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Research Article

Rapid immuno-monitoring of inoculant plant growth-promoting microorganisms

Rapid, specific techniques are essential to monitor the quality of inoculant plant growth-promoting strains at all stages of manufacture from starter culture to the final product in its carrier medium. In this study, colony immunoblotting was evaluated for the specific detection and enumeration of *Citrobacter freundii*, one component of a Vietnamese commercial inoculant plant growth-promoting product used to improve the yield and nutrient efficiency of paddy rice. For quality control of either sterilised or unsterilised carrier media in commercial products colony immunoblotting proved to be a promising tool. Furthermore, it was possible using this technique to measure the survival of this strain in soil and the rhizosphere.

Keywords: Plant growth-promoting bacteria / Colony immunoblotting / ELISA

Received: November 20, 2008; *revised:* July 31, 2009; *accepted:* August 7, 2009

DOI: 10.1002/elsc.200800101

1 Introduction

Plant-associative bacteria can enhance plant development by fixing atmospheric nitrogen to the soil, by producing phytohormones, by improving the uptake efficiency of nutrients and water, and/or by inhibiting pathogens *via* the excretion of antibiotics or siderophores (see [1] for a recent review). These plant growth-promoting (PGP) characteristics can be exploited for amplified biomass accumulation and increased yields of agricultural crops by rhizosphere inoculation of beneficial microbes [2–4].

Inoculant PGP microbes are delivered to crops by various techniques, including applications directly to seed as slurries of cells, often with stickers. In Vietnam [5] unsterilised solid carriers referred to as peat, actually soils with high organic matter around 20% by weight, are often used. The product concerned, BioGro, is prepared in small factories located in the field near the site of application to rice or other crops by adding sugar to moistened peat and inoculating with starter culture containing the PGP bacteria. This process is repeated by two 10-fold increases yielding a product about 100 times as great by weight. The strains used [5] were selected partly on the basis of their robust growth, enabling the PGP strains to outgrow native bacterial strains in the soil.

However, a limitation for quality control of these inoculant PGPs is the choice of technical methods for tracing number of microbes in mixed cultures of inoculant products using carriers such as peat or in the field following application to seed or seedlings. Expression of reporter genes cannot be used and most microbiological or molecular tools for characterising mixed cultures are inefficient as they require lengthy and expensive testing, beyond the means of biofertiliser producers. This situation is undesirable for practical application of these microbial strains as inoculant PGPs, either for guarantees of the quality of the product in terms of viable cell numbers or the credibility of the commercial application.

To help resolve this issue we have assessed the use of colony immunoblotting (CIB) and supporting enzyme-linked assay because of their relative simplicity and speed, to verify their practical potential for testing large sample volumes of diverse media. Similar methods have been widely used in molecular cloning techniques as a way of differentiating mutants or to detect human, animal and plant pathogens [6–9].

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2 Materials and methods

2.1 Microbiological methods

A culture of the *Citrobacter freundii* (3C) was obtained from Professor Nguyen Thanh Hien of the Hanoi College of Science, Vietnam National University, Hanoi as one of three unidentified strains of the PGP inoculant, BioGro, in 2003. This diazotroph was identified by a combination of nutritional tests and analysis of ribosomal DNA [5], confirmed by a later analysis using similar techniques by the DSMZ (Deutsche Sammlung für Microbiologische Zellkulturen, Braunschweig, Germany) as *C. freundii*. *Pseudomonas fluorescens* (1N) and *Klebsiella pneumoniae* (4P) were also identified as components of BioGro [5] by similar methods and their identities verified by the DSMZ. These bacterial strains were grown using Nutrient agar (NA)/broth or Modified Nutrient media (Bacto Laboratories, Sydney) for 24–48 h at 32°C.

2.2 Antisera production, immunoglobulin purification and immunoglobulin-horseradish peroxidase conjugation

Polyclonal antibodies against *C. freundii* were raised in New Zealand White rabbits at the University of Sydney. Immunisation of rabbits was performed using heat-killed cells of *C. freundii*. The immunogen was diluted in 0.9% saline and emulsified in Freund's complete (first immunisation) or incomplete adjuvant (subsequent immunisation). After an initial injection, a booster injection was given monthly for 3 months. A small amount of blood from each rabbit was collected to check for specific antibody production. A non-competitive, solid-phase enzyme immunoassay with an immobilised antigen was applied. The specific antibody of rabbits was detected with goat anti-rabbit immunoglobulins (IgG)-horseradish peroxidase (HRP) conjugate. IgG were purified from antiserum using protein A-Sepharose affinity chromatography provided by Sigma (Sydney, Australia). IgG was conjugated to HRP (Sigma) using a sodium periodate method modified from Tsang *et al.* [10].

2.3 Immunoassay and blotting reagents

Concentrated antibody stock (as prepared above) was stored in sealed plastic vials at −20°C. Working antibody solutions were prepared at concentrations as needed by diluting stock with PBS. PBS contained 8.7×10^{-3} M Na_2HPO_4, 1.7×10^{-3} M NaH_2PO_4 and 0.15 M NaCl, adjusted to pH 7.4. Carbonate buffer was prepared by dissolving 5.8 g $NaHCO_3$ and 3.2 g $NaCO_3$ in 2 L of ultrapure water. Washing solution contained 2 g/L Tween 20 in PBS. Blocking solution contained 50 g/L skim milk powder in washing solution. HRP-conjugated swine anti-rabbit antibodies (HRP-conjugate) were purchased from DAKO, stored at 4°C and diluted to specified volumes with nanopure H_2O for working stock as necessary. HRP-substrate contained 2.5 g/L β-cyclodextrin and 50 µL/L 30% H_2O_2 in 0.1 M sodium acetate, adjusted to pH 5 with acetic acid. Chromogen contained 10 mg/mL TMB (3,3′,5,5′-tetramethylbenzidine) in DMSO, and stored sealed in an amber glass vial at room temperature.

2.4 Sandwich ELISA testing of purified IgG

Pure cultures of *C. freundii* 3C, *Citrobacter koseri*, *Escherichia coli* sp, *Pseudomonas fluorescens* 1N, *Klebsiella pneumoniae* 4P and *Brevibacter* sp. were grown for 48 h at 30°C on NA plates. Colonies were washed from the plate using PBS solution and suspended in a final volume of 3 mL. Serial dilutions were made of this stock suspension and the original cell density calculated by plating each dilution on solidified NA. These dilutions were frozen at −20°C and thawed when required for standard curves in the sandwich ELISA. The sandwich ELISA was conducted with 96-well maxisorb plates that had previously been coated with 1 µg purified anti-3C IgG per well by incubation overnight in carbonate buffer (pH 8) at 25°C. Subsequently, plates were washed three times with washing solution and incubated for 1 h at 25°C with 200 µL of blocking solution per well. After washing again three times with washing solutions, 150 µL of bacterial antigen solution as prepared above was incubated in each well at 25°C for 30 min. Plates were again washed (six times) and 150 µL 3C HRP-IgG conjugate added to each well as a 1/500 dilution from the original stock. After incubation for 30 min at room temperature, plates were washed six times and 100 µL of chromogen substrate added to generate colour. Stop solution (50 µL) was added after 5 min of incubation and the final absorbance read at 450 nm on a plate reader.

2.5 Optimisation of CIB

CIB was initially carried out by following the method described by Duez *et al.* [11], and the method optimised thereafter. Briefly, pure culture bacterial colonies were grown for 24 h on NA plates as mock colonies from toothpick smears, or serially diluted colonies from broth cultures. The initial procedure involved taking colony blots by applying nylon membranes (82 mm diameter, cut no. 11699075001 Roche, Sydney, Australia) to plates for 30 min. Membranes were then removed from the plate and the blot was heat-fixed at 80°C for 30 min. Following cooling, membranes were incubated in freshly prepared blocking solution for 1 h on a rocking shaker. Membranes were then transferred into antibody solution (10 µg/mL) without drying and incubated with shaking for 60 min at room temperature. After this, membranes were washed three times in blocking solution for 5 min each time, and then incubated in HRP-conjugate solution (0.75 µL/mL of commercial stock in blocking solution) for 1 h. Three more 5 min wash steps followed, twice with washing solution and once with PBS, before colour was developed by incubation with HRP-substrate:chromogen (95:5) mix solution for 5 min. Finally, the background was de-coloured with 1 M H_2SO_4 and the membrane rinsed with distilled water.

Optimisation experiments were performed to increase sensitivity, reduce reagent volumes and reaction times, by varying solution concentrations and incubation times.

Parameters were varied individually in triplicate and visually assessed for colour intensity and blot resolution.

2.6 Inoculant recovery experiment

Plastic pots of 6 in. diameter were filled with sterilised sand (autoclaved for 30 min at 121°C) or non-sterile sand and placed inside a glasshouse. Each pot was planted with five, one-week-old pre-germinated wheat seeds (variety 'Dollarbird'), using sterile water to moisten the soil. Two days later, seedlings were inoculated by infiltrating the root zones with 5 mL of a 24-h liquid nutrient culture of 3C containing 10^8 cfu/mL. The suspension was prepared by picking a colony of 3C from NA into a conical flask containing 250 mL of sterilised modified nutrient broth. The wheat was grown in the sterile and non-sterile sand for 6 weeks before an attempt was made to recover and estimate the density of surviving inoculum from its rhizosphere. Approximately 1 g of rhizosphere soil adhering to roots was collected, weighed and suspended into 100 mL sterile water. The suspension was shaken using an orbital shaker (Ika Labortechnik, Denmark) at 200 rotations per minute, followed by dilution plating and subsequent incubation at 32°C for 24 h. Colonies were then identified and counted using the CIB technique described above.

In another experiment, a non-sterile sandy loam soil (pH 5.36, organic matter 5.1%, sand 60%, silt 26%, clay 14%) from Camden, New South Wales, Australia, was potted without plants and infiltrated with strain 3C (5 mL of 10^8 cfu/mL). Each pot was monitored weekly for five weeks in the greenhouse and again CIB was used to follow the fate of the *C. freundii*.

3 Results

3.1 Characterisation of anti-3C IgG antibodies

The purified IgG raised against antiserum prepared from strain 3C showed a high degree of specificity in a sandwich ELISA format, with insignificant cross-reaction observed against the closely related enterobacterial cells of *C. koseri* and *E. coli*, or the other three unrelated biofertiliser strains (Fig. 1). The limit of detection, calculated by interpolating three-fold the standard deviation of the blank absorbance [12], was 5.91 $\log_{10}$(cfu)/mL.

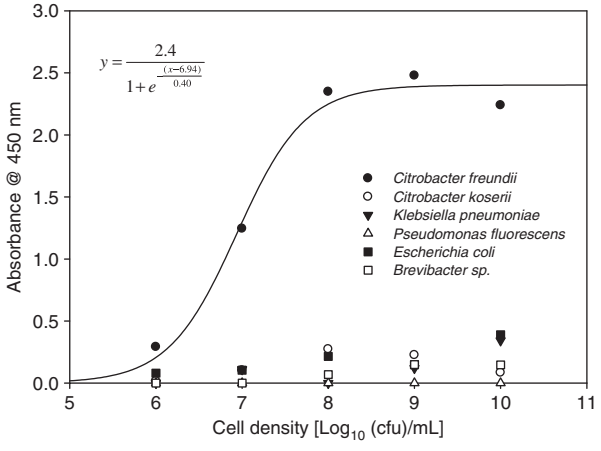

Figure 1. Sensitivity and specificity of purified anti-3C IgG measured by sandwich ELISA. IgG was diluted to 2 μg of protein per milliliter.

3.2 Optimisation of presentation of CIB

The development of the immunoblotting procedure was based on the work of Duez *et al.* [11]. After optimisation, the original fixation time and antibody concentration were reduced, concentration and incubation of the chromogen increased and the incubation time with the antibody was left unchanged. For example, the best results were given by incubating membranes for 1 h at room temperature (about 18 C) with IgG solution. After one hour, the intensity of the spots became less intense. (Fig. 2, Table 1). The final step of the colony immunoblot procedure was also modified to enhance the uniformity of de-colourisation and colony blot visualisation. This involved using a perfume spray bottle (Sydney Essential Oil, cat. nos: PGAB73-000, 018CPWM) to apply H_2SO_4 to the membrane, providing much finer droplet sizes and even coverage, thus avoiding smearing of the chromogen and giving greater resolution of blots. The optimised method was then used for the identification of 3C in inoculant recovery experiments in sand or soil.

3.3 Monitoring inoculant in sand and soil with CIB

The root rhizosphere of wheat plants both in the sterile and non-sterile sand samples contained a low background microbial population when counted on NA dilution plates of sand samples. CIB performed on inoculated samples after 6 weeks of wheat seedling growth was effective for the identification and counting of surviving *C. freundii* as compared to indigenous microorganisms (Fig. 3).

In soil treatments, changes both in total microbes and *C. freundii* populations over the 5-week period were observed. In Fig. 4 a spread plate and the resulting immunoblot show that 12 out of 27 bacterial colonies at this dilution were *C. freundii.* The background concentration of indigenous microorganisms increased directly after inoculation of *C. freundii* culture, but declined slowly after the second sample taken at 7 days (Fig. 5). The concentration measured of *C. freundii* was less consistent, increasing in concentration until week 2 to nearly 10^8 cfu/g soil, followed by a decline to approximately 2×10^6 cfu/g, where it remained stable for the next week. The densities of the *C. freundii* inoculant and the background microbial population were only significantly different at the 4-week measurement, at which time *C. freundii* numbers were less.

4 Discussion

The use of immunological methods for the identification and quantification of microorganisms is well established [13], but

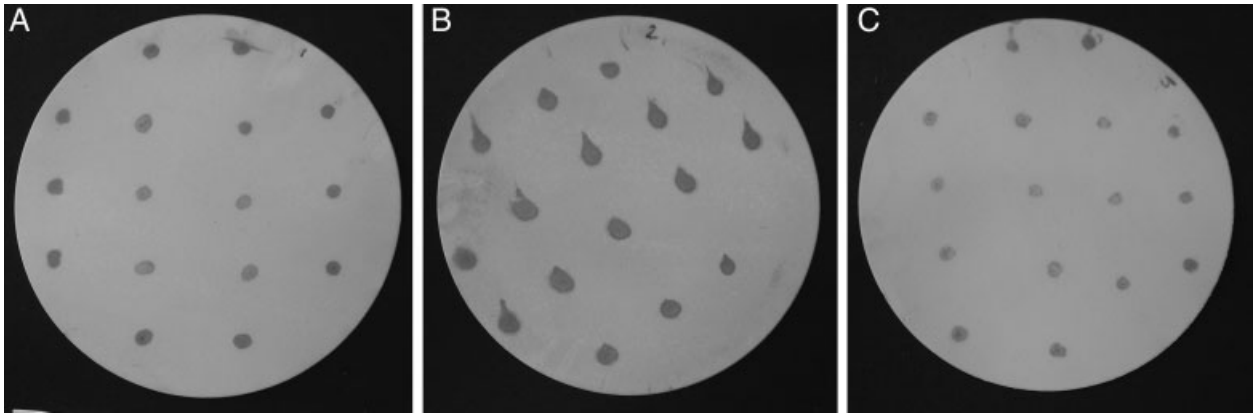

Figure 2. Comparison of the effect on colour development of immunoblots with different anti-3C IgG incubation times (0.5, 1 and 2 h). Colonies of *C. freundii* were prepared by direct inoculation of agar plates.

Table 1. Optimisation of the immunoblotting protocol.

Parameter	Original protocol	Improved protocol
Fixation time	30 min	15 min
Antibody incubation	1 h at room temperature	1 h at room temperature or more at 4°C
Antibody (IgG) concentration	10 µg/mL	5 µg/mL
HRP incubation	1 h	1 h or more
Chromogen concentration	3%	5%
Chromogen incubation	5 min	≤5 min

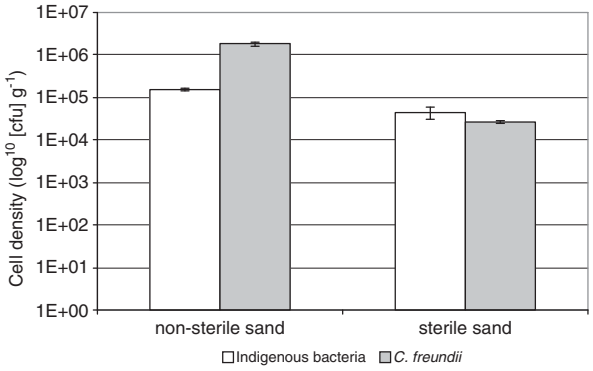

Figure 3. Survival of *C. freundii* in wheat seedling rhizospheres in sterile and non-sterile sand 6 weeks after inoculation measured by the optimised CIB procedure. In case of trials with sterile sand indigineous bacteria mainly introduced during plant growth. Error bars represent 95% confidence levels ($n = 3$).

it has only recently begun to be validated in earnest for non-medical or environmental studies [14]. We perceived our investigation to be necessary because of the rapidly expanding PGP industry, comprising diverse microbial inoculant species, but lacking specific, consistent methods for product quality control, particularly in developing countries.

The procedure of Duez *et al.* [11], which was used for studying *Bifidobacterium animalis* in faeces, was optimised for studying *C. freundii* in pure cultures and successfully used to trace inoculants in sterile and non-sterile sand against an indigenous population of approximately 10^5 cfu/g. Subsequently, the method was used to monitor the survival of *C. freundii* inoculated onto wheat seedlings against a background microbial density of 10^6 cfu/g. One week after inoculation, the indigenous population density was significantly higher, probably as a result of nutrients added from the 5 mL of culture medium in which the *C. freundii* was introduced. A similar result was also observed by Nybroe *et al.* [15], who used immunoblotting to assess the survival of *Bacillus licheniformis* in seawater: measured increases in phosphate and ammonia concomitant with *B. licheniformis* inoculation to seawater resulted in the 39 times more total biomass one day after inoculation. As with our experiment, the indigenous population returned to original background levels, although this occurred after only 7 days in seawater, compared with approximately 5 weeks in our soil experiment. While the relative decline in viable *C. freundii* cell numbers can be accounted by competition, the stabilisation of *C. freundii* numbers after 4 weeks may be explained by the establishment of the inoculant strain in the rhizosphere of wheat plant. This experiment shows the utility of the procedure to also be successfully used in soil as well as inoculant media such as peat, although much more data is required for such ecological studies. However, our results give support to the recommendation of other authors for using immunoblotting in similar environmental microbiology research [16–18].

Possible limitations of this approach inherent in immunological methods should be acknowledged; antigenic cross reactions to some of the native cultivable strains present in unsterilised carrier media may reduce the potential of the method [12]. For inoculant products as prepared in reference [5] this should not cause a significant problem given the relatively low numbers of such background strains in peat compared to the inoculant strains used in higher numbers. For

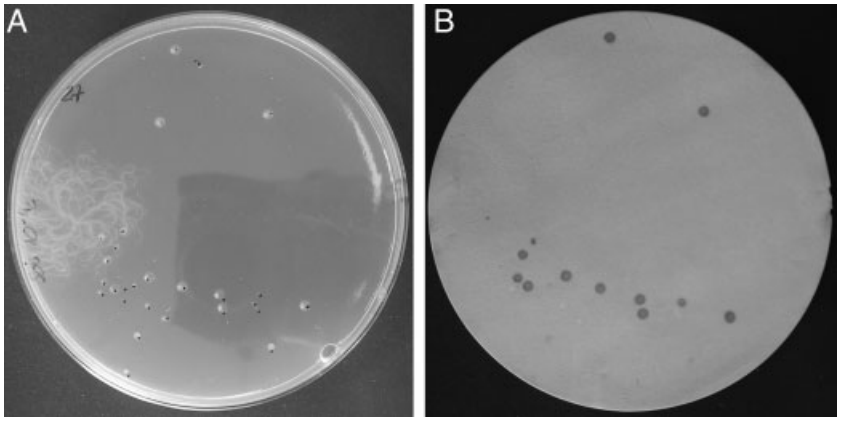

Figure 4. Modified NA spread plate and immunoblot of *C. freundii* recovered 4 weeks after inoculation into non-sterile sandy loam soil (pH 5.36, organic matter 5.1%, sand 60%, silt 26%, clay 14%) from Camden, New South Wales, Australia).

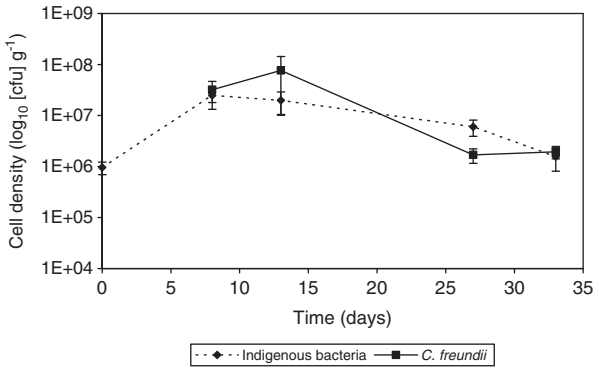

Figure 5. Monitoring the survival of *C. freundii* (3C) against indigenous bacteria in non-sterile sandy loam soil (pH 5.36, organic matter 5.1%, sand 60%, silt 26%, clay 14%; Camden, New South Wales, Australia) for five weeks after inoculation with 3C. Each pot was infiltrated with inoculant strain (5 mL of 10^8 cfu/mL) and kept in the greenhouse. Error bars represent 95% confidence levels ($n \geq 2$).

biofertilisers and soil analysis, a combination of selective media, incorporation of antibiotics into the culture media, together with the use of correct concentration of antibody preparation and the study of the polyclonal or even monoclonal antibody recognition sites would enhance the sensitivity and specificity of these immunoassays. In addition, confirmation by sandwich ELISA can be recommended to determine sufficient level of antibody specificity.

This study has shown under laboratory conditions that CIB with serial dilution can be used successfully to monitor numbers of PGP strains such as *C. freundii* in environmental media like peat or soil. This method was chosen for investigation as it integrates the benefits of counting live (colony-producing) cells with the identification of the target strain. In principle, this approach resolves the basic problem of monitoring inoculant PGP strains in carrier materials and in soil after application in the field. Optimised protocols, as developed here for *C. freundii*, could be established for many other microbial strains employed in such products to minimise the time required and to economise on reagents such as antibodies, while still providing reliable cell counts. Further improvements in the reliability of immunoblotting and its user-ease are expected, aiding in its applicability as a simple and rapid tool for monitoring inoculant PGPs, thus ensuring quality products and farmer confidence in their use.

Abbreviations: CIB, colony immunoblotting**; HRP,** horseradish peroxidase**; IgG,** immunoglobulin**; NA,** nutrient agar**; PGP,** plant growth-promoting

Acknowledgements

This work was partially sponsored by the Australian Research Council (Projects ARC LP0347940 and DP0771664) and Australian Centre for International Agricultural Research (ACIAR SMCN/2002/073). We thank Professor Nguyen Thanh Hien (previously Hanoi University of Sciences, Biofertilizer Action Research Center) for providing the BioGro strains and to Dr. J. Mengaud, Danone, France for providing us with the laboratory protocol of his original procedure.

Conflict of interest

The authors have declared no conflict of interest.

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