The identification of *Bradyrhizobium japonicum* strains isolated from Italian soils

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Pyrolysis mass spectrometry (PYMS) and PCR using arbitrary primers were used to characterize strains of *Bradyrhizobium japonicum* isolated from fields in northern Italy. The combination of techniques allowed us to identify bacteria derived from inoculants and to demonstrate that information about the nature of one inoculant was incorrect. PYMS also indicated that the derivatives from one inoculant formed two distinct populations: one like the parent strain and the other altered phenotypically.

Keywords: *Bradyrhizobium japonicum*, pyrolysis mass spectrometry, polymerase chain reaction, strain identification, soil bacteria

INTRODUCTION

Leguminous crop plants have been inoculated with strains of *Rhizobium* and *Bradyrhizobium* since the turn of the century in order to improve their growth in soils which do not contain appropriate species of these bacteria. Much effort has been put into developing simple and reliable techniques for identifying isolates from soils so that the fate of inoculants can be determined and plant responses can be related to the bacteria forming the nodules (Dudman, 1971; Mielenz *et al.*, 1979; Kamicker & Brill, 1986; Hartmann *et al.*, 1992).

Recently there has been considerable interest in studies of the survival of organisms released to the environment because of the development and intended use of genetically modified organisms. This has increased the

need for good methods to study soil populations of bacteria. We decided to take advantage of the introduction of a new legume crop, soybeans, to Italy to initiate studies of the fate of non-indigenous bacteria released into soils. *Bradyrhizobium japonicum* strains capable of nodulating soybeans must be introduced to allow nodule formation. Because few inoculants have been used, and we have access to the strains used in them, we are in a position to look at specific fields and see what has happened to the strains that were introduced.

In order to facilitate these studies we have used two techniques that have only recently become available for ecological studies. These are pyrolysis mass spectrometry (PYMS) and the use of the polymerase chain reaction (PCR). With PCR we fingerprint strains using arbitrary primers annealing at low stringency to the template DNA, producing fragments of DNA from the regions between closely spaced primers (RAPD; Williams *et al.*, 1990).

PYMS is a procedure in which samples from colonies are thermally degraded under vacuum (pyrolysis). Fragments produced are ionized and then analysed by a mass

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Abbreviations: MPN, most probable number; PYMS, pyrolysis mass spectrometry; RAPD, randomly amplified polymorphic DNA.

spectrometer. Using multivariate analysis the proportions of different mass classes are used to discriminate strains (Gutteridge, 1987; Goodacre *et al.*, 1991b). This technique is able to discriminate derivatives of the same strain of *Escherichia coli* which do, or do not, have fimbriae (Goodacre *et al.*, 1991a), or which carry different plasmids (Goodacre & Berkeley, 1990). The great advantage of this technique is that it characterizes strains on the basis of their phenotype and thus should be able to detect genetic drift, even though the number of base pair differences between strains is very small. In this respect it has been used to study fossil material (Meuzelaar *et al.*, 1982).

RAPD is a fingerprinting system based on the genotype because the bands produced reflect the presence of short sequences of DNA having sufficient homology with the primers used to enable *in vitro* DNA synthesis under low-stringency annealing conditions. With this technique derivatives of the same strain are unlikely to be discriminated because the chance that the priming sites share the same DNA sequence is very high.

METHODS

Isolation of bradyrhizobia. In 1990 about 100 nodules were selected at random from roots of soybeans nodulated by resident bradyrhizobia in a field near Udine (field A) and near Perugia (field B). Nodules were surface-sterilized and bradyrhizobia were isolated by streaking liquid from crushed nodules on YMA plates to produce single colonies. Once colonies had formed, the YMA plates were posted to Bristol and the bacteria were frozen at approximately -20°C in 20% (v/v) glycerol by taking a loopful of culture from a Petri dish and emulsifying it directly in the glycerol. When required for analysis, samples from glycerol were cultured once on YMA agar and were incubated at 28°C for 5–10 d on PSY agar.

Standard strains of *B. japonicum*. Strain CB1809 is used worldwide in inoculants. We also used strain NPPL, which is an isolate of CB1809 obtained by us from an inoculant produced by New Plant Products Ltd, Cambridge, UK, as a control strain to monitor the reproducibility of our assay techniques. Strain USDA110, like CB1809, is a 'well-known' *B. japonicum* strain. The cultures used as standards in this study were from our laboratory culture collections. They were maintained routinely on YMA, with incubation at 28°C .

Two inoculants were used in the sites we studied: Nitragin and Azofix. The Nitragin inoculum (obtained from Helibioagri, Gaiba, Rovigo, Italy) contained six different *B. japonicum* strains: 61A101_u, 61A148, 61A212, 61A118_u, 61A152 and 61A124_u, which we have named I1–I6 respectively. Azofix is a single-strain inoculant and was obtained from SES, Massa Lombarda, Italy. We isolated *B. japonicum* from the Azofix inoculant which was marketed prior to 1986 and call the strain Azo, which should be identical to USDA110. Having completed the research we were informed by Dr P. Rubboli (SES, Massa Lombarda, Italy) that the Azofix inoculant was formulated with *B. japonicum* strain USDA110 up to 1986, and from then strain CB1809 was substituted.

Medium. YMA is yeast-extract mannitol agar. It was prepared as described by Beringer *et al.* (1984) and was used for routine culturing of *B. japonicum* strains. PSY is peptone salts yeast and was used for the growth of colonies for RAPD and PYMS analysis because they produce less polysaccharide, which interferes with these assays, on this medium. It was prepared as described by Regensburger & Hennecke (1983).

Most probable number (MPN) counts. These were done in triplicate for each sample according to the procedure recommended by Vincent (1970) using Leonard jars in a glasshouse, with the soybean as host plant. Soils were sampled just before soybeans were sown in 1989.

PYMS of *B. japonicum* cultures. A Horizon Instruments PYMS 200X machine was used (Horizon Instruments, Heathfield, East Sussex, UK; Aries *et al.*, 1986), at the Mass Spectrometry Unit, University of Newcastle-upon-Tyne and the Department of Biological Sciences, University of Wales, Aberystwyth. Materials for PYMS, such as sample glass tubes, Curie-point foils and Viton O-rings were supplied by Horizon Instruments. The foils had a nickel:iron ratio of 50:50, which produced a pyrolysis temperature of 530°C .

Clean foils were inserted into pyrolysis tubes, so that about two-thirds of the foil was protruding from the mouth of the tube. After incubation (7 d), bacteria were picked up carefully from the top of a colony, avoiding the agar surface, by means of disposable plastic loops and smeared on the exposed foil to give a thin uniform surface coating. All bacteria for one analysis were cultured for the same length of time. Three samples for analysis were taken from each plate. The samples were oven dried at 80°C for 5 min, then the foils were pushed into the tubes using a stainless steel depth gauge so as to lie 10 mm from the mouth of the tube. Finally, Viton 'O'-rings were placed on the tubes.

The bacterial samples were processed by the machine auto-sampler. Individual tubes were heated to 80°C for a few seconds under vacuum before pyrolysis. Curie-point pyrolysis was at 530°C for 2.4 s, with a temperature rise time of 0.5 s. The pyrolysate then entered a gold-plated expansion chamber heated to 140°C , whence it diffused down a molecular beam tube to the ionization chamber of the mass spectrometer. Low-voltage electron-impact ionization (25 eV) was used to ionize the pyrolysate, which was then focussed by the electrostatic lens of a set of source electrodes, accelerated and directed into a quadrupole mass filter. The ions were separated by the quadrupole on the basis of their mass-to-charge (m/z) ratio, and detected and amplified with an electron multiplier. The mass spectrometer scans the ionized pyrolysate of mass between 50 and 200 over 20 s. The IBM-compatible personal computer used to control the PYMS-200X was also programmed (using software provided by the manufacturers) to record spectral information on ion count for the individual masses scanned and the total ion count for each sample analysed.

Only samples whose total ion counts were between 5×10^5 and 6×10^6 were used so that pyrolysis mass spectra were representative of the sample. It is considered that if total ion counts below 5×10^5 are observed, too little sample was analysed; above 6×10^6 , saturation effects can be seen in spectra and catalytic effects or heat transfer problems may have occurred during pyrolysis (Windig *et al.*, 1979). Finally, data were normalized by an iterative procedure to remove the effect of sample size differences and sorted by characteristicity, which is based on the masses produced. Masses are ranked on the basis of their pre-determined importance. For each mass the programme looks at masses showing most variance between groups (replicates), but least within the group. The 30 most characteristic masses were subjected to further statistical analysis.

The statistical analysis of the data was performed using GENSTAT version V, using the program codes provided by Horizon Instruments with the software PYMENU. The method of data analysis has been described by MacFie & Gutteridge (1982) and Gutteridge *et al.* (1985). In essence, the first stage was the reduction of the data by principal-components analysis (PCA) (Chatfield & Collins, 1980; Causton, 1987; Gutteridge, 1987;

Fig. 1. RAPD fingerprint of inoculant strains. Lane number and strain: 1 and 2, Azox; 3 and 4, USDA110; 5 and 6, NPPL; 7 and 8, CB1809; 9 and 10, I1; 11 and 12, I2; 13, no sample; 14, I3; 15 and 16, I4; 17 and 18, I5; 19 and 20, I6.

Flury & Riedwyl, 1988; Martens & Naes, 1989), which is a well-known technique for reducing the dimensionality of multi-variate data whilst preserving most of the variance. Data were reduced by keeping only those principal components (PCs) whose eigenvalues accounted for more than 0.1% of the total variance. Canonical variates analysis (CVA) then separated the samples into groups on the basis of the retained PCs and some *a priori* knowledge of the appropriate number of groupings (MacFie *et al.*, 1978; Windig *et al.*, 1983). The next stage was the construction of a percentage similarity matrix by transforming the Mahalanobis' distance between *a priori* groups in CVA using the Gower similarity coefficient S_g (Gower, 1971). Finally, cluster analysis was employed to produce a dendrogram, using average linkage clustering (Gutteridge *et al.*, 1985).

Ordination diagrams of the PYMS data were produced by plotting the eigenvalues derived from the canonical variate analyses (Figs 2, 4 and 6). The first three axes usually accounted for between 92 and 97% of the total variation in the datasets and the simple two-dimensional representation was sufficient for demonstrating the relationship between the datapoints in multidimensional space.

RAPD. Bacteria from isolated colonies of 6-d-old cultures were lysed in proteinase K as described by Young & Blakesley (1991). Samples (9 µl for each PCR reaction) were added to 16 µl of the following reaction mixture: 10× Super-Taq reaction buffer (HT Biotechnology), 2.5 µl; dNTP mix (Pharmacia), 2.5 µl of 2 mM stock; primer P25 (5'-TATTTATTCG-GGCCAATCATCCGC-3'), to a final concentration of 2 µM; Super-Taq (HT biotechnology) at 0.5 units per reaction (added last); and water to give final volume. The lysate was mixed with the reaction mix in an Eppendorf tube and a drop of mineral oil (Sigma) added to the surface to prevent evaporation during the reaction.

Samples were placed in a thermal reactor (Hybaid) and run on the following programme with a temperature change regime of 1 °C s⁻¹: 94 °C for 3 min; (94 °C for 45 s; 30 °C for 2 min; 72 °C for 2 min) × 4; (94 °C for 45 s; 36 °C for 1 min; 72 °C for 2 min) × 26; 72 °C for 5 min.

Samples were stored in a refrigerator and run on 1% (w/v) agarose gels for separation of bands. These were visualized by staining with ethidium bromide (50 µg l⁻¹) for 1 h and photographing the stained gels under UV light.

RESULTS

Characterization of strains used in inoculants

All inoculant strains were examined using PYMS and RAPD (Figs 1 and 2). The RAPD results show that different strains can be distinguished. Fortunately, for this study, the strain used in the Azofix inoculant (USDA110) was readily distinguished from strain CB1809 and the strains used in the Nitragin inoculant.

Field A

The recent cropping history of this field has been: 1982–1985 maize, 1986 beet, 1987–1989 soybeans. The soybeans were inoculated by seed dressing with inoculants in 1987, 1988 and 1989. In 1987 the inoculant was Nitragin. In 1988 and 1989 the inoculant was Azofix. In 1989 the field was examined to record nodulation by bradyrhizobia persisting from previous inoculations of soybean, which was excellent, and the population of

Fig. 2. PYMS of inoculant strains. Axes represent 92.85% of the total variation.

Fig. 4. PYMS of strains in Fig. 3. Axes represent 81.38% of the total variation. Triangles represent isolates; rectangles represent reference strains.

bradyrhizobia at the time of sowing, which was between 2 and 4×10^6 per g soil on the basis of an MPN count.

Sample results for the determination of the different types of bradyrhizobia isolated from the field using RAPD and PYMS are shown in Figs 3 and 4. These results, and

others not shown, found no isolate corresponding to that which should have been in the Azofix inoculum, even though it was the strain used in the inoculants for the year of sampling and the previous year. Examination of a

Fig. 3. RAPD of field A isolates and standard strains. Lane number and strain: 1, Azo; 2, I2; 3, CB1809; 4, I6; 5, isolate M7; 6, isolate I7; 7, isolate O8; 8, isolate N8; 9, isolate D8; 10, isolate C6; 11, λ Bgl/I size marker; 12, isolate C7; 13, isolate B4; 14, isolate C1; 15, isolate B1; 16, isolate M1; 17, I1; 18, I3; 20, I5. Lane 19 is too faint to use for characterization, probably because of a loading problem.

Fig. 5. RAPD of field B isolates and standard strains. Lane number and strain: 1, USDA110; 2, NPPL; 3, A4; 4, A8; 5, B5; 6, G1; 7, L6; 8, N5; 9, P8; 10, I7; 11, λ BglI; 12, B6; 13, E2; 14, E4; 15, H8; 16, L7; 17, N2; 18, P7; 19, E6; 20, E8.

number of RAPD gels (not shown) indicated that some of the Nitragin strains were still present in the field. For example, in Fig. 3 isolates M7, I7 O8 and D8 appear to be derived from inoculant strain I6. PYMS analysis shows inoculant I6 to be very different from these isolates (Fig. 4), perhaps indicating genetic drift in the field, or that strain I6 used in the original inoculant was phenotypically different from our strain.

Field B

The recent cropping history of this field has been: 1983 sunflower, 1984 wheat, 1985 maize, 1986 soybeans, 1987 and 1988 maize, 1989 soybeans. The soybeans were inoculated by seed dressing with the Azofix inoculant in both 1986 and 1989. In this field, nodulation by bradyrhizobia remaining in the soil from previous inoculations of soybean was very good, even though no bradyrhizobia were detected by MPN counting later in the year.

Analysis of bacteria isolated from nodules using RAPD and PYMS in no case showed any strain to be similar to strain USDA110 (Figs 5 and 6) even though this was the strain we expected to find from the Azofix inoculant. By chance we included strain NPPL in these tests and found that most isolates had the same banding pattern using RAPD (Fig. 5). PYMS analysis (Fig. 6) showed that one group of isolates was very similar to NPPL and that there was another large group which was much less similar to it. Three strains, L6, A2 and N7, formed yet another small

Fig. 6. PYMS of strains in Fig. 5. Axes represent 79.88% of the total variation. Symbols as in Fig. 4.

group which were distinguishable from the main cluster around strain NPPL. Isolates E6 and E8 were found to be outliers in the PYMS analysis, and when analysed by RAPD were shown to be similar to each other but to have different banding patterns from the other strains tested. N3, the other outlier in PYMS, did not grow well and was lost before it could be studied further.

DISCUSSION

Our results indicate that PYMS and RAPD can be used to discriminate strains of *B. japonicum* and have value in studying the fate of inoculants. The banding patterns produced by RAPD tend to show a number of bands in common between most strains, indicating that they are closely related. This relationship could be demonstrated by sequencing DNA from bands and using the sequenced DNA in hybridization assays to see whether the bands do indeed represent the same fragments of the genome. There was no need to demonstrate whether bands were the same or not in this study.

Both techniques have the advantage that hundreds of isolates can be analysed if required. Both have the disadvantage that they are very sensitive to the cultural state of the organisms concerned. We overcome this problem by making comparisons of organisms that are cultured on the same medium, under the same growth conditions at the same time, and do not assume that the techniques can be used to identify organisms without appropriate standard control strains cultured at the same time. The main advantage of the two techniques is that they allow us to determine if isolates are derivatives of the inoculum (RAPD), and if so whether they are phenotypically the same (PYMS). Fingerprints produced by RAPD are easy to generate and the cost per sample is low. PYMS analysis depends on expensive equipment, which limits its availability and means that the cost per sample is relatively high (£1–2).

The choice of primer for PCR fingerprinting was entirely arbitrary; P25 was available in the laboratory, it works well, and gives a reasonable number of reproducible bands (Coutinho, 1993). The annealing conditions and other aspects of PCR fingerprinting are those which work well with bradyrhizobia in our laboratory. Our experience indicates that published procedures for species other than the one reported upon will need to be modified (Coutinho, 1993). For example, we have found that the age of colonies used for PCR is very important (Coutinho *et al.*, 1993); young colonies (5–6 d old) of these slow-growing bacteria are ideal. As other workers have found (Caetano-Anolles *et al.*, 1991, 1992; Young & Blakesley, 1991; Mazurier *et al.*, 1992; Brousseau *et al.*, 1993) the amount of DNA, concentration of primers, method for preparing DNA and various other factors affect the efficiency with which strains can be discriminated (Coutinho, 1993).

Initially the results from the two fields studied were surprising because no bradyrhizobia isolated from them were like the strain we expected to find from the Azofix inoculant. Our results indicated that strain CB1809, or something very much like it, had been used as an inoculant in both fields. Further checking with the suppliers of the inoculant in Italy revealed that strain USDA110 had been replaced by CB1809 in Azofix inoculants used in Italy from 1986. This demonstrated the value of our techniques for characterizing inoculant strains.

Our analysis of strains from field B by PYMS indicated that there were three populations of *B. japonicum*. RAPD indicated that they were all the same as NPPL, which is

the same as CB1809. We assume that the PYMS analysis is showing change as a result of natural selection in the soil, although it cannot be excluded that it represented three populations present in the initial inoculum. An exciting challenge facing microbial ecologists in the immediate future is to find ways of identifying which characteristics change during adaptation, and why.

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