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Review article

Detection of Rhizobia by DNA-DNA-Hybridization From Soil Samples: Problems and Perspectives

AIMO SAANO and KRISTINA LINDSTRÖM
Department of Microbiology, University of Helsinki
00710 Helsinki, Finland
Tel. 90-7085302, Telex 122352 HYMK-SF

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Abstract

The following main steps are included in the detection of soil bacteria using DNA-DNA-hybridization: (1) isolation of the probe DNA sequence; (2) labeling of the probe DNA; (3) extraction of bacterial cell fraction followed by isolation of the bacterial DNA (cell extraction method), or isolation of the total DNA from crude soil samples (direct lysis method); (4) hybridization of the labeled probe DNA with the sample DNA; and (5) detection of the hybrids.

Keywords: *Rhizobium*, DNA-DNA-hybridization, identification, GEM monitoring

1. Introduction

Bacteria from the genus *Rhizobium* are widely studied for their symbiotic nitrogen fixation with many agriculturally important legumes. *R. loti* form nitrogen fixing nodules on roots of *Lotus*; *R. meliloti* on roots of *Medicago, Melilotus* and *Trigonella* species; *R. leguminosarum* biovar *trifolii* nodulates *Trifolium* species; *R. leguminosarum* biovar *viciae* nodulates *Lathyrus, Lens, Pisum* and *Vicia* species (Jarvis et al., 1986; Spaink et al., 1989); *R. leguminosarum* biovar *phaseoli* nodulates *Phaseolus*; *R. fredii* nodulates *Glycine* (Scholla and Elkan, 1984), and *R. galegae* nodulates *Galega* species (Lindström, 1989). The slow-growing symbiotic bacteria form a separate genus *Bradyrhizobium*, with one

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species, *B. japonicum*, which nodulates *Glycine* plants (Jordan, 1984). The legumes provide food or feed of high nutritional value, in addition to improving soil fertility and replacing chemical nitrogen fertilizers. If suitable rhizobia are not already present in the soil, leguminous crops can be inoculated with compatible rhizobia. With the rapid development of genetic engineering, genetically modified rhizobia will be released in the environment in the near future. For these reasons it is of increasing importance to develop an easy-to-use and inexpensive method for reliable identification of rhizobia from soil samples.

From the existing methods to identify *Rhizobium*, microbiological cultivation (Weaver and Frederick, 1972) even when compiled with antibiotic resistance-marking of strains is both time-consuming and laborious. The antibiotic resistance-marking is, moreover, complicated by the possible change of the properties of the microbe caused by the introduced mutation. The marker can also be lost in exchange of the genetic material with natural populations of bacteria. Serological methods, such as enzyme linked immunosorbent assay (ELISA) (Swaminathan et al., 1985) and the fluorescent antibody technique (FA), suffer from cross-reaction and non-specific binding of the antibody. These problems are caused by the main principle of these techniques, which is based on detection of antigens, i.e. gene products by antigen specific antibodies. FA has been used to identify *Bradyrhizobium japonicum* (Schmidt, 1974; Vidor and Miller, 1980; Crozat et al., 1982), *R. leguminosarum* biovar *phaseoli* (Robert and Schmidt, 1983), and other, taxonomically unclassified strains of *Rhizobium* (Kingsley and Bohlool, 1981).

Because the DNA-DNA-hybridization technique is aimed at detecting the genes themselves, not their products, it can provide a more specific tool both for the detection of *Rhizobium* in soil and, more generally, for monitoring the fate of genetically engineered microorganisms (GEMs) in the environment.

2. DNA-DNA-hybridization

Isolation of probe DNA

The probe DNA is chosen depending on the following goals: (1) the probe should be genus specific, i.e. all species and strains of the genus *Rhizobium* should be detected; (2) the probe should be species specific; (3) the probe should be strain specific. In all three cases the choice also depends on availability of genome sequence information.

The whole cellular DNA from different species of rhizobia has been used as probe DNA in determining occupancy of nodules by strains of *Rhizobium*,

both in colony hybridizations (Hodgson and Roberts, 1983) and in direct DNA hybridization of crushed root nodules (Cooper et al., 1987). Total DNA probes have also been used in cross hybridizations to determine percentages of relative homology between species of *Rhizobium* and *Bradyrhizobium* (Crow et al., 1981; Lindström et al., 1983, Wedlock and Jarvis, 1986). The same principle of labeling total DNA from a species has also been used for other microorganisms, for example, for *Bacillus* (Seldin and Dubnau, 1985). This approach is easy to apply because it does not require any genome sequence data. However, there is a high risk of non-specificity which can cause problems in the assessment of the degree of homology.

A whole *Escherichia coli*-originated vector plasmid pCU101 as well as its Tn5-derivative (pGS9) were used as probe DNA for enumeration of *R. leguminosarum* cells containing pCU101 or carrying Tn5 in its genome (kanamycin-resistant mutants), which had been added to soil. Sensitivity of colony DNA-hybridization, which was used to detect the rhizobia, was found to be approximately the same as for FA or estimations from plant infectivity compiled with most-probable-number-tests (Fredrickson et al., 1988). The use of foreign gene material as a marker for DNA-probing of agricultural strains causes doubts in terms of the environment and of technical reliability. Cells harboring foreign plasmid or Tn5 may lose them or suffer from lower competitiveness.

A significant part of the genetic research on rhizobia has dealt with nodulation (*nod*) and nitrogen fixation (*nif*) genes. Thus, a large amount of data on the sequences of these genes has become available. Some common sequences have been found in several species within the genus *Rhizobium* in association with the nodulation genes *nodC, nodD, hsnB* and *hsnD* (about 1 kb in length) (Rodriquez-Quinones et al., 1987). In addition, a very interesting sequence, measuring 25 base pairs, has been found within the regulatory region of the nodulation genes. The fragment, which is called the *nod*-box, is very well conserved and it has been shown to be present in many different species of *Rhizobium* (Rostas et al., 1986). Some of the sequences have proved species specific, such as the RtRS sequences (about 100 base pairs in length) next to the transcription initiation site of the *nif*HDK for *R. trifolii* (Watson and Schofield, 1985). A segment of the insertion sequence IS*Rm*1 has been found in more than 20 strains of *R. meliloti* as well as in one strain of *R. leguminosarum* and one unspecified soil bacterium (Wheatcroft and Watson, 1988a). The IS*Rm*1-derived probe can be used for strain differentiation for *R. meliloti* Southern blots of total-DNA digestion patterns (Wheatcroft and Watson, 1988b).

For isolation of *R. loti* strain specific probe sequences, Bjourson and Cooper (1988) used a method called subtraction hybridization. In this method total

DNA from the prospective probe strain is repeatedly hybridized to a mixture of DNA from cross-hybridizing strains (subtracter DNA). The subtracter DNA is either immobilized on an epoxy-activated cellulose matrix (heterogeneous method), or is biotinylated and mercurated (homogeneous method). In both cases the unique probe strain sequences can be separated from the hybridized sequences which share homology with the subtracter DNA.

Holben et al. (1988) used an *rbc*L gene probe (a cloned gene of parental strain) and an *npt*II gene probe (a sequence engineered into a *Bradyrhizobium* strain) in order to detect and differentiate between wild-type *B. japonicum* BJ110 and an engineered derivative of this strain. The sensitivity of the *npt*II probe was high: 0.02 pg of the sequence of interest in slot-blot, 0.1 pg in Southern blot, and 10^3 *Bradyrhizobium* cells/g of soil. Richaume et al. (1989) followed intergeneric plasmid transfer in soil from *E. coli* to *R. fredii* using a Tn5 probe in Southern hybridizations of restriction digested genomic DNA from *R. fredii*. The plasmid transfer was found to depend on soil type.

Extraction of bacterial cells and their total DNA from crude soil samples

General principles

Two main approaches have been used to liberate the total DNA from soil samples for subsequent purification, concentration and hybridization. The first approach is to first isolate the bacterial fraction from the soil particles, and then lyse the cells (cell extraction method). The other is to lyse the cells directly without fractioning (direct lysis method).

The lysis problem

Keeping in mind the main aim, i.e. the detection of *Rhizobium* among the diversity of soil bacterial communities, it becomes evident, that one of the crucial steps of the procedure is the reliable release of the total DNA from these bacteria. In fact, it is almost as important to be sure that all kinds of soil bacteria are disrupted using one single procedure. Otherwise the initial steps of searching for convenient probe molecules by comparative hybridizations with different soil bacteria will become complicated, demanding variations in the test conditions.

Since the important impact of Grunstein and Hogness (1975) into the simplification of hybridizations *en masse* by colony hybridizations, many improvements of lysis procedures for different groups of bacteria have been introduced. The most commonly used lysis treatments employ NaOH and proteinase K (Grunstein and Hogness, 1975), which has been directly applied to the identification of *Rhizobium* strains from leguminous root nodules (Hodgson and

Roberts, 1983). NaOH and SDS (Maniatis et al., 1982), SDS + proteinase K and NaOH (Cooper et al., 1987) have been used for the identification of *Rhizobium* from crushed root nodules. Lately some new approaches have been tried for a variety of gram-negative and gram-positive microorganisms (though not including *Rhizobium*) such as microwaves (Datta et al., 1987) and acetone together with proteinase K, lysozyme and SDS (Heath et al., 1986).

Our laboratory is also working on improvement of the lysis procedure for the identification of rhizobia by DNA-DNA-hybridization. The problem can be illustrated by comparison of autoradiograms of two filters, one with total-DNAs extracted directly on the filter from *E. coli* cells, the other with total-DNAs extracted on the filter from different soil bacterial cells. The probe was in both hybridizations radioactively labeled *nod*-box. Wherever the DNA extracted from *E. coli* cells carries cloned fragments of *nod* genes of *R. meliloti* or *R. galegae*, it gives clear spots of hybridization, whereas the DNA extracted from a variety of soil bacteria, including *R. meliloti* and *R. galegae*, gives spots that are difficult to interpret unambiguously. It is evident from the autoradiograms that a quantitative estimation is possible in case of *E. coli* samples: the three strongest spots are from the cells that carry a fragment of the common nodulation genes of *R. meliloti* in a multicopy plasmid pBR322, whereas all the smaller spots are from cells that harbour a low-copy-number plasmid (pRK290 or pLAFR1) with the nodulation genes of either *R. meliloti* or *R. galegae* (Fig. 1).

Cell extraction method versus *direct lysis method*

Torsvik (1980) proposed a procedure which involves the following steps: (1) separation of the bacterial fraction (repeated dilution of the sample in Winogradsky's salt solution and repeated centrifugation with collection of the supernatants); (2) lysis of the cells with lysozyme and SDS; (3) extraction of the DNA by urea and KCl; (4) purification of the DNA on hydroxyapatite or dialysis. The yield from a 60–90 g wet weight sample ($1 - 2 \times 10^{10}$ cells/gram dry sediment) was 90–187 micrograms of DNA/gram dry sediment.

The protocol by Holben et al. (1988) follows Torsvik's principle of isolation of the bacterial fraction by centrifugation, but improves the separation of the bacteria by using polyvinylpyrrolidone, which removes humic acid contaminants. This way the authors succeeded in detecting *B. japonicum* at densities as low as 4.3×10^4 cells/gram of dry soil.

Ogram et al. (1987) isolated DNA from sediments by direct lysis of cells in the sediment using SDS. The preparation was purified with alkaline extraction of the released DNA from the sediments and repeated centrifugation.

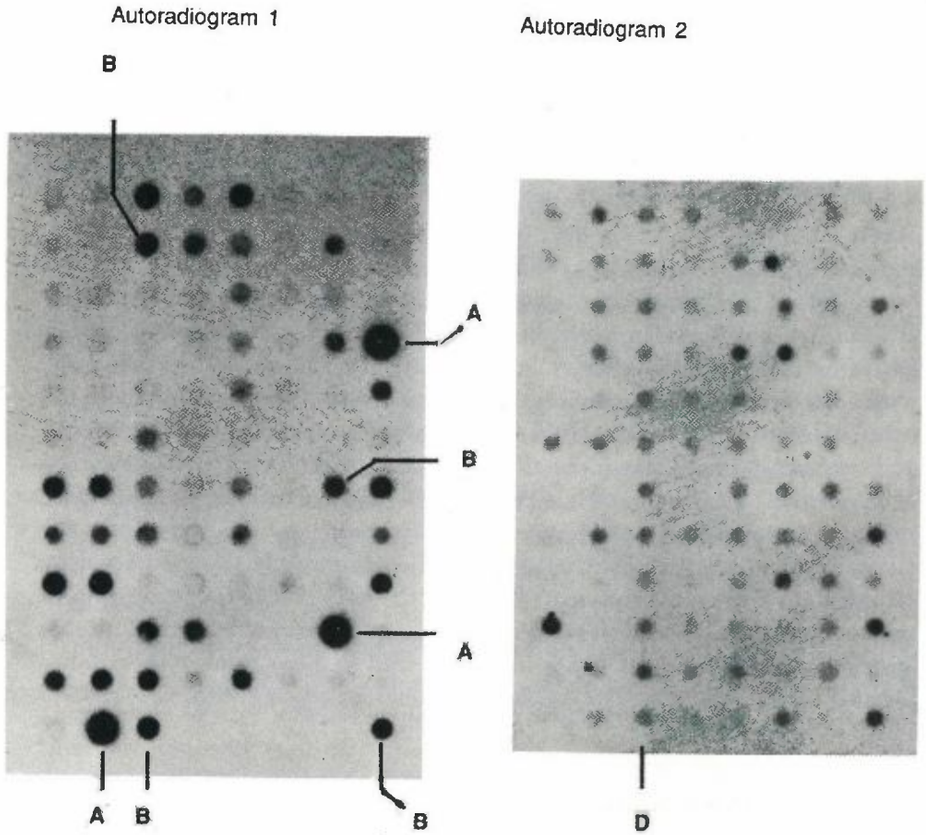

Figure 1. Autoradiograms of two hybridizations on nitrocellulose filters. The bacterial suspensions at the same growth stage were pipetted into a 96-well Bio-Dot apparatus and lysed with lysozyme and proteinase K, mainly according to Meade et al., 1982. The released DNAs were immobilized by baking them 2 hr at 80°C in vacuum. The 25 bp long *nod*-box probe DNA was labeled with ^{32}P-dATP using end-labeling by T4 kinase. The hybridizations and the washings of the filters were performed according to Maniatis et al. (1982).
Autoradiogram 1. Every DNA dot is from an *E. coli* suspension. (A) common nodulation genes of *R. meliloti* in pBR322 (Egelhoff et al., 1985); (B) host specific nodulation genes of *R. meliloti* in pRK290 (Rostas et al., 1986); the rest of the dark spots carry fragments with nodulation genes of *R. galegae* in pLAFR1.
Autoradiogram 2. The DNA dots are from suspensions of different species of *Rhizobium, Erwinia, Pseudomonas, Agrobacterium, Klebsiella* and *E. coli*. (D) the whole vertical line contains DNA dots from *R. galegae* suspensions.

Finally the DNA was precipitated with either ethanol and potassium acetate, or with polyethylenglycol followed by phenol-chloroform extraction, and further purification by CsCl-EtBr gradient centrifugation and/or hydroxyapatite chromatography. The yield of intracellular DNA was 8–26 micrograms/gram wet weight, and of extracellular DNA 1 microgram/gram wet weight. The origin of the DNA in terms of genus or species was not studied.

Steffan et al. (1988) performed a series of experiments in order to evaluate the effectiveness of the different extraction protocols. They concluded that for both methods the use of polyvinylpyrrolidone was important for the removal of humic compounds. In their hands the direct lysis method gave more than 1-order of magnitude higher yield than the cell extraction method. They also came to the conclusion that the yield from the direct lysis method might have contained both intra- and extracellular nucleic acids, whereas preparations recovered by the cell extraction method were derived primarily from active bacterial cells. The estimated DNA yields were 0.03–0.04 mg/100 g dry sample for the cell extraction method, and 0.85–1.99 mg/100 g dry sample for the direct lysis method, when the samples contained $3.5 - 6.6 \times 10^9$ cells/gram dry weight. Both methods yielded DNA of approximately the same purity. The origin of the isolated DNAs was not determined.

It can be claimed that the cell extraction method may selectively remove cells that are easily dislodged, while leaving untouched those species that are more tightly bound, resulting in false estimation of the species composition (Ogram et al., 1987). This would favor the direct lysis method.

However, a serious problem still remains to be solved concerning the reliability of these methods when the soil samples contain a high proportion of polysaccharide-producing bacteria, which may not be easily lysed by lysozyme and SDS (Steffan et al., 1988). This is very important both for *Bradyrhizobium* (Holben et al., 1988) and *Rhizobium* (Lipsanen and Lindström, 1989). When Ogram et al. (1987) estimated the efficiency of recovery for DNA by the direct lysis method to be as high as 90% from sediments (part of which is probably extracellular or from eukaryotic cells), Steffan et al. (1988) estimated the recovery from both soil and sediment by the cell extraction method to be 33%. Because it was impossible to exclude the presence of eukaryotic or extracellular DNA in the extracts from the direct lysis method, the question of efficiency of cell recovery was left open. Holben et al. (1988) could recover only 33% of the bacterial DNA (including inoculated *B. japonicum* cells) even when using polyvinylpyrrolidone to release the bacteria from the soil particles and using an extensive lysis procedure including sarkosyl-lysozyme-pronase-sarkosyl treatment.

Hybridization of labeled probe DNA with sample DNA

The hybridization rate depends on universal physical and chemical factors, such as the concentration and the complexity of the probe, the temperature, ionic strength and pH of the hybridization solution (Hames and Higgins, 1985).

Aiming at the specific detection of rhizobia from soil samples, two main approaches can be applied: the sample DNA is immobilized on a solid support, or it is kept in solution. Immobilization of the target DNA on solid support may be performed either by baking the nitrocellulose filters with the denatured DNA for two hr at 80°C with vacuum, or by short-wave UV radiation, which takes from 30 sec for nylon filters to 2–5 min for nitrocellulose filters (Khandijan, 1987).

No reports have been published on usage of solution hybridization to identify rhizobia. So far the hybridizations have been performed with sample DNAs immobilized on solid support despite the advantages of solution hybridizations. The main advantage of solution hybridization is the better access of the probe molecules to the target molecules, which makes the reaction about three times faster and reduces the background given by non-specific binding of the probe to impurities (Syvänen et al., 1986).

Detection of the hybrids

The hybrid formation can be detected after the hybridization and washing procedures by scintillation (counts per minute, quantitative results), autoradiography (visual, qualitative results), color reaction (visual, qualitative results) or by fluorometry (quantitative measurement). To detect the hybrids from solution hybridization, it is necessary to introduce chemical groups into nucleic acid probes which are then used as affinity labels for chromatography or collection on, for example, microplates.

Application of color precipitation or fluorometry to detect rhizobia or any other bacteria from soil samples is complicated by the tendency of the antibodies or the color compounds to bind to the impurities present in large amounts in soil samples. No experience of using non-radioactive labeling and hybrid detection of rhizobia DNA directly from soil samples has been published so far.

3. New Techniques

The polymerase chain reaction (PCR), a relatively new technique in the analysis of specific nucleotide sequences (Saiki et al., 1985), has significantly

contributed to sample diagnosis. The method is based on exponential amplification of specific target DNA fragments in the sample. The quickly produced surplus amount of target DNA makes it easily detectable even though it may have been a single and relatively short fragment in the original sample. In PCR two oligonucleotide primers that flank the DNA segment to be detected hybridize to opposite strands of that segment. The procedure further involves repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase so that DNA synthesis proceeds across the region between the primers (Erlich et al., 1988). Until now, PCR has been mainly applied to medical diagnostic purposes (Bugawan et al., 1988), but lately it has been successfully used for the monitoring of GEMs (herbicide degrading strain of *Pseudomonas cepacia*) as well as making target DNA detectable in river sediment samples initially containing as little as 0.3 pg of target (Steffan and Atlas, 1988; Bej et al., 1989). However, PCR has its own drawbacks. It may multiply some background DNA along with the target. Therefore, the absolute prerequisite for the use of PCR is highly specific primers which means that the exact sequence data on the ends of the target DNA segment must first be obtained.

Another new approach to improve the sensitivity of DNA-DNA-hybridization is to amplify the signal-generating capacity of the system by: (1) concentrating more label at the site of the target molecule; (2) designing each label to produce a stronger signal. Some commercial manufacturers of diagnostics kits have made these efforts by attaching multiple enzyme molecules to each of the probes, or, by using multiple probes for each of the targets, or multiple "secondary" probes that hybridize to multiple, target-specific "primary" probes (Fahrlander and Klausner, 1988). To our knowledge, no reports on the application of these ideas for the monitoring of GEMs have so far appeared.

An interesting approach to detect 100% complementary target sequences in the sample in solution hybridization conditions is to use the very specific property of ligase to join pairs of oligonucleotides annealed head to tail (Landegren et al., 1988).

4. Conclusions

Mainly the complexity of the methods have prevented DNA-DNA-hybridization to be adapted to large-scale or field analyses. Current nucleic acid-based tests (DNA *versus* RNA probes: better stability *versus* higher sensitivity) are still far from automation, because they tend to be complicated to run. They often require laborious sample preparation, multiple wash steps, and long

incubation times. Frequently, expensive or potentially dangerous reagents, such as radioisotopes, are employed.

What we rely on when using DNA probes is their ability to directly recognize genetic material — in contrast to antibody-based enzyme tests which detect gene products. This is their advantage in terms of specificity, but their disadvantage in terms of sensitivity. Immunoassays benefit from using two levels of *in vivo* target amplification: (1) transcription of the target DNA to yield many molecules of messenger RNA; and (2) translation of the messengers to yield many copies of the particular protein. To respond to this challenge, caused by nature itself, the DNA-DNA-hybridization technique has to make benefit from amplifying target DNA *in vivo*. Here we hope that PCR and techniques of amplifying the signal will be of help.

When the main shortcomings of DNA-DNA-hybridizations are overcome, the striking affinity of one strand of DNA for its complementary sequence will likely play a major role in future diagnostics, also for the detection of *Rhizobium* in soil samples and for the monitoring of GEMs, in general.

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