A recA gene phylogenetic analysis confirms the close proximity of Frankia to Acidothermus

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The closer proximity of Frankia and Acidothermus cellulolyticus relative to the morphologically close Geodermatophilus found previously was confirmed by resequencing the rrs gene of Acidothermus cellulolyticus and the housekeeping gene, recA. The diagnostic sugar 2-O-methyl-D-mannose was detected only in Frankia, while hopanoid lipids were present at high levels in both Acidothermus and Frankia.

Keywords: Acidothermus, Frankia, Geodermatophilus, hopanoid, recA

Frankia is one of the few bacterial genera to have developed a symbiosis with plants suitable for growth. The nitrogen-fixing actinorhizal symbiosis allows 24 genera of plants belonging to eight dicotyledonous plant families to colonize soils that are poor in nitrogen, e.g. sandy beaches, mine spoils, burned forests or glacial moraines (Benson & Silvester, 1993). The variety of the plants concerned has generated discussion about whether the symbiosis has appeared once and been subsequently lost in several lineages, or if several independent appearances occurred (Soltis et al., 1995).

Discussion on the evolutionary origin of the symbiosis has also been fuelled by recent phylogenetic reconstructions using the rrs (also designated the 16S rRNA) gene. The first such work (Hahn et al., 1989) concluded that Geodermatophilus was the closest neighbour of Frankia, which was consistent with the fact that they shared a common morphological feature (the presence of multilocular sporangia) and a common habitat (soil). Later, as the number of sequences in databases increased, another study of the genus Frankia (Normand et al., 1996) came to the conclusion that the closest neighbour of the genus Frankia was not Geodermatophilus (with a genetic distance of 5.3–7.5%) but was a recently described cellulytic bacterium from an acidic hot spring in Yellowstone National Park; this bacterium, Acidothermus cellulolyticus (Moghagheghi et al., 1986), was lying at a genetic distance of 4.8–6.5% (Normand et al., 1996).

Rainey & Stackebrandt (1993), as part of their work on the reorganization of the phylogeny of the actinomycete line of descent, determined the rrs sequence of A. cellulolyticus and came to the conclusion that this bacterium was phylogenetically part of the actinomycetes although it was not closely related to any known bacterium. The high number of undetermined positions in the A. cellulolyticus rrs sequence could be construed as an indication that errors had been made elsewhere. Thus, a resequencing of the rrs gene was thought to be appropriate. Furthermore, since lateral transfer of genes has been demonstrated in bacteria (Hakenbeck et al., 1998; Tamanai-Shacoori et al., 1995), analysis of another ubiquitous but unlinked housekeeping gene was deemed useful to provide an independent confirmation of the unexpected proximity of Acidothermus to Frankia. Of the various genes recently used for that purpose, e.g. gyrB, rpoB and tuf, we selected recA (encoding a protein involved in repairing damaged DNA in the SOS regulon) because it is ubiquitous, highly conserved and has a phylogeny congruent, on the whole, with that of the rrs gene (Lloyd & Sharp, 1993; Eisen, 1995). Finally, strains belonging to the genus Frankia have been shown to have two diagnostic phenotypic features [i.e. the sugar

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence (with 21 corrections and 71 ‘N’) described in this work is AJ007290.
2-O-methyl-d-mannose (Mort et al., 1983) and large amounts of hopanoid lipids relative to the total cell lipid content that distinguish members of this genus from most other bacteria described to date (Berry & Kleemann, 1994; Berry et al., 1991). Consequently, these compounds were looked for in the Frankia phyletic neighbours.

Frankia strains were grown in liquid propionate BAP medium (Murry et al., 1984). A. cellulolyticus was grown at 55 °C according to Mohagheghi et al. (1986), while Geodermatophilus obscurus subsp. obscurus was grown on both solid and liquid ATCC no. 172 media according to Luedemann (1968). Genomic DNA was released from thermal-shocked (three liquid nitrogen passages) sonicated cells without further purification. Amplification and sequencing of the 16S rRNA gene was done as described previously (Normand et al., 1996). The signature sequences defined by Stackebrandt et al. (1997) to differentiate family Actinomycetaceae from other families in the class Actinobacteria remain valid. The distances obtained (not shown) and the topology of the resulting tree (Fig. 1) did not change significantly from those obtained before (Normand et al., 1996). Acidothermus remaining the closest neighbour to Frankia, with Geodermatophilus obscurus subsp. obscurus being further away. These results were confirmed by the maximum-likelihood and parsimony methods. Thus, none of the GenBank sequences from cultivated microorganisms is closest to Frankia, although it must be kept in mind that unisolated bacteria detected in the rhizosphere of Alnus viridis are closer to Frankia (Normand & Chapelon, 1997).

The rrs gene has become the golden standard of bacterial phylogeny, more than 23000 sequences having been determined so far with no sign of saturation yet. Numerous works have sought to confirm surprising results obtained with it using housekeeping protein genes such as rpoB (Mollet et al., 1998), gyrB (Yamamoto & Hayarama, 1996), tuf (Cousineau et al., 1992) and recA. The general conclusion that emerges from such comparisons is that although there are slight modifications, such as the non-monophyly of Actinobacteria (Stackebrandt et al., 1997) with low G + C Gram-positives (Eisen, 1995; Galtier & Gouy, 1994), the overall topology remains. A set of primers was selected in conserved areas of the recA gene: primer FGPR-476 (5'–GARTCN-TCN-GGN-AAR-ACN-AC-3') corresponds to the conserved motif ESSGKTAT coordinates 68–74 of the Streptomyces lividans sequence, (accession no. X76076) and primer FGPR-477 (5'–GGN-GCNY-ACY-TTR-TTY-TTR-AC-3') corresponds to the complement of the conserved motif VKNKVA (at coordinates 247–253 of the S. lividans sequence). Amplifications were performed according to Mullis & Faloona (1987) and the optimal temperature was 55 °C. An amplicon of about 550 nucleotides was expected, corresponding to 50% of the whole gene. Sequencing was done on an automatic ABI sequencer (Applied Biosystems) using the amplification primers. Nearly full-length double-stranded sequences were
obtained for all strains tested. These have been deposited in EMBL and the accession numbers are given in Fig. 1. GenBank was scanned for related sequences using the algorithm BLAST (Altschul et al., 1997) and the related sequences (S. lividans, Mycobacterium tuberculosis, Corynebacterium glutamicum and Bacillus subtilis) were included in the analyses. Sequences were aligned using CLUSTAL X (Thompson et al., 1997). Indel-containing regions were excluded from the analyses. Matrix pairwise comparisons of nucleic acid sequences were corrected for multiple base substitutions according to Kimura (1980). Pairwise evolutionary distances between encoded polypeptides were computed using the Poisson correction for multiple substitutions ranging from 0.8–19.5% substitutions/site and 1.9–10%. A. cellulolyticus is closest to the different Frankia strains, with DNA distances ranging from 25.3% to 28.9% and amino acid distances ranging from 93% to 158%. The tree obtained when all available Actinobacteria sequences were included and Bacillus subtilis was used as the outgroup is given in Fig. 1. It shows that the A. cellulolyticus strain and the five Frankia strains form a cluster but that G. obscurus is not closer to Frankia than either Streptomyces or Mycobacterium. This difference in topology between the rrs and recA trees was found to occur with nucleic acid sequences and with all phylogenetic reconstruction methods used. The two Elaeagnus-infective Frankia strains were clustered together, as were the two Alnus-infective strains and the Casuarina-infective strain. The topology of the nucleic acid distance tree obtained was similar to that obtained with amino acid distances except that the M. tuberculosis sequence was found within the Frankia–Acidothermus cluster. Study of the recA gene thus confirms the close proximity of A. cellulolyticus to Frankia. A lack of congruence was noted between the rrs and recA tree topologies (Fig. 1) with regard to the position of Geodermatophilus and that of the pair Mycobacterium–Corynebacterium. This may be due to the short length of the recA gene studied (550 nucleotides or about half of the gene), which can be misleading especially in a highly conserved gene.

Whole-cell sugars were determined on alditol acetate derivatives according to Sawardeker et al. (1965) on 8 mg lyophilized cells. Gas-phase chromatography was performed on Intersmat 120FL equipment with a silica SP2380 (Supelco) capillary (0.025 mm × 30 m) column and a flame-ionization detector. Large amounts of 2-O-methyl-d-mannose, as well as rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, glucose and inositol, were found in the two Frankia strains studied. Of the two Frankia phylectic neighbours, a faint peak barely above the detection threshold was found only in Geodermatophilus grown on solid medium (and not in Geodermatophilus from liquid cultures). No 2-O-methyl-d-mannose was detected at all in Acidothermus. The sugar 2-O-methyl-d-mannose has not been found as a major component in the two phylectic neighbours analysed. Its diagnostic status for strains belonging to the genus Frankia (Mort et al., 1983) thus remains valid.

Whole-cell lipids were determined according to Bligh & Dyer (1959) from 0.1 g lyophilized and sonicated cells. Total extracted lipids were dried, weighed and subjected to H2IO–NaBH4 treatment before derivatization by acetylation. Hopanoid lipids were identified by GC/MS and comparison with synthetic standards (Rohmer et al., 1984). Quantification of total hopanoid lipids was performed using peak area integration. Large amounts of hopanoid lipids were found in the two Frankia strains studied (7–25% w/w and 22% w/w of whole-cell lipids in ArI3 and in ULFA, respectively) as well as in Acidothermus, in which 15% (w/w) of the total lipid content was hopanoid. In Geodermatophilus, the amounts found (3% w/w) were much lower.

According to the molecular clock calibration of Moran et al. (1993), the genus Frankia (which has 4.8–6.5% divergence in the rrs gene) would have shared a common ancestor with A. cellulolyticus 250–300 million years ago. This time estimate would predate the emergence of the earliest actinorhizal plants, i.e. Myrica (Maggia & Bouquet, 1994), which is estimated (from studies of the pollen record) to have appeared 100–110 million years ago in the Cenomanian period (Macdonald, 1977)] but would follow the appearance of the first land plants 400 million years ago. The presence of large amounts of hopanoid lipids in a Frankia phylectic neighbour may indicate that they were also present in the common ancestor and that their protective function, the purpose of which remains unknown, later evolved towards protection of nitrogenase from oxygen diffusion in Frankia (Berry & Kleemann, 1994) and protection of A. cellulolyticus from the acidic thermal environment in which it grows. Large amounts of hopanoid lipids, such as those reported for Frankia and Acidothermus, have been found so far only in Zymomonas mobilis, which grows at high alcohol concentrations (Hermans et al., 1991), and in Alcyclobacillus acidocaldarius, which also grows in thermal acidic springs (Poralla et al., 1980). The presence of multiple cellulases in A. cellulolyticus highlights the ability of this hypothetical ancestor to thrive in the cellulose-rich rhizosphere of primitive land plants. The presence of comparatively low levels of cellulase in the interface capsule that surrounds Frankia in symbiotic plant cells has already been described (Berg, 1990) and could be due to the ability of Frankia to degrade cellulose (Safa-Sampah &
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