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CONFIRMATION OF AN ISOLATE FROM *DATISCA CANNABINA* AS ATYPICAL *FRANKIA* STRAIN USING PCR AMPLIFIED 16S rRNA SEQUENCE ANALYSIS

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Abstract

An actinomycete (Dc2) was isolated from the nitrogen fixing root nodules of *Datisca cannabina*. The actinomycete formed hyphae and sporangia, like typical *Frankia* strains but failed to infect host plants. This noninfective isolate was confirmed as *Frankia* by sequence analysis of 16S rRNA gene, amplified by polymerase chain reaction (PCR).

Introduction

Actinorhizal root nodule formation involves the symbiotic association of an actinomycete *Frankia* and the roots of dicotyledonous plants belonging to 8 plant families and 24 genera (Benson & Silvester, 1993). Actinorhizal plants have current and potential applications in reforestation and soil improvement projects (Gordon & Wheeler, 1983). In Pakistan, 12 species belonging to 6 genera (*Alnus, Casuarina, Coriaria, Datisca, Elaeagnus* and *Hippophae*) of actinorhizal plants have been reported (Chaudhary *et al.*, 1981). The actinomycetous nature of the root nodule endophyte of *Datisca cannabina* as well as the nitrogen-fixing ability of the endophyte has been shown (Hafeez *et al.*, 1984a,b).

The isolation of nitrogen fixing endophytes from actinorhizal nodules proved difficult for many years. *Frankia* strains from a number of actinorhizal species are now available in pure culture (Benson & Silvester, 1993). Besides the infective and effective *Frankia* strains, a number of atypical isolates have been obtained from several hosts. These non-infective strains have been included in the genus *Frankia* on the basis of their similar morphology, fatty acids pattern or sequence homology of 16S rRNA (Mirza *et al.*, 1991, 1992).

In the present paper, isolation and characterization of atypical *Frankia* strains from root nodules of *Datisca cannabina* is discussed.

Materials and Methods

Isolation of the endophyte: The field collected root nodules were surface sterilized with 3% OsO_4 and used to inoculate Qmod medium (Hafeez *et al.*, 1984c). To study morphology of the isolate, it was grown in P-N (nitrogen-free propionate), as well as in P+N medium (Meesters *et al.*, 1985).

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Extraction of nucleic acids: Nucleic acids from *Frankia* pure cultures and *Datisca* nodules were extracted by bead beating. Cells from pure culture of Dc2 were collected by centrifugation at 5000 xg for 10 minutes and washed with TE buffer. Root nodules of *Datisca cannabina* were collected from plants inoculated with soil suspensions collected from Swat, Pakistan. The nodules were washed with liquid detergent (Decondi 118, Otares, Enschede, The Netherlands), for five minutes followed by three washings with sterile water. For extraction of nucleic acid, 200 mg nodules were homogenized in 500 µl TE buffer and transferred to an Eppendorf tube. After adding 200 mg of glass beads (0.1-0.11 mm dia.), the homogenate was shaken vigorously in a bead beater (Braun, Melsungen, Germany) for 10 minutes. The nodule debris and glass beads were pelleted by centrifugation at 13000 xg for 10 minutes. The supernatant was extracted twice with phenol/chloroform, followed by two extractions with chloroform/ isoamylaloohol (24:1). After adding 1/10 vol. of sodium acetate (3M, pH 5.2) and 0.5 vol. of isopropanol, the supernatant was incubated at -70°C for 20 minutes. The nucleic acids were precipitated by centrifugation at 13000 xg for 20 minutes and the pellet was washed with 70% ethanol and dried under vacuum. The nucleic acids pellet was dissolved in 50-100 µl of TE buffer and used for filter hybridizations and PCR amplifications.

PCR amplifications and cloning: Amplification of partial 16S rRNA gene was carried out using template DNA of Dc2 and nodules. Each reaction mixture (100 µl) contained, 0.2 µl Super Tth Taq polymerase (50/ µl; HT biotechnology, UK), 10 µl of 10X Super Tth buffer, dNTPs (final concentration 200 µM each), 1 µl (100 ng/ µl) of each primer (124 & 1115) and 1 µl nucleic acid preparation. Thirty-five rounds of temperature cycling (94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes) were followed by incubation at 72°C for seven minutes (Mirza *et al.*, 1992).

The amplification products were treated with proteinase K (BRL, final concentration 60 ng/100 µl) at 37°C for 15 minutes (Crowe *et al.*, 1991), phenol/chloroform extracted and precipitated with ethanol. The DNA pellets were redissolved in 15 µl of TE and digested with restriction enzymes *Bam*HI and *Hind*III (BRL). The DNA-fragments were obtained by TAE-agarose gel electrophoresis followed by excision of the fragments and subsequent purification by the Gene-Clean procedure (Biol 101, La Jolla, California). Cloning of the amplification products into pUC19 was done by standard methods (Maniatis *et al.*, 1982).

Filter hybridization: Plasmid DNA was isolated by the alkaline lysis method (Maniatis *et al.*, 1982). Dot blot hybridizations were performed on Hybond-N^+ nucleic acid transfer membranes (Amersham), using 60 clones obtained from nodules. Plasmid DNA was denatured with equal volumes of 0.5 N NaOH. The denatured plasmid DNA (100 ng/dot) was applied with a Hybri.Dot manifold (BRL). The nucleic acids were immobilized by UV light and hybridized according to Church & Gilbert (1984). Oligonucleotide probes were 5'-labeled using phage T_4 polynucleotide kinase(BRL) and 10-20 Ci of [τ^{-32}P] adenosine-5'-triphosphate (3000 Ci/mmol; Amersham) (Maniatis *et al.*, 1982). The blots were initially washed with 5X SSC/1% SDS at 30°C for 15 minutes and then with 1% SSC/0.1% SDS at 50°C for 15 minutes (Maniatis *et al.*, 1982).

Cloned PCR products were sequenced, using the T7 DNA polymerase kit (Pharmacia) following the manufacturer's instructions.

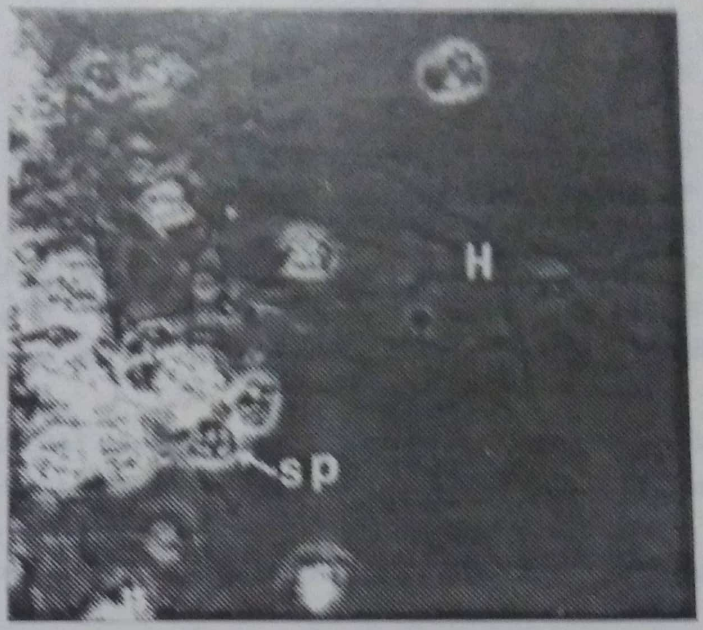

Fig.1. Strain Dc2 grown for 3 weeks on Qmod medium, showing branched hyphae (H) and characteristic sporangia (SP). X1000.

Results and Discussion

The isolate Dc2 from the nodules was characterized by hyphae and sporangia formation (Fig.1). The actinomycetous hyphae were branched and septate. The hyphal diameter on Qmod medium was approximately 0.2-0.4 μm but 0.6-0.8 μm on P+N medium. The sporangia were terminal on the hyphae with varied sizes and shapes. The young sporangia were pear shaped which became subglobose, elongated or irregular on maturity. In young sporangia the spores were roughly polyhedral but the released free floating spores were ovoid to spherical in shape.

The actinomycetous isolate (Dc2) showed all morphological features of the genus *Frankia* but failed to form vesicles in nitrogen-free medium. These structures are known to contain nitrogen-fixing enzyme nitrogenase (Meesters *et al.*, 1985).

Part of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) from the isolate Dc2. The sequence showed very high homology to 16S rRNA gene sequences of confirmed *Frankia* strains (Mirza *et al.*, 1994). This high sequence homology gives strong evidence that the isolate Dc2 belongs to the genus *Frankia*. However, relatively low homology values of about 95% were obtained between the sequences of Dc2 and the nodule endophyte of *Datisca cannabina* that were directly amplified from the nodules. This indicates that Dc2 is a different *Frankia strain* than the predominant strain present in the nodules.

To detect association of Dc2 with the *Datisca* nodules collected from Swat area of Pakistan, 16S rRNA gene was amplified directly with PCR and the products were screened with 16S rRNA targeted oligonucleotide probes (Fig.2). Strong hybridization signals were obtained with most of the clones using both the *Frankia* genus probe (Fig.2, S-I) and the probe developed against strain nodulating *Datisca* (Fig.2, S-II). Three clones (e4, e8 and g4) hybridized with the *Frankia* genus probe (Fig.2, S-I) but

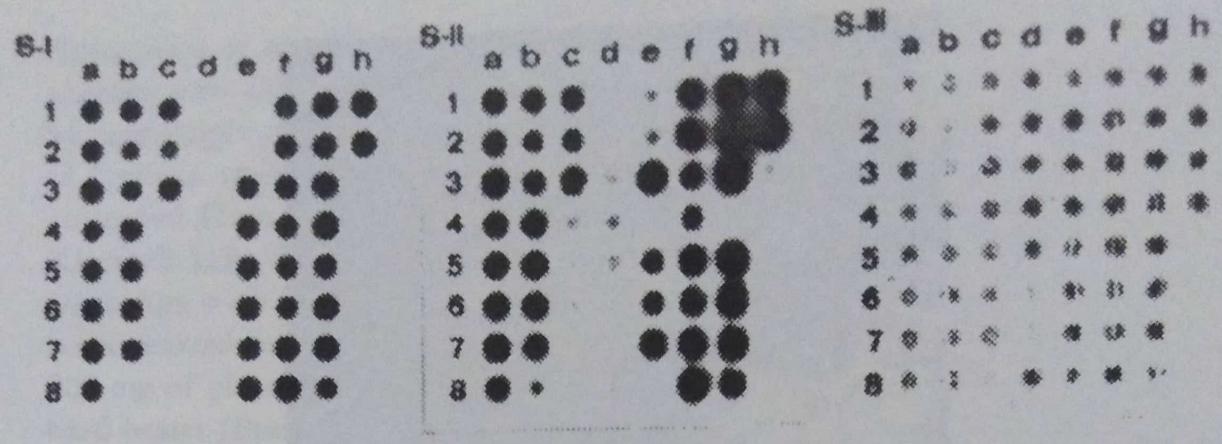

Fig.2. Dot blot hybridization of the oligonucleotide probes with the cloned 16S rRNA gene PCR-amplified from *Datisca cannabina* nodules. Filters representing Swat area were prepared with the plasmid DNA from 60 clones and hybridized with the *Frankia* genus probe FP (S-I), COR/DAT probe (S-II) and the Dc2 probe (S-III). No hybridizing signals were obtained with the Dc2 probe, indicating absence of Dc2 strains in the nodule samples.

failed to hybridize with the COR/DAT probe. This indicates presence of *Frankia* strain(s) other than the dominant strain against which COR/DAT probe was developed. The probe Dc2 did not hybridize with any clone (Fig.2, S-III), suggesting that Dc2 strain belongs to a minor population of the *Frankia* strains associated with the nodules.

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