovine pancreatic (Sigma): See description under DNA isolation buffers and reagents.

Ribonuclease T_1, from Aspergillus oryzae (Sigma): See description under DNA isolation buffers and reagents.

Formamide: De-ionized. Kept at room temperature.

Denhardt's preincubation mixture: Contains the following ingredients dissolved in 2X SSC: 0.02% Bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.); 0.02% Polyvinylpyrrolidine (Calbiochem-Behring, La Jolla, Calif.); 0.02% Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden). To two ml of concentrated Denhardt's mixture add 10 ml 20X SSC and distilled water to a volume of 50 ml.

Procedure for rRNA hybridization experiments

The rRNA hybridization experiments were performed in the following manner. Six x 22 mm vials were used for the rRNA hybridization. Four tubes were used for each competitor strain (heterologous binding) and eight tubes for the reference strain (homologous binding). Eight tubes contained only labeled reference rRNA and the immobilized DNA (direct binding) as a control for total number of radioactive counts possible if all the labeled rRNA were to bind to the DNA immobilized on the membrane. The reaction mixtures contained 10 µl of labeled rRNA, 25 µl 17.6X SSC-10^{-3} HEPES, 50 µl de-ionized formamide and either 25 µl 1X SSC-0.5% SDS for the direct binding vials or 25 µl homologous

competitor rRNA (2 mg/ml) or 25 µl heterologous competitor rRNA (2 mg/ml). The labeled rRNA was always added to the vials first. The reaction mixtures were mixed thoroughly. Nitrocellulose membranes containing the immobilized DNA were incubated for at least 30 minutes in Denhardt's preincubation mixture at 50°C before adding to each reaction vial. The experiment was incubated completely immersed in a 50°C waterbath for 17 hours. After the incubation, the membranes were removed from the vials and placed in a washing chamber. The 6 x 22 mm vials were cleaned out by first collecting the radioactive incubation mixture into a single test tube outfitted with a suction device made expressedly for this purpose. This waste was dicarded into a radioactive waste container. Three 400 ml beakers were filled with 30 ml 20X SSC and filled with distilled water to the 300 ml mark, each to be used as the membranes washes. The membranes were washed in two 300-ml volumes of 2X SSC at 50 °C for five minutes each. The first wash was discarded into a radioactive waste container. Then the membranes were washed in 2X SSC containing 10 µg/ml of RNase A and 0.25 units of RNase T1 for 1 hour at 37 °C. The membranes were then washed in a 300-ml volume of 2X SSC at room temperature for 5 minutes. The membranes were then taken out of the washing chamber and layed on paper towels and dried under a heat lamp for 15 minutes. The membranes were placed in pairs into polypropylene tubes for counting. Radioactivity of the membranes was measured with a Beckman model 5500 gamma scintillation counter as in the DNA homology procedure. After the experiment, the membranes were discarded into a radioactive waste container, and the empty tubes counted. Tubes with more than 50 cpm were placed in a separate container.

Phenotypic characterization of strains

Nitrogenase activity

Cultures of Conglomeromonas largomobilis strains UQM 2041, UQM 2042 and UQM 2043 were inoculated into nitrogen-free malate medium (semisolid), nitrogen-free malate medium (liquid) and malate medium containing 0.1% ammonium sulfate (semisolid). Five ml quantities of the media were used in 16 mm diameter tubes, capped with Ka-Puts. Cultures were inoculated 48 hours prior to the experiment and incubated at 37°C, except for 2042 which was incubated at 30 °C because of its inability to grow at a higher temperature. After 48 hours, an acetylene generator apparatus was set up under a hood by connecting a tube of water with 2 lumps of calcium carbide in it to a large Erlenmeyer flask half filled with water, by means of a rubber hose inserted into rubber stoppers in the mouth of the tube and the flask. Acetylene was generated by the reaction of the carbide with the water and bubbled into the Erlenmeyer flask. The rubber hose was filled with pure acetylene gas. The caps on the cultures were replaced with serum-bottle stoppers. With a syringe and a 26 gauge needle, 1.5 cc of air was removed from the culture tube, and 1.5 cc of acetylene was removed from the hose and injected into the culture tube. These culture tubes were incubated at the temperatures the cultures were grown at for at least one hour. During the incubation, a tank of ethylene gas was attached to a rubber hose, which was inserted into a beaker of water. Some ethylene was passed through the rubber tubing and bubbled into the water. Using a 3-cc syringe with a 26-gauge needle, 1 cc of ethylene was removed from the rubber hose and injected into a 520 ml Florence flask stoppered with a serum bottle stopper. After 5 minutes, using a fresh 10-cc syringe and needle,

10 cc from this flask was injected into a second 520 ml Florence flask stoppered with a serum bottle stopper. Nanomoles of ethylene per cc of flask #2 were calculated. One cc from flask #2 was injected into a Beckman GC-4 gas chromatograph. The height of the ethylene peak (third peak) was measured. This peak height corresponds to the nanomoles of ethylene calculated. The first two peaks are of no consequence. When the 1 hour incubation period for the cultures was over, 1 cc from each tube was injected into the gas chromatograph. If the peaks went off the chart, the attenuation of the chromatograph was increased (decrease the sensitivity). The total volume of the gas phase was determined by filling an empty tube with water and pouring it into a graduated cylinder and subtracting 5 ml for the volume of the culture. The following formula was used to calculate nmoles ethylene produced per culture per hour:

(height of peak x nmole ethylene at attenuation used x volume of gas phase)/ hours incubated.

Starch hydrolysis

Cultures were streaked once down the center of a starch agar plate and incubated at their optimal growth temperature for two weeks. Plates were incubated in a closed, lined can to minimize evaporation of the medium. After two weeks, Gram's iodine was poured on the surface of the plate. A positive test was a zone of hydrolysis shown by a non-purple area where there was no starch to react with the iodine. A negative test was the absence of this zone.

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Curriculum Vitae

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