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Study the diversity of Nif H genes in the communities of nitrogen fixing bacteria in soil

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Abstract
The specific primers for the Nif H genes were tested with different pure nitrogen fixing strains. A PolF/Polk primer set provided successful amplification and the restriction enzyme NdelIl chosen for restriction fragment length polymorphism (RFLP) analyses. These were the most discriminating for the study of Nif H genes diversity as they resulted in differences between strains at the species level. The amplification by selected primers and RFLP were applied to assess the genetic diversity of the Nif H genes pool in soil. The soils one under cultivation and other under permanent pasture were found to harbor a contrasting diversity of Nif H genes. Pure strain profiles could not be recognized in the Nif H genes soil patterns. This was shown that the structure of nitrogen fixers in soil was influenced by soil functioning.

Keywords: PCR-RFLP, Nif H genes, restriction enzyme, soil and genetic diversity

Introduction

The nitrogen fixation is a process that enables reduction of the atmospheric nitrogen in ammonium by nitrogenase, a universal enzyme. This process introduces nitrogen into the biosphere. The natural fixing process is responsible for 65% of annual fixation, while industrial processes represent only 25% [4]. The natural fixation is accomplished by fixing microorganisms belong to the archaea and bacteria. The bacteria have ability to fix nitrogen shown in organisms with various metabolisms such as anaerobes and aerobes, cyanobacteria and actinomycetes. Such diversity in physiology and ecology renders impossible the use of a universal selective culture medium for all nitrogen-fixing microorganisms [1].

These various microorganisms share the same operon in which the Nif H genes encodes for the Fe protein subunit of the nitrogenase. It has been shown by Young [10] that many features of a Nif H genes based phylogenic tree are entirely consistent with the 16S rRNA-based phylogeny of the nitrogen-fixing bacteria. Therefore, the diversity of the Nif H genes permits bulk representation of the taxonomic diversity of fixing bacteria and can be used to study the diversity of the bacterial community that can fix nitrogen [7]. Through molecular biology techniques, the diversity of nitrogen fixers in a complex environment such as the soil can be estimated, avoiding the bias of culturing the organisms on synthetic media, a procedure known to select a low percentage of soil populations.

There is no work has been reported on the different Nif H genes primers their specificity on pure cultures and their use for describing the diversity of nitrogen fixers in soils [8]. The objective of our study was to test several primers on different telluric bacterial genera well known to fix nitrogen or on microorganisms that fail to fix nitrogen. We suggest a simple protocol for polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), limiting the number of cycles to minimize the possible bias is Nif H genes amplification on the widest range of nitrogen fixing species. The specificity of amplification and in an amplification size large enough so as to obtain restriction fragment profiles representative of the nitrogen fixer diversity.

The few regions of the Nif H genes have been conserved among species of nitrogen-fixing bacteria. The forward primers were chosen between nucleotides 112 and 134 with the corresponding amino acid sequences being YGKGGIGK and GCDPKADS respectively. The reverse primers were chosen be between nucleotides 457 and 494 corresponding to the SGEMMAML and TIYAANNI amino acid sequence. All primers had a similar sequence with change in the number and type of degeneracies only.

First we tried to amplify the selected nitrogen fixing strains, *Azospirillum brusilense, Azospirillum lipoferam, Rhizobium leguminosarun, Sinorhizobium melloti and Frankia alni* with already published primers.

Materials and Methods

1. PCR Primers

Primers were chosen taking into account three parameters considered to be essential for the study of diversity by RFLP

2. PCR Amplification

The PCR amplifications from pure strains with 1ul of cells or 50 ng of DNA were performed in a total volume of 50 ul. The final concentrations were 0.5 µM of each selected primer and 4 uM for highly degenerate primers defined by Zehr and Mac Reynolds [11]. The other reagents were 200 mM of each dNTP, 2 U DNA polymerase. The PCR conditions consisted of 30 cycles at 90 °C 1 min for the annealing step and with 2 min extension at 72°C for the last cycle [3]. The amplification products were submitted to electrophoresis in 2% agarose gel and stained with ethidium bromide. Depending on the primer combinations used we obtained either no PCR product, or expected product size with additional unexpected band. The amplification was considered successful when only one PCR product with the correct size was detected. This was obtained with the PolF/Polk primer set successfully on DNA from all five tested trains

To evaluate primer specificity and universality this preliminary experiment was complemented with other reference nitrogen-fixing strains representative for diverse bacterial groups and non- nitrogen-fixing strains. The Pol F/ Pol R primer set was able to amplify the Nif H genes

fragment out of the totality tested fixing strains. As expected, no amplification was obtained with negative control strains.

The selected primers Pol F/Pol R were tested on bacterial DNA extracted from soil. The soil samples were collected from the upper layer of paired soils, i.e. soils of the same type under permanent pasture (PP), and under maize cultivation (MC) in close field situation. One hundred nanograms of DNA extracted and purified were used for soil DNA amplification [5]. The specificity of this band was verified by hybridization of the PCR product with a probe for the Nif H genes.

Table 1: Result of Nif H genes amplification with different primer sets using nitrogen fixing bacteria. I. *A. brasilense* II. *A. lipoferum* III. *R. leguminosarum* IV. *S. meliloti*

Primer sets	Conc.	Temp °C	I	II	III	IV
IGK 1/FGPH 273	0.5 µM	55	−	--	+	−
IGK 1/Pol R	0.5 µM	55	+	--	−	+
469/470	0.5 µM	45	−	−	+	−
Zf /Zr	0.5 µM	57	−	−	+	−
Pol F/AQE	0.5 µM	48	+	-	+	--
Pol F/Pol R	0.5 µM	55	+	+	+	+

3. Enzymes for Nif H genes

To use PCR-RFLP analysis on Nif H pool genes from nitrogen-fixing populations as a tool to study the community structure, it was necessary to choose endonucleases providing fragments in the range of 50 to 430 bp for adequate separation, the resulting fragments must be distributed throughout the size range. Enzymes that generated the same size band from various nitrogen fixing species must be rejected as this may hide the complexity of the Nif H genes pool.

Ten microliters of each PCR product were directly used for restriction enzyme cleavage by Mnl1, Haelll and NdeIll, the digestions were performed overnight to ensure that complete fragmentation was achieved. The digested DNAs were analyzed in a 4% polyacrylamide gel and staining with 1x Syber Green. This experiment was completed with theoretical digestions simulated from 15 sequences of the Nif H genes found in the GenBank database*: Alcaligenes faecalis, Azospirillum brasiliense, Azotobacter chroococcum, Bradyrhizobium japonicam, Klebsiella pneumoniae, Rhizobium meliloti* and *Thiobacillus ferrooxidans* [2, 6].

Results and Discussion

All enzymes discriminated pure strains which gave similar patterns with the various bacterial strains. Obviously. This endonuclease could be used to confirm the Nif H genes origin of a PCR product, but not to discriminate between strains. Three enzymes HaelII, NdeIll and Mnl1 were selected on the basis of the above arguments to study Nif H genes pool diversity: Haelll and MnlI was used in their study on the Nif H genes pool by Widmer *et al*. [9] while NdeIll was applied here for the first time to the Nif H gene study.

1. RFLP Results on Pure DNA

The pure strains from nitrogen fixers from various genera were discriminated by RFLP resulting from the three selected enzymes Haelll, NdeIll and Mnll. Slight differences between nif H profiles of *Azospirillum irakense, Aznarcus tolulyticus, Rhizobium leguminosarum* and *Mesorhizobium loti* resulted from the presence of small fragments. Taking into account the <40-bp size fragments. HaelII, Mnll and NdeIll RFLP showed differences in profiles not only between closely related genera such as *Rhizobium, Sinorhizobium and Mesorhizobium*, but also between species of the same genera such as *Azospirillum brasilense. Azospirillum*. lipoferum and *Azospirillum*. irukense. The depending on the enzymes used and the species considered, strains of the same species could be discriminated as well. Haelll profiles differentiated two strains of *Frankia alni* and two strains of *Paenibacillus polymita*. Haelll digestion did not distinguish between strains of *Azospirillum brasilense*, whereas these two strains were distinguished by Mnll and NdeIll digestion. In contrast, Mnll and NdeIll presented identical profiles of the two strains of *P. polymixa*. The complementary use of Haelll, Mnll and NdeIll restrictions was needed to access the differences in Nif H genes sequences.

Two strains of *Azospirillum irakense* showed the same profiles for three of the assayed enzymes. The common origin of these two strains isolated from rice roots in the same field could explain such a similarity in their nif H gene. The sum of the molecular weights of all fragments higher than the PCR products was obtained in *Sphingomonas pancimobilis* and *Enterobacter cloacar* RFLP profiles. The presence of only one band in the PCR product and the positive hybridization with nigh probes excluded a nonspecific amplification. Such a discrepancy would indicate a substantial contamination of DNA from *S. paucimobilis* and *E. cloacar* by DNA of other nitrogen fixers.

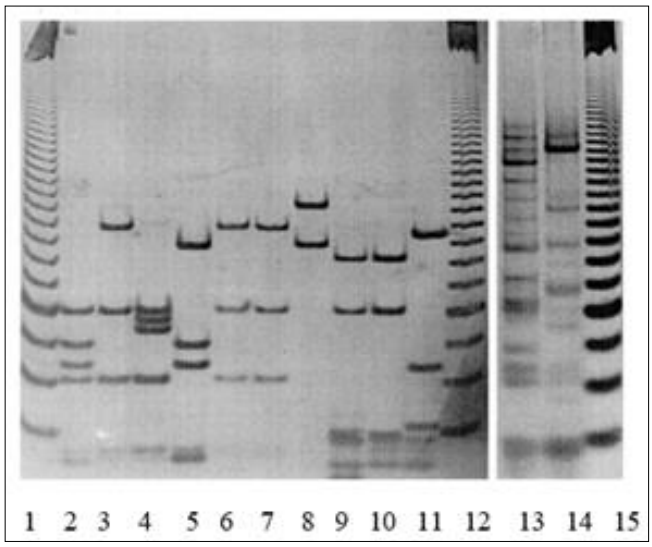

Fig 1: Restriction enzyme NdeIII RFLP of Nif H gene. PCR products from pure strain and soil DNA. 1. 20 bp ladder, 2. *A. lipoferum*, .3 *A. brasilense*, 4. *A. brasilense*, 5. *A. brasilense*, 6. *A. irakense*, 7. *A. irakense*, 8. *S. meliloti*, 9. *R. leguminosarum*, 10. *M. loti*, 11. *K. oxytoc*a, 12. 20 bp ladder, 13. Lcsa-C soil, 14. Lcsa-P soil, 15. 20 bp ladder.

2. RFLP Results on Soil DNA

The RFLP data concerning the amplification products from soil DNA digested by either endonuclease Haelll, Mnll and NdeIll were reproducible. Each enzyme provided a specific profile for each soil DNA. The comparison of soil and strain profiles showed that some strain profiles were found in soils such as *Azospirillum* brasilense and *Azospirillum*. tolulyticus with Mnll restriction in MC soil or *S. meliloti* and

Parnibacillus pofymcua with NdeIll restriction in PP soil. However, soil DNA RFLP never resulted in profiles identical to a pure strain on the three restriction enzymes. *Azospirillum brasilense* profiles were found in MC soil with Haelll and NdelIl but not with Mnll. Moreover, several hands in soil DNA profiles did not correspond with any band of the pure strains. These observations could lead to the conclusion that the pure strains used were not dominant in the environment and that there existed a number of nitrogen fixers [9]. The protocol used revealed that the Nif H gene pool and probably the nitrogen- fixing community were different under cultivation and under permanent pasture, even in the same soil type.

A simple protocol with specific primers of the Nif H gene and with three discriminating endonucleases was described here which allow a rapid assessment of the structure of nitrogen fixers in an ecosystem through the diversity of Nif H genes. This molecular tool revealed that long-term cultivation with fertilization, pesticide treatments or specific plant cover would result in a structure of the Nif H gene pool different from permanent pasture. This approach could be used to compare the diversity of nitrogen fixers according to soil characteristics such as texture, structure, plant cover, etc. It should be a powerful tool for monitoring the community of nitrogen fixers in soil in order to investigate seasonal changes, variations due to modification in the plant cover or effects of the input of chemical products in soils.

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