

## CHAPTER 2 ISOLATION AND IDENTIFICATION OF PHOSPHORUS MOBILISING BACTERIA

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### 2.1 Introduction

Plants acquire P from soil solution as phosphate anions, mostly as  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  and possibly  $\text{PO}_4^{3-}$  depending on the pH of the soil solution. It is possible that the uptake systems of some plants may prefer a specific phosphate ion. The most predominant phosphate ion available for use by plants in the pH range 5-6.5 is  $\text{H}_2\text{PO}_4^-$  (Troeh and Thompson, 1993). Phosphate anions in the soil solution are extremely reactive and become unavailable through precipitation with cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{3+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$ , depending on the soil pH (1.2.7.2, Figure 1.9) or adsorption to Fe-oxides and Al-oxides, Al-silicates and Ca-carbonates, depending on the particular properties of a soil (Brady and Weil, 2002). Deficiency of available P in soil was identified as one of the most important chemical factors restricting plant growth in the soils of the southern wheat belt of New South Wales (NSW), Australia (Colwell, 1963).

The importance to plants of soil P mobilisation by fungi has been described (Tinker, 1984; Kucey *et al.*, 1989). Mycorrhizal fungi associated with plants are an important part of the soil microbial system and these associations with plants are very common under natural soil conditions. Endomycorrhizae and ectomycorrhizae have economic value in terms of mobilisation of unavailable soil P for several crops (Gerdemann, 1968). Fungal P mobilisation is well established (Kucey *et al.*, 1989; Whitelaw *et al.*, 1999) and *Penicillium radicum*, a P-solubilising fungus has also shown promise in plant growth promotion (Whitelaw *et al.*, 1999).

The existence of soil bacteria capable of transforming unavailable P to forms available for plants has also been reported although the degree of specific P-mobilising ability by bacteria is less well-documented than for fungi (Kucey, 1983; Kucey *et al.*, 1989; Chabot *et al.*, 1993; Rodríguez and Fraga, 1999). Bacteria that have been identified by their ability to make 'halo' zones around their colonies while growing on agar plates containing insoluble P can be described as P-mobilising bacteria (Molla *et al.*, 1984; Seshadri *et al.*, 2000).

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The objectives of the work described in this Chapter were, (1) to isolate potential P-mobilising bacteria from the rhizosphere, (2) to identify them by conventional/biochemical classification methods and (3) to confirm their identification by molecular classification.

### 2.2 Materials and methods

#### 2.2.1 Soils

Three soils were used for experiments to isolate P-mobilising bacteria. They were collected from the top 15 cm containing plant roots.

##### (a) Narrabri, NSW, Auscott

This soil is considered a Vertisol (Northcote, 1971) from Auscott cotton field No 5; cropping patterns have been cotton-wheat-fallow. The soil was obtained from fallow land.

##### (b) Wagga Wagga, NSW

This is a soil from a wheat-fallow-wheat cropping area. It was obtained immediately after a wheat harvest, including plant rhizospheres as shown by plant roots (1.4.1). It is a Red Kandosol (McKenzie *et al.*, 2004).

##### (c) Wee Waa, NSW, Ivanhoe

This is a natural grassland soil collected from 12 km east of Wee Waa. It also included plant rhizosphere soil (1.4.1). It is classified as a red-brown earth/transitional red-brown earth (Inceptisol) (Triantafilis *et al.*, 2002).

The soil samples (about 2 kg) were collected from these three sites, placed in plastic bags and kept in a refrigerator at 4°C until isolation of bacteria was carried out. The soils were used within two days of collection.

The physico-chemical properties of the soils were determined following different standard methods. For the Wagga (Red Kandosol) and Wee Waa (Inceptisol) samples, the soils were separated using a 2mm mesh from root material and plant debris before

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analysis. Organic matter was determined by the potassium dichromate and  $\text{H}_2\text{SO}_4$  digestion method (Walkley and Black, 1934). Total P was determined by the ICP (acid digest) method. Total N was determined by the LECO combustion method (LECO-3336). Soil pH was measured in a 1:5, soil:water suspension by a glass electrode (Peech, 1965). Available P was extracted using 0.5 M  $\text{NaHCO}_3$  according to Colwell (1963) and available P in the extractant was determined according to Murphy and Riley (1962). The CEC was determined using atomic absorption spectrometry, the soils being extracted by ammonium chloride at pH 7.0 (Tucker, 1974). Soil texture was determined using the hydrometer method.

### 2.2.2 Soil dispersing medium and agar medium for bacterial isolation

#### 2.2.2.1 Soil dispersing medium

Phosphate peptone buffer (PPB) contained peptone (1.0 g),  $\text{K}_2\text{HPO}_4$  (1.21 g),  $\text{KH}_2\text{PO}_4$  (0.34 g) in 1 L deionised water (final pH 7), autoclaved for 20 min at  $121^\circ\text{C}$ .

#### 2.2.2.2 Agar medium

A minimal medium (Nguyen *et al.*, 2003) was used for preparing sterile agar plates. The agar medium contained per L, 0.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g NaCl, 10 g glucose, and 5 g  $\text{Ca}_3(\text{PO}_4)_2$ ; 1 mL of micronutrient solution containing  $\text{H}_3\text{BO}_3$  ( $5 \text{ g L}^{-1}$ ),  $\text{Na}_2\text{MoO}_4$  ( $5 \text{ g L}^{-1}$ ),  $\text{ZnSO}_4$  ( $0.2 \text{ g L}^{-1}$ ) and  $\text{AlCl}_3$  ( $0.15 \text{ g L}^{-1}$ ); and 15 g agar. The pH was adjusted to 7.0 and the solution was autoclaved for 20 min at  $121^\circ\text{C}$ .

In order to prevent fungal growth, after autoclaving and before the plates were poured cycloheximide (actidione) was added (0.1 g in 5 mL ethanol to 1L medium) to a final concentration of  $100 \mu\text{g mL}^{-1}$ .

### 2.2.3 Isolation of phosphorus mobilising bacteria from soils

#### 2.2.3.1 Preparation of bacterial suspensions from soil samples

For each soil a 10 g sample containing root debris (rhizospheric soil) (1.4.1; 2.2.1) was mixed with 95 mL PPB in a 200 mL jar. Some glass beads were added into the jar and the suspension was shaken mechanically (100 rpm) for 1h at  $24^\circ\text{C}$ . Without

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allowing the soil to settle, a 10 times dilution series was made to 1:1,000,000 using 0.85% NaCl solution. For each solution 1 mL suspension was diluted to 10 mL. Before each dilution step the suspension was vortexed to prevent soil from settling.

### **2.2.3.2 Culture of bacterial suspensions on agar plates**

Each of the diluted samples was vortexed and a 100  $\mu$ L drop was placed onto the surface of minimal agar medium (2.2.2.2) with a micropipette (three replicates for each sample). A glass spreader shaped like a hockey stick was used to aseptically spread the suspension onto the surface of the plates. After spreading, the plates were incubated at 28°C for 48 h. The plates were examined for P-mobilisers characterised by 'halo' or clear zones around individual colonies.

### **2.2.3.3 Preparation of pure cultures and preservation of phosphorus mobilising bacteria**

In order to obtain pure cultures, bacteria that created 'halo' zones around their colonies were isolated and sub-cultured by streaking with a loop on agar plates. The plates were incubated at 28°C for 24 or 48 h.

About 5 mL of a 10 per cent sucrose/5 per cent peptone broth was added to each plate (Fry, 1954; Annear, 1956). The bacteria were scraped from the surface of the plate and mixed with the sucrose/peptone broth using a sterile pasteur pipette bent by flame. Using another sterile pasteur pipette the suspension was transferred into 10 mL sterile bottles. Ampoules containing a paper label and absorbent cotton at the bottom and a non-absorbent cotton plug at the top were sterilised for preserving the bacteria. The top plug was removed aseptically and after vortexing, 5-6 drops of each bacterial suspension were transferred to the label and absorbent cotton wool in the appropriate ampoule. There were two stages of freeze drying (Dynavac 840); in the first drying stage, samples were dried for about 30 min in a centrifuge at -40°C to -45°C while the vacuum gauge read below 0.1 torr. Then, using aseptic techniques, the sterilised non-absorbent cotton plugs were trimmed to size and re-inserted in the ampoules just below the top level. In the second drying stage, using vacuum tree suction through a mini freeze drier, the samples were dried until the vacuum gauge reached 0.05 torr (a

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few hours to overnight). The samples were then sealed under vacuum using a natural gas and air flame.

### **2.2.3.4 Phosphorus mobilising activity of freeze-dried samples**

To examine the relative P-mobilising ability of the bacteria, samples were incubated for two weeks, and the size of the 'halo' zones compared.

Ampoules were opened aseptically and agar plates streaked with the bacterial contents. The samples were sub-cultured three times using minimal agar plates containing  $\text{Ca}_3(\text{PO}_4)_2$ . After the third sub-culture of isolated bacteria, sterile tooth picks were used to inoculate another set of plates to confirm P-mobilising ability and assess the level of activity. Three replicates were prepared for each bacterial sample. The inoculated plates were incubated for three days at 30°C followed by 11 days at room temperature (20°C). A visual assessment of the 'halo' size was then made and one plate of each strain was photographed.

### **2.2.4 Characterisation of phosphorus mobilising bacteria by conventional classification (growth, morphology and biochemical reactions)**

Of the ten bacteria isolated, attempts have been made to identify seven of them. Morphological characterisation of an unknown organism can provide some indication about the group to which it belongs. Results from Gram staining, motility tests, culture tests (aerobic or anaerobic growth) can also provide information about the proper genus of an organism.

The most widely used system of a conventional classification is described in "Bergey's Manual of Systematic Bacteriology" (Krieg and Holt, 1989). In order to identify the isolated bacteria several conventional tests such as Gram stain, motility, growth in air, growth in aerobic conditions, catalase test, oxidase test, glucose utilisation, oxidation-fermentation and biochemical tests were performed using API 20E strips.

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The API system from BioMérieux (Appendix 2.2) was designed to give rapid identification of bacterial species. The strips consist of microtubes containing dehydrated media to which bacterial suspensions are added. During incubation the bacterial metabolism results in colour changes that are either spontaneous or revealed by the addition of reagents. The reactions are read according to colour production as positive or negative and identification is obtained by referring to the Analytical Profile Index or identification software (API 20E V4.0 and API 20 NE V6.0).

An incubation box was prepared (tray and lid) and about 5 mL of distilled water was distributed into the honeycombed wells of the tray to create a humid atmosphere. Bacteria from the freeze-dried samples were grown for 24 h on agar plates. Using a pipette a single well-isolated colony was removed and emulsified in 0.85% NaCl solution (5 mL) to achieve a homogeneous bacterial suspension. Using a sterile pasteur pipette the API strips were filled and results read following the BioMérieux (Appendix 2.2) instructions.

### **2.2.5 Characterisation of phosphorus mobilising bacteria by molecular methods**

#### **2.2.5.1 Amplification of DNA**

16S rRNA consisting of about 1,500 nucleotides is a structural component of the small subunit of ribosomes. It is encoded for by the 16S rDNA gene (<http://pilbara.mq.edu.au>, 2006). Bacterial identification by the use of 16S rDNA is based on sequence variation within the 16S rRNA molecules of individual species (<http://scicentr2.tc.cornell.edu>, 2006). Since ribosomal molecules and their corresponding DNA sequences occur in all cellular organisms, they are a convenient universal target. Molecular identification of bacteria using 16S rDNA is facilitated by the use of PCR with specific primers to isolate and amplify the 16S rDNA gene. The gene is then sequenced and compared with other known sequences in a possibly accessible database (<http://www.ncbi.nlm.nih.gov>, 2006)). For bacteria, 16S rDNA gene sequence analyses have completely redefined the phylogenetic relationships previously determined using biochemical and morphological characteristics.

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Bacteria from a single colony from a plate inoculated with a freeze-dried sample and incubated for 24 h was used for DNA amplification. Using a sterile toothpick, a small amount was transferred to an Eppendorf tube containing 50  $\mu\text{L}$  of DNA free sterile MilliQ water. The suspension was heated at  $86^{\circ}\text{C}$  in a water bath for 10 min to lyse the cells and release their DNA. In a sterile PCR tube, 5  $\mu\text{L}$  DNA extract, 45  $\mu\text{L}$  DNA-free water, primer (1) 10  $\mu\text{L}$  ( $1 \text{ pmol } \mu\text{L}^{-1}$ ), primer (3) 10  $\mu\text{L}$  ( $1 \text{ pmol } \mu\text{L}^{-1}$ ), in one case primer 5 10  $\mu\text{L}$  ( $1 \text{ pmol } \mu\text{L}^{-1}$ ) and Taq Mastermix 30  $\mu\text{L}$  were mixed (<http://www1.qiagen.com>, 2007). The sequence of Primer 1 (forward primer) was GTGCCAGCMGCCGCGG;Primer3(reverse primer) was GACGGGCGGTGTGTRC and Primer 5 (reverse primer) was AGAGTTTGATCMTGGCTCAG (Sambrook *et al.*, 1989). Where M = AC and R = AG.

A small layer of mineral oil was added to the surface of the mixture. The PCR was set at an initial denaturation temperature of  $94^{\circ}\text{C}$  for 3 min, followed by 38 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $55^{\circ}\text{C}$  for 30 sec, extension at  $72^{\circ}\text{C}$  for 1 min, a final extension at  $72^{\circ}\text{C}$  for 7 min followed by holding at  $4^{\circ}\text{C}$ .

The amplification products were then separated by using agarose gel electrophoresis. The 1.1% agarose gels were prepared in TAE buffer (40 mM Tris base, 0.114% (v/v) glacial acetic acid, 2 mM EDTA, pH 8) containing  $0.5 \mu\text{g mL}^{-1}$  ethidium bromide. Loading dye (4 $\mu\text{L}$ ) and PCR product or marker (10  $\mu\text{L}$ ) were mixed with the samples and the gels run at 100 V for 45 min to 1 h at constant voltage.

After completion of the electrophoresis, the DNA was visualised with a UV light and photographed using a Polaroid camera (Hoefen's PhotoMan).

### 2.2.5.2 Purification of DNA

For further purification, slices of the agarose gels containing DNA bands of about 900 bp in length were cut from the gels, weighed and placed in 2.0 mL tubes. Three volumes of Ultra Salt was added and mixed well with the gel. The mixture was incubated at  $55^{\circ}\text{C}$  to melt the gel. Then Ultra Bind solution (5  $\mu\text{L}$  plus 1  $\mu\text{L}$  per  $\mu\text{g}$  of DNA) was added and the contents resuspended by vortexing at high speed. After

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centrifuging for 5 sec, the pellets were resuspended in 1 mL of Ultra Wash by vortexing for 5-10 sec. After centrifuging for 5 sec at in a microcentrifuge (SIGMA CE; Model 1014) set at the maximum 10,000 rpm, the supernatant was discarded. Finally the pellet was resuspended in DNAase free water. After incubating the resuspended pellet at room temperature for 5 min it was centrifuged for 1 min. The supernatant was transferred into two 0.2 mL PCR tubes for each sample, adding forward primer (1) in one and reverse primer (3) or (5) to the other and despatched to SUPAMAC Laboratory, the University of Sydney, for sequencing.

After obtaining the sequence, strains were identified through the BLASTN (ver 2.2.14), software for classification of bacteria (Altschul *et al.*, 1997).

### 2.3 Results

#### 2.3.1 Soil physico-chemical properties

The physico-chemical properties of the three soils used for isolating soil P-mobilising bacteria are shown in Table 2.1. These three soils varied in their physico-chemical properties. The Wee Waa soil (Inceptisol) contained twice as much total P (300 mg P kg<sup>-1</sup>) as the Wagga (Red Kandosol) soil (150 mg P kg<sup>-1</sup>). The highest (17 mg P kg<sup>-1</sup>) available P however was found in the Wagga soil (Red Kandosol) while the lowest

**Table 2.1** Physico-chemical properties of three soil samples used for isolating P-mobilising bacteria

The following three soils were used for isolating soil P-mobilising bacteria. Physico-chemical properties of these three soils were determined using different standard methods of analysis (2.2.1).

Soil physico-chemical properties	Soils		
	Narrabri (Vertisol)	Wagga (R. Kandosol)	Wee Waa (Inceptisol)
Total P (mg kg <sup>-1</sup> )	280.0	150.0	300.0
Total N (mg kg <sup>-1</sup> )	1300.0	890.0	1500.0
OM (%)	2.5	3.0	4.6
Available P (mg kg <sup>-1</sup> )	11.1	17.0	3.8
pH	7.4	4.8	5.7
CEC (cmol <sup>c</sup> kg <sup>-1</sup> )	32.9	4.5	3.3
Sand (%)	33.1	63.0	83.0
Silt (%)	17.3	17.3	7.3
Clay (%)	49.5	19.7	9.7
Soil texture	C	SL	LS

OM = organic matter; C = clay; SL = sandy loam; LS = loamy sand



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(3.8 mg P kg<sup>-1</sup>) available P however was found in the Wee Waa soil (Inceptisol). The Narrabri soil (Vertisol) contained the highest clay content, and had the highest pH and CEC (Table 2.1). The Wee Waa soil (Inceptisol) had the lowest clay content and CEC, and the Wagga soil (Red Kandosol) had the lowest pH (Table 2.1).

### 2.3.2 Isolation of potential phosphorus mobilising bacteria from three soils

Bacterial cultures grew on agar plates containing minimum medium and insoluble Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> from all dilutions of the initial soil solutions from the Wee Waa (Inceptisol), Narrabri (Vertisol) and Wagga Wagga soils (Red Kandosol). Some clearing of the opaque agar caused by dissolution of the insoluble Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> was evident on all plates, but discrete, flat, circular colonies were present only on the plates from the more diluted samples.

#### (a) Wee Waa soil (Inceptisol)

There were two colonies on the agar plate prepared from the most diluted sample from the Wee Waa soil (Inceptisol) suspension, which were surrounded by a 'halo' zone. One of them was yellow in colour and viscid, and was named FA001. The other one was white and butyrous and was named FA002.

#### (b) Narrabri Soil (Vertisol)

On an agar plate prepared from the most diluted sample from the Narrabri soil (Vertisol) there were two colonies surrounded by 'halo' zones. These two colonies were both cream. They were named FA003 and FA004.

#### (c) Wagga Wagga soil (Red Kandosol)

Six colonies surrounded by 'halo' zones were found on an agar plate prepared using the most diluted samples from the Wagga soil (Red Kandosol). They were named FA005, FA006, FA007, FA008, FA009, and FA010. Five (FA005, FA006, FA007, FA008 and FA009) were cream in colour and butyrous; the other one (FA010) was deep yellow in colour, odorous and more viscid than the FA001 colony.

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### 2.3.3 Culturing the ten strains and preparing freeze-dried culture

When the ten isolated strains FA001 to FA010 were cultured on agar plates they retained their characteristic colour and butyrous or viscid character. The strains FA001 and FA010 were yellow and viscid and the remaining eight strains (FA002 to FA009) were cream and butyrous. The cultures from the agar plates were freeze-dried in 8-10 ampoules for each strain.

### 2.3.4 Determination of phosphorus mobilising ability by measurement of 'halo' zone for seven bacterial strains

Seven of the ten isolated strains of bacteria were used to assess P-mobilising activity by inoculation of a freeze-dried sample, re-cultured three times. The strains selected were the two strains isolated from the Wee Waa soil (Inceptisol), FA001 and FA002; the two strains from Narrabri soil (Vertisol), FA003 and FA004; and three strains from the Wagga Wagga soil (Red Kandosol), the cream strains FA005 and FA009, and the yellow strain, FA010. Photographs of agar plates cultured for a total of 14 d (2.2.3.4) are shown in Figures 2.1 to 2.7.



**Figure 2.1** 'Halo' zone created by strain FA001 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .



**Figure 2.2** 'Halo' zone created by strain FA002 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .

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**Figure 2.3** 'Halo' zone created by strain FA003 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .



**Figure 2.4** 'Halo' zone created by strain FA004 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .

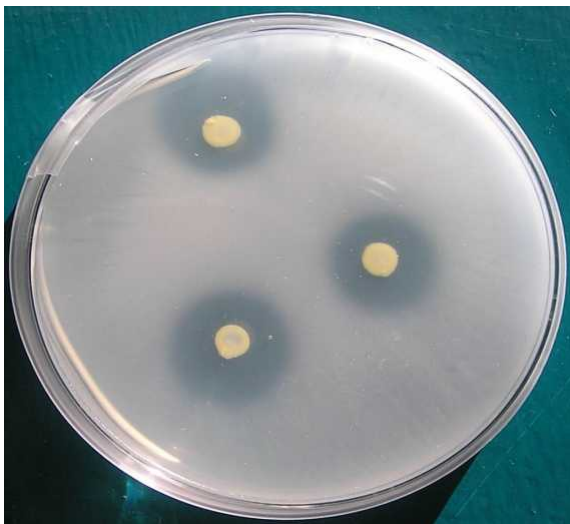


**Figure 2.5** 'Halo' zone created by strain FA005 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .



**Figure 2.6** No 'halo' zone was visible when strain FA009 was applied to minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .

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**Figure 2.7** ‘Halo’ zone created by strain FA010 on minimal agar plate containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$ .

The diameters and clarity of the ‘halo’ zones for these seven bacterial strains are shown in Table 2.1

**Table 2.2** Evaluating different strains based on the size of ‘halo’ zone created on agar plates.

The diameter of the ‘halo’ zone is the mean value calculated from three replicates.

Strains	FA001	FA002	FA003	FA004	FA005	FA009	FA010
Diameter (mm)	18	11	8.5	7.3	11	0	18
sd	± 0.5	± 1.0	± 0.5	± 0.3	± 1.0	± 0	± 1.0
Clarity	very clear	clear	clear	clear	very clear	not clear	very clear

The two yellow strains, FA001 and FA010, created the largest diameter (18 mm) ‘halo’ zones. Strain FA009 did not create a halo zone as shown in Figure 2.6.

### **2.3.5 Characterisation of potentially P-mobilising bacteria**

#### **2.3.5.1 Characterisation by conventional classification (growth, morphology and biochemical reactions)**

The seven bacteria examined by two week incubation on agar plates (2.3.3) have been assessed using conventional tests. The results are presented in Table 2.3. The results of the oxidation-fermentation tests of the seven bacteria, 48 h after inoculation and incubation at 28°C, are shown in Figure 2.8.

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**Table 2.3** Results of conventional tests performed on seven bacterial strains.

Tests	FA001	FA002	FA003	FA004	FA005	FA009	FA010
Gram strain	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Shape	R	R	R	R	R	R	R
Motility	+	+	+	+	+	+	+
Growth in air	+	+	+	+	+	+	+
Growth anaerobically	+	+	-	-	-	-	+
Catalase test	+	+	+	+	+	+	+
Oxidase test	-	-	+	+	-	-	-
Glucose (acid)	+	+	+	+	+	+	+
Carbohydrate (O/F/-)	F	F	O	O	O	O	F
MacConkey Medium	+ve	+ve	+ve	+ve	+ve	-ve	+ve

+ = strain positive to the test

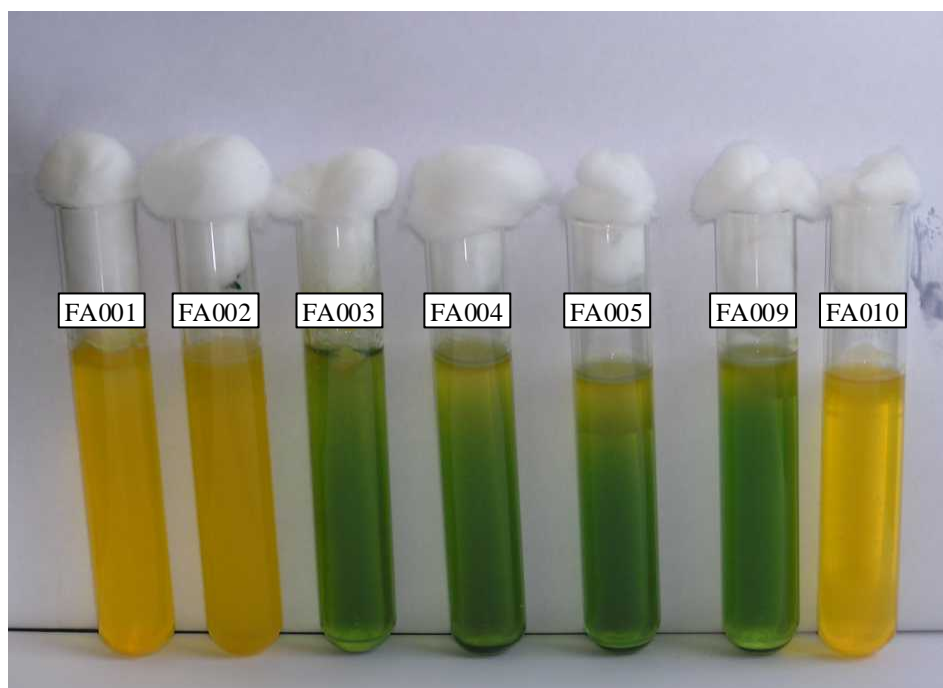
- = strain negative to the test

R = rods

O = oxidation

F = fermentation

Based on these initial tests, FA001 (yellow and viscid), FA002 (white) and FA010 (yellow, odorous and viscid) were identified as belonging to the Enterobacteriaceae family, whereas the remaining four strains (FA003, FA004, FA005 and FA009) did not belong to this family.



**Figure 2.8** Oxidation fermentation test using bacteria FA001, FA002, FA003, FA004, FA005, FA009 and FA010.

The OF test is used to determine whether a bacterium has the enzymes necessary for the aerobic breakdown of glucose (ie oxidation) and/or for the fermentation of glucose. Inoculation is carried out as a stab to within 1 cm of the bottom of the tube containing Hugh-Leifson's medium. After incubation at 37°C for 48 h yellow at the top of a tube (i.e. in air) indicates oxidative (aerobic) metabolism. Yellow throughout a tube indicates fermentative metabolism.

### **2.3.5.2 Characterisation of bacteria using API strips**

The three strains, FA001, FA002 and FA010 belonging to the Enterobacteriace Family were tested with API 20E strips. The remaining four strains were tested with API 20NE strips.

The data from the API strips and conventional data (Table 2.3) were analysed by the API specific software. The results did not indicate single species of bacteria for any of the strains. The strains FA001, FA002 and FA010 were found to be fermentative in the Oxidative/Fermentative test, which is a preliminary indication of bacteria in the Enterobacteriaceae family. However, the results of the analysis in all cases show more

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than one possible genus for these strains. The API 20E strip for FA001 after 48 h incubation at 30°C is shown in Figure 2.9.



**Figure 2.9** API 20E test strip for FA001 strain.

The results of the API analysis for the seven bacteria are shown for each strain.

**FA001:** Analysis of the API 20E strip for FA001 (Figure 2.9) did not confirm the preliminary result of the Enterobacteriaceae family for this strain. The possible genres indicated were *Pantoea* spp. or *Erwinia* spp.. There were also counter arguments against *Pantoea* spp..

**FA002:** The API 20E strips suggested ‘unacceptable profile’ with the possibility of *Enterobacter cloacae* or *Enterobacter sakazakii*, although there were some tests against these two strains.

**FA003:** Despite some counter results, the API 20 NE strip suggested the possibility of *Agrobacterium radiobacter* (79.8%) and less probably *Sphingomonas paucimobilis* (19.9%) for this strain.

**FA004:** The result of API 20 NE analysis for this strain using V6.0 software suggested the possibility of *Agrobacterium radiobacter* or *Sphingomonas paucimobilis* or *Burkholderia cepacia*. The Software also suggested that *Burkholderia gladioli* should be considered.

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**FA005:** The results of API 20 NE suggested, with some counter results, the possibility that this strain is *Sphingomonas paucimobilis* (45.9%), or *Chryseomonas luteola* (44.2%).

**FA009:** The API 20 NE strip result suggested the possibility, with some counter results, that this strain is *Agrobacterium radiobacter*, *Chryseomonas luteola*, *Burkholderia cepacia* or *Sphingomonas paucimobilis*. The software also suggested that this strain may be *Burkholderia gladioli*.

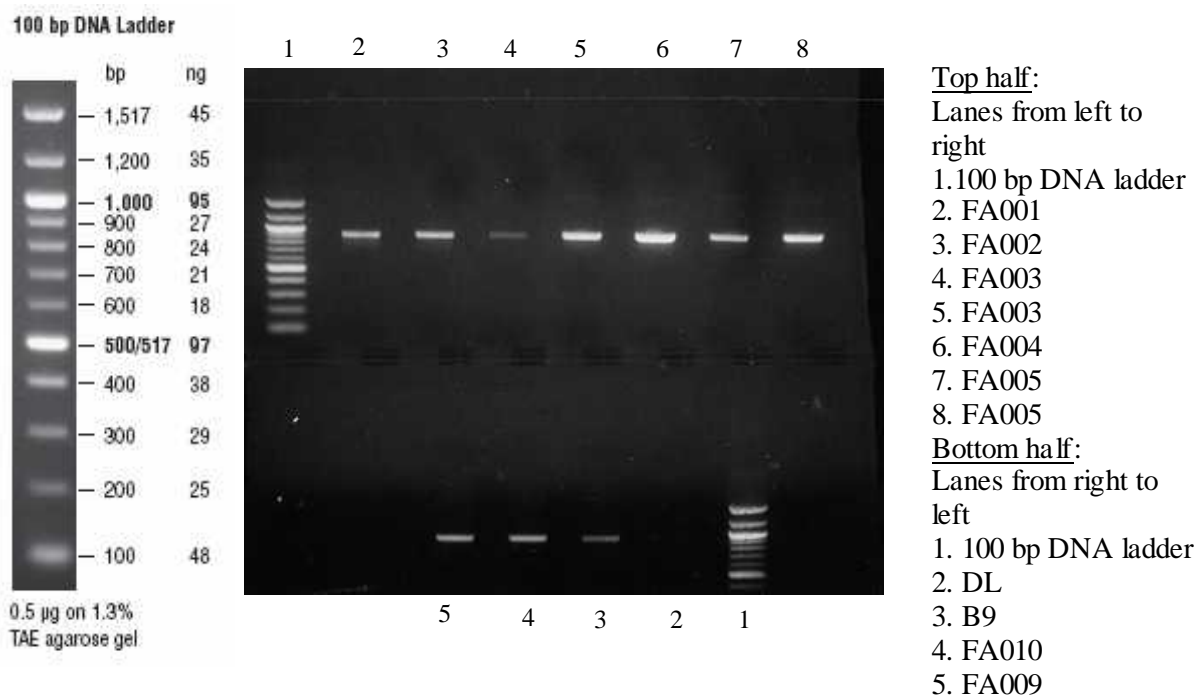
**FA010:** The results of the API 20E strip, suggested ‘unacceptable profile’ with the possibility of *Pantoea* spp. 3, *Serratia rubidaea*, or *Erwinia* sp. but there were some tests against two strains (*Pantoea* spp. 3, *Serratia rubidaea*) as well.

### 2.3.6 Molecular classification of isolated bacteria using 16S rDNA technique

After amplification of the 900 bp DNA fragments of the 16S rDNA extracted from the seven strains, samples were run together with a 100 bp ladder (Figure 2.10). Samples of FA003 and FA005 were taken from two replicates from a colony from a single freeze-dried sample. Two different strains, DL and B9, were included, but are not discussed in this thesis.



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**Figure 2.10** Agarose gels of PCR products using forward (1), reverse (3) and reverse (5) primers.

Two sets of DNA samples and marker were run in the same agarose gel. The size marker (top half) shows 100 bp DNA ladder (sizes from the top left side are 1517, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). The second marker shows the same sizes as the first marker, but starting from the right bottom half.

The alignment of the unknown 16s rDNA sequences to the *E. coli* K-12 16 s rDNA, determined using Genius pro 3.03 software are presented in Table 2.4 with the forward and reverse sequencing obtained using the BLASTIN software.

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**Table 2.4** Identification of isolated bacteria with per cent similarity by 16S rDNA sequencing (forward and reverse).

"Maximum identity" is the score of the high-scoring segment pairs (HSP) from that database sequence. "Coverage" is the percentage of the query that has been matched by that database sequence.

Strains	Forward sequence	Coverage (%)	Maximum identity (%)	Alignment (forward) bp*	Reverse sequence	Coverage (%)	Maximum identity (%)	Alignment (reverse) bp *
FA001	<i>Pantoea ananatis</i>	100	100	56-400	<i>Pantoea ananatis</i>	100	100	1006-1308
FA002	<i>Pantoea agglomerans</i>	100	100		NI			
	<i>Enterobacter cloacae</i>	100	100		NI			
FA003	<i>Burkholderia</i> sp.	100	99					
FA004	<i>Burkholderia</i> sp.	100	99	601-1238	<i>Pantoea agglomerans</i>	100	100	620-1238
					<i>Enterobacter</i> sp.	100	100	
FA005	<i>Burkholderia</i> sp	100	100	628-1151	<i>Burkholderia</i> sp.	96	98	531-1367
FA009	<i>Burkholderia</i> sp.	100	100	576-814	<i>Burkholderia</i> sp.	100	99	889-1355
FA010	<i>Pantoea</i> sp.	100	100	564-664	<i>Pantoea agglomerans</i>	100	99	674-1304

"E value" is the traditional expected value that exists for matches between sequences, thus the lower the E-value, or the closer it is to "0" the more significant the sequences are. 'E' value of top ten matching isolates from all bacteria was between 0.0 to 1e-78

\*alignment of forward/reverse unknown 16 srDNA (bp) to *E. coli* K-12 16srDNA

NI = not identified, the extracted DNA sequences were very short thus BLASTIN software did not match with a specific bacterium

**FA001:** Using the BLASTN software both forward (1) and reverse (5) sequences give similar results. The reverse base pair gave a longer sequence, and is presented in the Appendix 2.1a. The output of the BLASTN analysis suggested the possibility of several known bacteria, including *Pantoea ananatis*, *Enterobacter* sp. and *Enterobacter agglomerans*, in this order of probability.

**FA002:** The forward sequence in the BLASTN showed characteristics of many bacteria including some uncultured bacterial clones that coincided with the genetic constituents of FA002. This software predicted the possibilities of *Enterobacter cloacae*, *Pantoea agglomerans*, *Enterobacter* sp. XW110 (Appendix 2.1b).

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**FA003:** The forward sequence predicted different species of *Burkholderia* sp including some uncultured bacterial clones (Appendix 2.1c). Therefore there is a possibility that FA003 is *Burkholderia* sp.

**FA004:** The result of the forward sequence suggested the possibility that FA004 is *Burkholderia* sp., *B. graminis* or *B. cepacia* (Appendix 2.1d).

**FA005:** The results of the forward sequence predicted that FA005 is *Burkholderia* sp, *B. graminis* or *B. caryophylli* (Appendix 2.1e).

**FA009:** The results of the forward and reverse complemented sequences suggested that FA009 is also *Burkholderia* sp., an uncultured *B. sp.*, or another uncultured soil bacterium clone (Appendix 2.1f).

**FA010:** The results of the forward sequence suggested that FA010 is most similar to bacteria such as *Pantoea agglomerans* strain ATCC, *Pantoea* sp. 62HP12, *Pantoea* sp. pfb25 and an uncultured strain from the Enterobacteriaceae (Appendix 2.1g).

For strains FA001, FA005, FA009 and FA010 there is similarity in the results obtained using forward and reverse sequences. This is not the case for the strain FA004. The forward sequence of the strain FA004 matched with *Burkholderia* sp. whereas the reverse sequence matched with *Pantoea agglomerans*, *Enterobacter* sp. and some other bacteria. This is not consistent with the results shown in Figure 2.8. These results show that the strain FA004 can carry out oxidative fermentation. *Enterobacter* sp. would show fermentative breakdown of sugars. The reverse sequences of the strains FA002 and FA003 were very short, thus they were not included in the table. The top 10 similar strains for these seven isolates determined using the BLASTN software are presented in detail in the Appendices.

### 2.4 Discussion

Ten P-mobilising bacteria were isolated from three soil samples from Wee Waa (Inceptisol), Narrabri (Vertisol) and Wagga (Red Kandosol). They were isolated from

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three soils with varying physico-chemical properties (Table 2.1). In terms of clay content, CEC, pH, total P and available P, these three soils varied substantially and P-mobilising bacteria were isolated from all of them. Six colonies surrounded by 'halo' zones were found on an agar plate prepared using an extract from Red Kandosol, whereas only two colonies were found on the plates prepared using extract from Inceptisol and Vertisol. However, there is not sufficient information available to suggest that the Red Kandosol is a better source for P-mobilising bacteria. The P-mobilising capacity of these bacteria was determined by their ability to create a 'halo' zone surrounding a colony on agar plates containing insoluble  $\text{Ca}_3(\text{PO}_4)_2$  (2.3.3). Seven of these isolated bacteria were studied to identify them and quantify their P-mobilising capacity. Of these seven bacteria, after a total of five sub-cultures including three after freeze-drying, six bacteria (FA001, FA002, FA003, FA004, FA005 and FA010) could create a 'halo' zone around their colonies on agar plates containing insoluble P. The strain FA009, which was initially selected from the Wagga Wagga soil (Red Kandosol) as a P-mobiliser did not produce a 'halo' after five sub-culture steps. This shows that it had lost its P-mobilising capacity. Kucey (1983) has reported that some P-mobilising bacteria lose their P-mobilising ability after a few subculture steps and this loss is irreversible. The strains that gave the largest 'halo' in the 14 days incubation experiment were FA001 from the low pH, low clay Wee Waa soil (Inceptisol), and FA010 from the low pH, low clay Wagga soil (Red Kandosol).

It has been reported that in many cases bacteria that can mobilise P in the laboratory do not mobilise P in field (Richardson, 2001). Although there could be several reasons for this, loss of P-mobilising ability in field culture could be an important factor. More research on genetic studies might be a promising way to find the reason and resolve this problem.

The selection method used here to isolate P-mobilising bacteria is not comprehensive. It was based on the use of an agar-based medium containing  $\text{Ca}_3(\text{PO}_4)_2$ , using glucose in luxury amounts and  $(\text{NH}_4)_2\text{SO}_4$  as a nitrogen source under aerobic conditions. This possible limitation in the range of P-mobilising strains selected should be kept in mind for future research. In this isolation technique, incubation was allowed for 48 h.,

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which is suitable only for fast growing bacteria. There could have some slow growing potentially P-mobilising bacteria which were not screened due to the short incubation time used.

From the results presented it can be seen that reasonable similarity was found between classification by conventional and molecular methods. In the case of FA001, the conventional result gave tentatively *Pantoea* spp, and the molecular test predicted *Pantoea ananatis* most strongly. By both conventional and molecular classification, the strain FA002 is most probably *Pantoea agglomerans* or *Enterobacter cloacae*. For the strain FA003, while the conventional experiments suggested probably *Agrobacterium radiobacter* (79.8%), but *Burkholderia* sp. was suggested by molecular methods. The conventional classification for FA004 suggested *Burkholderia cepacia* and the molecular classification suggested *Burkholderia* sp. from the forward sequencing, while using reverse sequencing it indicated *Pantoea agglomerans*. The forward sequence is very long and consistent in comparison to the reverse sequence. Together with information from conventional classification, it can be presumed that FA004 was *Burkholderia* sp. While the conventional classification suggested FA005 is most probably *Sphingomonas paucimobilis*, using molecular classification with both forward and reverse sequencing *Burkholderia* sp. was suggested. Conventional classification of FA009 was indecisive between *Agrobacterium radiobacter*, *Chryseomonas luteola*, *Burkholderia cepacia*, *Sphingomonas paucimobilis* or *Burkholderia gladioli*. However, *Burkholderia* sp. was suggested by molecular classification. The conventional classification suggested that FA010 is *Pantoea* spp., and molecular classification confirmed it as *Pantoea agglomerans*.

The two bacterial strains, FA001 and FA010, identified as *Pantoea* spp, were shown to give the largest diameter halo on agar plates incubated for 14 d (Table 2.2). *Pantoea* spp with P-mobilising ability have been isolated from Korean soils (Chung *et al.*, 2005; Son *et al.*, 2006). Among the other P-mobilising bacteria isolated two *Burkholderia* strains were obtained from Narrabri (Vertisol) and two from Wagga (Red Kandosol) soils. *Burkholderia* strains with P-mobilising capacity have been isolated from rice rhizosphere in Indonesia (Purnomo *et al.*, 2004).

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Amy *et al.* (1992) described a comparison of the identification systems, API-NFT strips, BIOLOG and MIDI for a wide range of bacteria. Each system identified only a small percentage of the total isolates, and in only seven cases were the isolates identified the same way by more than one system. All systems used to identify bacteria, whether phenotypic or genotypic, have limitations, because no single test methodology can provide results that are 100 per cent accurate. Many classification systems have been developed by humans for study of bacteria. Because of this, the terminology and nomenclature are not consistent between organisms when they are studied by conventional methods. A useful test to distinguish between two genera in one system might have no use in distinguishing between any other two genera (<http://www.ratsteachmicro.com>, 2007).

Phenotypic systems, the most common approach used in clinical laboratories to identify bacteria, have several drawbacks. In contrast to the properties determined by molecular methods such as DNA hybridization, biochemical properties determined do not accurately reflect all the characteristics of a given species. In addition, phenotypic properties can be unstable at times and expression can be dependent upon changes in environmental conditions such as growth substrate, temperature, and pH (Russell and Amann, 2001). A further problem with the commercial systems available is the construction of the data base, and the biochemical tests included on the panels. Once biochemical tests are commercially produced, panel configurations are rarely changed. For example, the tests included on the API 20E strip in 2001 were the same as tests on the strips in 1975 (Janda and Abbott, 2002).

Before the development of genetic methods for the classification of bacterial relatedness based on the comparison of nucleic acid sequences, the taxonomic classification of bacteria was difficult, arbitrary, variable, and often contradictory. Other classification systems can be used in conjunction with conventional classification of bacteria such as Fourier-transform infrared spectroscopy (FT-IR) (Helm *et al.*, 1991). FT-IR spectroscopy measures dominant vibrations of functional groups and highly polar bonds. Thus these (bio)chemical 'fingerprints' are made up of the vibrational features of all the sample components. For microbial samples these include DNA/RNA, protein, membrane and cell-wall components. These authors

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concluded that: (1) FT-IR patterns could be used to type bacteria; (2) FT-IR provided data which could provide classifications that are similar and/or complementary to conventional classification schemes; and (3) FT-IR could be used as an easy and safe method for the rapid identification of clinical isolates.

16S rDNA gene sequencing is a powerful tool and by far the single most common molecular technique presently used for bacterial species identification (Wilson, 1995). Although the conventional basis of classification of bacteria remains important, the taxonomic basis of classification based on molecular sequences is now indispensable (Doolittle, 1999).

Many important pathogens, such as *Mycobacterium tuberculosis*, grow very slowly in culture. Others, like *Tropheryma whippelii*, the cause of Whipple's Disease, have not been successfully cultured. A number of rapid diagnostic tests have been developed in an effort to improve diagnostic accuracy. Some of these tests require a small amount of growth in culture, and then use cDNA probes for early identification of the causative agent. Organisms such as certain pathogenic fungi and mycobacteria can be identified using this technique. Other tests use PCR to directly identify a pathogen in tissue specimens. Molecular classification of an organism is considered an authentic method of identification (<http://www.ratsteachmicro.com>, 2007).

### 2.5 Conclusion

Isolated bacteria described in this Chapter were identified as P-mobilisers based on the 'halo' zone created by them around their colonies. Based on the size of 'halo' zones strains FA001 and FA010 were the best P-mobilisers. There was some similarity and dissimilarity between conventional and molecular classification of bacteria. In these identification procedures, the software BLASTN in some cases gave identification up to the genus and in some cases up to the species. Strain FA001 was identified as *Pantoea ananatis*, strain FA002 as *Pantoea agglomerans* (molecular) or *Enterobacter cloaca* (conventional), strain FA003 as *Burkholderia* sp., strain FA004 as *Burkholderia* sp., strain FA005 as *Burkholderia* sp., strain FA009 as *Burkholderia* sp. and strain FA010 as *Pantoea agglomerans*.

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In future experiments Bromocresol Purple could be used for identifying P-mobilising bacteria based on pH change as it has been shown that some P-mobilisers do not show 'halo' formation on agar plates (Kucey, 1983). It could be useful to isolate bacteria from other soils such as calcareous soil, acid soil and neutral soil.