Symbiosis, 13 (1992) 47–53
Balaban, Philadelphia/Rehovot

Characterization of *Azospirillum irakense* and *Azospirillum lipoferum* by Direct Sequencing of a PCR Amplified 16S rDNA Gene

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Received October 20, 1991; Accepted February 13, 1992

Abstract

Partial 16S rDNA sequence was determined for two *Azospirillum* species and compared to a set of ribosomal sequences from other bacteria belonging to the alpha subdivision of the Proteobacteria. The two *Azospirillum* spp. sequences were found to be 87% similar. They were closely related to *Aquaspirillum* and to a lesser extent to *Rhodospirillum* with which they share some morphological features.

Keywords: *Azospirillum*, *Aquaspirillum*, *Rhodospirillum*, PCR, ribosomal, phylogeny

1. Introduction

Nitrogen fixing bacteria of the genus *Azospirillum* are associated with roots of grasses from various geographical origins. The genus *Azospirillum* is generally described as composed of five species on the basis of physiological, morphological and molecular (DNA:DNA hybridizations) properties: *A. brasilense*, *A. lipoferum* (Tarrand et al., 1978). *A. amazonense* (Magalhaes et al., 1983), *A. halopraeferens* (Reinhold et al., 1987) and the recently described *A. irakense* (Khammas et al., 1989).

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The comparison of rRNA sequences is used to measure phylogenetic and evolutionary relationships among bacteria. The 16S rRNA gene is a patchwork of conserved and non-conserved regions making it a suitable molecule for employing new approaches such as PCR amplification and DNA sequencing. In this study we have used the same approach to measure the phylogenetic relationships between the genus *Azospirillum* and the bacteria belonging to the alpha subdivision of the Proteobacteria described by Woese et al. (1987). We also ascertained the position of *A. irakense*, the *Azospirillum* species recently described by Khammas et al. (1989), on the *Rhodospirillum* branch.

2. Materials and Methods

Bacteria: *Azospirillum irakense* strain KA3 (Khammas et al., 1989) and *A. lipoferum* strain BR17 (Tarrand et al., 1978) were grown in nutrient broth (Difco), pH 6.8 and maintained in Nfb malate semi-solid medium. The total DNA of mid-log phase bacteria was isolated according to Simonet et al. (1990). GGAGAGTTAGATCTTGGCTC-3′ and FGPS659′ (5′-CACCGCTACACCAGGAATTC-3′), using a standard protocol (Nazaret et al., 1991) yielding intense DNA bands of the expected size (i.e., 650 bp, not shown). These dsDNA fragments were sequenced in both directions according to Sanger et al. (1977) as modified by Winship (1989) using primers FGPS6 (described above) and FGPS 305′ (5′-CCAGTGTGGCCGGTCGCCCTCTC-3′) and T7 sequencing kit (Pharmacia, Sweden). These primers frame a structural domain (region "A") known to be among the most variable of the 16S rDNA gene (Pernodet et al., 1989) containing helices 6, 9 and 10 corresponding to hypervariable regions V1, V2 and V3 of Raué et al. (1988).

The 230 bp DNA fragments were aligned using the Clustal (Higgins and Sharp, 1988) and the lineup programs of the University of Wisconsin nucleic acid sequence analysis software (Devereux et al., 1984). Pairwise evolutionary distances between DNA fragments were computed according to Jukes and Cantor (1969) using perfect matches only and the the length of the shorter sequence without gaps as denominator with the Poisson correction for multiple substitutions (Nei, 1987). The sequence considered for distance calculation exclude positions 19 to 38 of the lineup (Fig. 1).

The distance method of Fitch and Margoliash (1967), the Neighbour-Joining (Saitou and Nei, 1987) and the modified UPGMA method of Li (1981) were used to obtain unrooted gene trees that do not assume strict homogeneity of substitution rates among lineages. Rooted trees were obtained using the KITSCH algorithm of the PHYLogeny Inference Package ("PHYLIP," Felsenstein, 1985) and the UPGMA (Sneath and Sokal, 1973) algorithm.

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                1                                                          60
A. lipoferum    ACACATGCAA GTCGAACG.. ..AAG.GCTT CGGCCTT.AG TGGCGCACGG GTGAGTAACA
 A. irakense    ACACATGCAA GTCGAACG.. ..CATCGC.. .AACATG.AG TGGCGCACGG GTGAGTAACA
A. magnetotac   ACACATGCAA GTCGAACG.. ..AAG.TCTT .CGGACTTAG TGGCGCACGG GTGAGTAACA
   E. longus    ACACATGCAA GTCGAACGAA CCCTTCG.G. ..GT...TAG TGGCGCACGG GTGGCTAACG
  R. meliloti   ACACATGCAA GTCGAGCG.. CCCCGCA.AG GGG.....AG CGGCAGACGG GTGAGTAACG
 B. japonicum   ACACATGCAA GTCGAGCGGG CGTAGCA.AT ACGT...CAG CGGCAGACGG GTGAGTAACG
A. caulinodan   ACACATGCAA GTCGAACGGG CCCTTCG.G. ..GT...CAG TGGCAGACGG GTGAGTAACG
P. thompsonia   ACACATGCAA GTCGAGCGGG CGTAGCA.AT ACGT...CAG CGGCAGACGG GTGAGTAACG
 R. palustris   ACACATGCAA GTCGAACGGG CGTAGCA.AT ACGT...CAG TGGCAGACGG GTGAGTAACG
    R. rubrum   ACACATGCAA GTCGAACG.. ..CATCCCTT CGGGATG.AG TGGCGCACGG GTGAGTAACA
 M. trichospo   ACACATGCAA GTCGAACGGG CGCAGCGCAT GCGTAGACAG TGGCAGACGG GTGAGTAACG
    consensus   ACACATGCAA GTCGAaCGgg cgcagcg-at -cgt---cAG tGGCagACGG GTGagTAACg

                61                                                         120
A. lipoferum    CGTGGGAACC TGCCTTTCGG TTCGGAATAA CGTCTGGAAA CGGACGCTAA CACCGGATAC
 A. irakense    CGTGGGAATG TGCCCTTTGG TTCGGAATAA CGTCTGGAAA CGGACGCTAA TACCGGATGT
A. magnetotac   CGTGGGAATA TACCTCTTGG TGGGGAATAA CGTCGGGAAA CTGACGCTAA TACCGCATAC
   E. longus    CGTGGGAACC TGCCTTTAGG TTCGGAATAA CTCAGAGAAA TTTGAGCTAA TACCGGATAA
  R. meliloti   CGTGGGAATC TACCCTTTTC TACGGAATAA CGCAGGGAAA CTTGTGCTAA TACCGTATGA
 B. japonicum   CGTGGGAACG TACCTTTTGG TTCGGAACAA CACAGGGAAA CTTGTGCTAA TACCGGATAA
A. caulinodan   CGTGGGAACG TGCCCTTCAG TTCGGAATAA CCCAGGGAAA CTTGGGCTAA TACCGGATAC
P. thompsonia   CGTGGGAACG TACCTTTTGG TTCGGAACAA CACAGGGAAA CTTGTGCTAA TACCGGATAA
 R. palustris   CGTGGGAACG TACCTTTTGG TTCGGAACAA CACAGGGAAA CTTGTGCTAA TACCGGATAA
    R. rubrum   CGTGGGAACG TACCTTGGAG TGCGGAATAA TCTTTGGAAA CGAGGACTAA TACCGCATAC
 M. trichospo   CGTGGGAACG TAC.TTTCGG TTCGGAATAA CTCAGGGAAA CTNAAGCTAA TACCGGATAC
    consensus   CGTGGGAAcg TaCctTttgg TtCGGAAtAA c-caggGAAA cttgtgCTAA tACCGgATaa

                121                                                        180
A. lipoferum    GCCCTTTGGG GGAAAG.TTT A.CGCCGAGA GAGGGGCCCG CGTCGGATTA GGTAG...TT
 A. irakense    CCCCTGCGGG GGAAAGATTT ATCGCCAAAG GAGCAGCCCG CGTCTGATTA GGTAG...TT
A. magnetotac   GCCCTTCGGG GGAAAGATTT ATCGCCGAGA GATTAGCCCG CGTCCGATTA GCTAG...TT
   E. longus    TGTCTTCGGA CCAAAGATTT ATCGCCTTTA GATGGGCCCG CGTTAGATTA GATAG...TT
  R. meliloti   GCCCTTCGGG GGAAAGATTT ATCGGGAAAG GATGAGCCCG CGTTGGATTA GCTAG...TT
 B. japonicum   GCCCTTACGG GGAAAGATTT ATCGCCGAAA GATCGGCCCG CGTCTGATTA GCTAG...TT
A. caulinodan   GTCCGAAAGG AGAAAGATTG ATCGCTGAAG GATCGGCCCG CGTCTGATTA GCTAG...TT
P. thompsonia   GCCCTTACGG GGAAAGATTT ATCGCCGAAA GATCGGCCCG CGTCTGATTA GCTAG...TT
 R. palustris   GCCCTTACGG GGAAAGATTT ATCGCCGAAA GATCGGCCCG CGTCTGATTA GCTAG...TT
    R. rubrum   GCCCTTAGGG GGAAAGATTT ATCGCTCCAA GATCGGCCCG CGTCCGATTA GCTAG...TT
 M. trichospo   GCCTTAAGGG GGAAAGATTT ATTCGCGAAA GATCGGCCCG CGTCCGATTA GCTAGCTGTT
    consensus   gcccttagGg ggAAAGaTTt Atcgccgaaa GAtcgGCCCG CGTctGATTA GcTAG---TT

                181                                                232
A. lipoferum    GGTGAGGTAA AGGCTCACCA AGCCTTCGAT CCGTAGCTGG TCTGAGAGGA TG
 A. irakense    GGTGGGGTAA AGGCCTACCA AGCCGACGAT CAGTAGCTGG TCTGAGAGGA TG
A. magnetotac   GGTGAGGTAA TGGCTCACCA AGGCGACGAT CGGTAGCTGG TCTGAGAGGA TG
   E. longus    GGTGGGGTAA TGGCCTACCA AGTCGACGAT CTATAGCTGG TCTGAGAGGA TG
  R. meliloti   GGTGGGGTAA AGGCCTACCA AGGCGACGAT CCATAGCTGG TCTGAGAGGA TG
 B. japonicum   GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TG
A. caulinodan   GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TG
P. thompsonia   GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TG
 R. palustris   GGTGAGGTAA TGGCTCACCA AGGCGACGAT CAGTAGCTGG TCTGAGAGGA TG
    R. rubrum   GGCGGGGTAA TGGCCCACCA AGGCGACGAT CGGTAGCTGG TCTGAGAGGA TG
 M. trichospo   GGTGAGGTNA AGGCTNACCA AGGCGACGAT CGGNAGCTNG TCTGAGAGGA TG
    consensus   GGtGaGGTaA tGGCtcACCA AGgCgaCGAT CagtAGCTgG TCTGAGAGGA TG
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Figure 1. Lineup of the 16S partial DNA sequences of *Azospirillum irakense* strain KA3 and *A. lipoferum* strain BR17 with *Aquaspirillum magnetotacticum* (GenBank no. M58171) (Eden et al., 1991) *Erythrobacter longus* ATCC 33941 (GenBank no. M55493), *Rhizobium meliloti* NZP 4017 (GenBank no. M55495), *Bradyrhizobium japonicum* USDA 59 (GenBank no. M55489), *Azohizobium caulinodans* ORS 571) (GenBank no. M55491), "*Photorhizobium thompsonianum*" BTAi1 (GenBank no. M55492), *Rhodopseudomonas palustris* 2.1.6 (GenBank no. M55496), *Rhodospirillum rubrum* ATCC 11170 (GenBank no. M55497) (Young et al., 1991), and '*Methylosinus trichosporium* (GenBank no. M29024) (Tsuji et al., 1989). The consensus line represents nucleotides present in all sequences with upper case letters,those occurring in a majority of sequences in lower case, and those positions where no majority exists with dashes. Numbering starts at position 51 relative to the *Escherichia coli* sequence given in Raué et al. (1988). Underlined bases at coordinates 94, 103, 105 and 203 are particular to *Azospirillum* and to *Aquaspirillum* species.

3. Results

DNA sequences

The partial DNA sequences of the two *Azospirillum* spp. strains 16S rDNA are given in Fig. 1. The two *Azospirillum* spp. strains have one nucleotide unique to them at position 203, however, there are 3 other nucleotides at coordinates 94, 103 and 105 which are shared by *Aquaspirillum* and *Azospirillum* spp. strains. These nucleotides may eventually be used to define generic oligonucleotide probes. The two *Azospirillum* spp. strains have 34 substitutions, resulting in a similarity of 87%.

The tree obtained with the above-given alignment using the Neighbour-joining method is presented in Fig. 2. The two *Azospirillum* spp. strains are grouped into a cluster which is close to *Aquaspirillum magnetotacticum* (Eden et al., 1991) and to *Rhodospirillum rubrum*. Other treeing algorithms gave a similar topology.

4. Discussion

This sequencing work was undertaken to determine the phylogenetic branching pattern of genus *Azospirillum* and its relation to other genera of the alpha

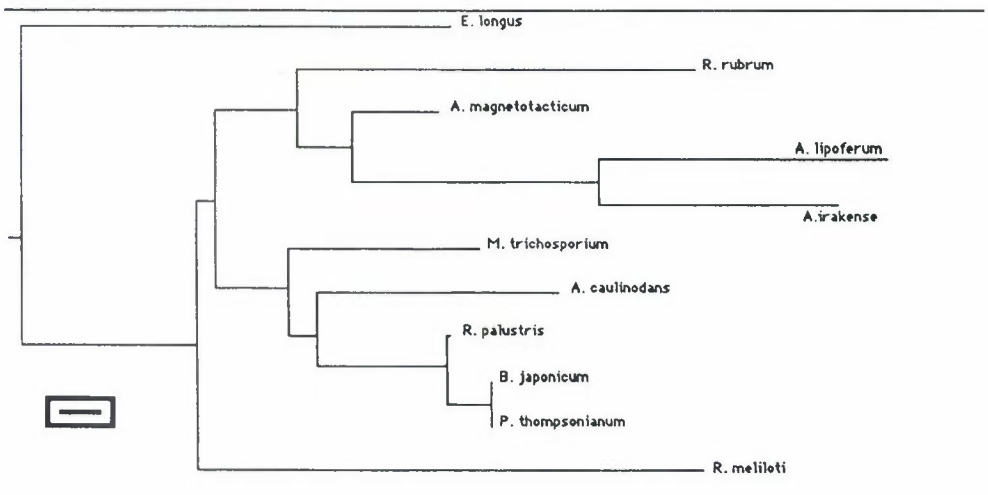

Figure 2. Tree obtained using the Neighbour-Joining method of Saitou and Nei (1987) from the lineup given in Fig. 1. The scale, given below left, is 0.01 substitution per site. The tree was rooted using as outgroup the *E. coli* sequences of Brosius et al. (1981).

subdivision of the Proteobacteria, and to see if generic nucleotides positions could be exploited to identify these bacteria in complex microbial communities as in soil or rhizosphere.

The genus *Azospirillum* appears to be coherent since the two strains tested are closer to one another than they are to the rest of the strains examined. A more comprehensive analysis of more *Azospirillum* species and of different genera in the same alpha subdivision of the Proteobacteria should help pinpoint the relation of *Azospirillum* to other taxonomical units. The % similarity detected between the two strains is rather low (87%, distance = 0.13 substitution/site), compared with the *Bradyrhizobium japonicum* – "*Photorhizobium*" genus (97%, 0.033 s/s, Young et al., 1991), with *Frankia* genus (92%, largest difference among 40 strains tested 0.097 s/s, Nazaret et al., 1991, unpublished) or with *Nitrobacter* genus (unpublished).

The resulting tree is entirely consistent with the one given by Young et al. (1991) which used a slightly different part of the 16S rDNA gene. Young et al. (1991) avoided using positions 19–38 because of uncertainties in the alignment. We found that the potential error is not very important since the trees obtained using the whole fragment or avoiding the gap-region were similar. The nucleotide sequence for *Aquaspirillum*, which proved later to be most similar to the sequence of *Azospirillum* was not available to Young et al. (1991). *Aquaspirillum* and *Azospirillum* share certain characteristics in common such as morphology and nitrogen fixation but this last feature may not be the most striking feature to highlight a recent common ancestry since it is widespread among bacteria and has a very ancient phylogeny (Normand and Bousquet, 1990).

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