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Characterization of phosphate solubilizing rhizobacteria associated with pea (*Pisum sativum* L.) isolated from two agricultural soils

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Abstract Phosphorous (P) availability is a major concern in European agriculture where reserves are limited. In the case of pea (*Pisum sativum* L.), one of the most important legumes in the human diet, P has specific effects on nodulation and N_2 fixation. Therefore, when biofertilization schemes are considered for pea cropping, it is very important to include symbiotic dinitrogen-fixing bacteria as well as phosphate-solubilizing bacteria (PSB). In this study sixteen PSB were isolated from the rhizosphere of two pea cultivars in two French soils with different characteristics. They were phenotypically and genotypically diverse displaying 9 different Two Primers-Random Amplified Polymorphic DNA (TP-RAPD) patterns. The 16S rRNA gene analysis of representative strains showed that they belong to four highly divergent phylogenetic groups. Most of the PSB strains belonged to the genus *Pseudomonas* and were closely related to *Pseudomonas baetica*, *P. lutea*, *P. azotoformans*, *P. jessenii* and *P. frederiksbergensis*. Other strains from the genus *Burkholderia* were closely related to *B. caledonica* and those from the genus *Rhizobium* to *R. grahamii*. The single strain of genus *Bacillus* was close to *Bacillus toyonensis*. Some phylogenetic groups to which our PSB strains belong are widely distributed in plant rhizospheres in different countries and continents. This is particularly interesting in the case of strains from the phylogenetic group of *P. fluorescens* which includes PSB strains with high ability to solubilize phosphate indicating that they may be used as biofertilizers in many soils.

Keywords Phosphate solubilizing bacteria · Insoluble phosphate · Soil bacteria diversity · Rhizosphere · Pea

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This paper is dedicated to the memory of Gisèle Laguerre

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1 Introduction

Pea (*Pisum sativum* L.) is one of the most important legumes in human diet. Its benefits for human health are derived from its protein, fiber, starch, vitamins, mineral and phytochemical contents, and these have been widely studied (Dahl et al. 2012). Moreover, pea is one of the legumes included in the last Common Agricultural Policy (CAP) reform to be used in greening practices (Hodge et al. 2015) since legumes are a source of biologically fixed nitrogen which helps to reduce the use and application of chemical nitrogen fertilizers and hence of the fossil fuels used to produce them (Bedoussac et al. 2015). Both fossil fuel and P sources are projected to be in serious decline by the end of the 21st century (Mohr et al. 2015; Withers et al. 2015), and their continued availability is a major concern in European countries where reserves are very limited. Therefore, plant biofertilizers are not only useful to produce healthier foods (García-Fraile et al. 2012; Flores-Félix et al. 2015), but they also constitute an alternative to chemical fertilization to provide essential elements for plant growth. Legumes are able to fix

atmospheric N in symbiosis with rhizobia and, in the case of some legumes such as soybean, their inoculation with these bacteria has been performed for decades in order to supply N to plants (Catroux et al. 2001). However, legumes fixing atmospheric nitrogen in symbiosis have more P requirements than plants receiving nitrogen fertilisers (Graham and Vance 2000; Gyaneshwar et al. 2002), and particularly in pea P has specific effects on N_2 fixation and nodule initiation, growth, development, and metabolic function (Jakobsen 1985). Therefore, biofertilization of pea with symbiotic N-fixing bacteria may be as important as with P solubilizing bacteria (PSB), which play a crucial role in soil P cycle and in the P-uptake by plants (Richardson and Simpson 2011). PSB have been identified on the basis of analysis of their *rrs* genes in several plant rhizospheres (Peix et al. 2003; Peix et al. 2004; Chung et al. 2005; Islam et al. 2007; Naik et al. 2008; Browne et al. 2009; Oliveira et al. 2009; Peix et al. 2009; Selvakumar et al. 2009; Collavino et al. 2010; Liu et al. 2011; Narveer et al. 2014; Xiang et al. 2011; Liu et al. 2014; Acevedo et al. 2014; Ruangsanka 2014), including those of some legumes, such as *Lotus* (Castagno et al. 2011), *Glycine max* (Li et al. 2008; Ndungu-Magiroi et al. 2012), *Phaseolus vulgaris* (Kumar et al. 2012a) and *Vigna unguiculata*. (Toro et al. 2013), but to the best of our knowledge there are no published studies on PSB from the rhizosphere of pea.

Therefore, the aims of this study were the phenotypic characterization of PSB strains isolated from the rhizosphere of pea in two French agricultural soils with different crop histories, the identification of these strains based on analysis of their 16S rRNA genes and on analysis of their phylogenetic relationships with previously reported PSB bacteria isolated from the rhizospheres of various plants in other countries and continents.

2 Materials and methods

2.1 Plant and soil conditions

The plants used for the experiment were peas cv. Frisson, which is a winter variety traditionally used in France. In order to study the occurrence and diversity of PSB associated with these plants two soils with different characteristics were chosen. Soil 1 (Epoisses) is a calcareous clay-loam soil from the East of France (Côte d'Or), in which pea was cultivated in 1982, and after which no legume has been cultivated. Soil 2 (La Bruyère) is a sandy soil which also comes from Eastern France (Côte d'Or), and it was last cultivated with pea in 2002. The samples were taken from different places in each plot, in the first 0–20 cm depth, sieved through a 5 mm mesh and homogenized. Jars of 2.5 L capacity were placed in a greenhouse containing 5 pea plants per pot. They were grown for 3 weeks.

2.2 Isolation of total and phosphate solubilizing bacteria (PSB)

Isolation and counts of total bacteria were performed in 3 different culture media to obtain the maximum culturable number and diversity of PSB. Medium A was a complex medium containing different C sources, with the following composition: 0.07 g l^{-1} K_2HPO_4, 0.2 g l^{-1} KH_2PO_4, 0.1 g l^{-1} $MgSO_4.7H_2O$, 0.05 g l^{-1} $CaCl_2.2H_2O$, 0.02 g l^{-1} $FeCl_3$, 0.5 g l^{-1} NH_4NO_3, 1 g l^{-1} mannitol, 1 g l^{-1} glucose, 1 g l^{-1} sucrose, 1 g l^{-1} sodium acetate, 1 g l^{-1} sodium L-lactate, 100 mg l^{-1} yeast extract, 0.5 mg l^{-1} biotin, 0.5 m gl^{-1} thiamine hydrochloride, 0.5 mg l^{-1} calcium pantothenate and 15 g l^{-1} agar. Medium B was Tryptone Soy Agar (TSA, Becton-Dickinson Co.) diluted 1:10, and Medium C was based on cold soil extract (Lilley et al. 1996) and was prepared with extracts from soil 1 and from soil 2, separately (C_1, C_2). The three media were supplemented with cycloheximide (50 μg ml^{-1}) to avoid fungal growth. Serial dilutions of both soils were performed by suspending 10 g of soil in 90 ml sterile water, and after shaking well for 30 min, decimal dilutions from 10^{-1} to 10^{-6} were obtained. From each one of them 200 μl were inoculated onto Petri dishes containing the three culture media and spread thoroughly with sterile glass balls. The plates were incubated at 28 °C for 7 days, and then counted in the dilution plates showing from 30 to 300 colonies.

PSB were selected in YED-P plates containing 1 % glucose, 0.5 % yeast extract, 0.2 % tricalcium phosphate and 2 % agar (Peix et al. 2001), by replica plating as indicated earlier (Lederberg and Lederberg 1952). The plates were incubated at 28 °C for 7 days, and the presence of PSB was detected by the halo produced surrounding the colonies. The strains showing high ability of phosphate solubilization by means of their halo diameter being 15 mm or larger according to De Freitas et al. (1997) were isolated and stocked in glycerol solution at −80 °C.

2.3 Phenotypic characterization

Physiological and biochemical tests were performed using the API 20NE system (BioMérieux, France) following the manufacturer's instructions. The strains *Pseudomonas fluorescens* DSM 50108 (1147555), *Burkholderia cepacia* LMG 1222^T (1067577) and *Rhizobium radiobacter* ATCC 19358^T (1467744) were used as positive controls. The data obtained were coded in binary form and Jaccard's coefficient was calculated to construct a similarity matrix. A dendrogram was obtained using the unweighted pair group method with arithmetic mean (UPGMA). Profile codes were obtained from the results of the tests using the

APILAB database system (https://apiweb.biomerieux.com/jsp/ident/index.jsp).

2.4 Two Primers-Random Amplified Polymorphic DNA (TP-RAPD) pattern analysis

To obtain TP-RAPD patterns, PCR was performed using an AmpliTaq Gold reagent kit (Perkin-Elmer Biosystems, California, USA) and the primers 879 F and 1522R at a final concentration of 2 μmol l^{-1} (Rivas et al. 2002). PCR conditions were as follows: pre-heating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 50 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. Electrophoresis was performed on 1.5 % agarose gels at 6 V cm^{-1}.

2.5 16S rRNA gene sequence analysis

The 16S rRNA genes were nearly full-length sequenced as described by Rivas et al. (2007). The sequences obtained were compared with those from the EzTaxon-e database (Kim et al. 2012). Sequences were aligned using the Clustal W software (Thompson et al. 1997). The distances were calculated according to Kimura's two-parameter method (Kimura 1980). A phylogenetic tree was inferred using the neighbour-joining method (Saitou and Nei 1987). Bootstrap analysis was based on 1000 re-samplings. The MEGA 5 package (Tamura et al. 2011) was used for all analyses.

2.6 Effect of carbon sources on phospahte solubilization

The effects of carbon sources on phosphate solubilization was tested following the methodology of Mardad et al. (2014) on the National Botanical Research Institute's phosphate growth medium (NBRIP) medium containing 1 % glucose, 0.5 % $Ca_3(PO_4)_2$, 0.5 %; $MgCl_2 \cdot 6H_2O$, 0.5 %; 0.025 % $MgSO_4 \cdot 7H_2O$, 0.02 % KCl, and 0.01 % $(NH_4)_2SO_4$. For the comparison, the glucose present in the medium was replaced by galactose or fructose and the bacterial cultures were incubated for 7 days at 28 °C and 160 rpm. Autoclaved uninoculated batch cultures were included as negative controls. The P released from tricalcium in the supernatants obtained after centrifugation at 10, 000 rpm for 10 min was analyzed by inductivelycoupled plasma-atomic emission spectrometry (ICP-AES) using a Varian 720-ES. The results were presented as a percentage of P released with respect to the control (glucose-containing NBRIP).

2.7 Pea inoculation assays with *Rhizobium* strains

The plant assays of *Rhizobium* isolates were performed on peas as was previously described (Ramírez-Bahena et al. 2009). The strain *Rhizobium leguminosarum* DSM 30132^T (currently *R. pisi*)^T which is able to nodulate pea but not to solubilize phosphate in YED-P plates was used as a reference. The negative controls were unninoculated pea plants. Seeds were sown into pots containing sterile vermiculite plus 0.2 % (w/w) tricalcium phosphate as substrate. After germination, inoculations were performed by adding 1 ml of a 10^6 cfu ml^{-1} bacterial suspension to each seedling. Nitrogen- and soluble phosphorous-free Rigaud and Puppo (1975) solution alternately with sterile distilled water was used to water the plants. Then they were placed in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m^{-2} s^{-1}; 400 to 700 nm), programmed for a 16 h photoperiod, day-night cycle, with a constant temperature varying from 25 to 27 °C, and 50 to 60 % relative humidity. Five replicates per treatment were set and the plants were harvested 6 weeks after planting. P content was analyzed by ICP-AES. Nitrogen was determined by combustion using a Leco CN-628 analyzer. Data were analyzed by one-way analysis of variance, and mean values compared by Fisher's Protected LSD test (Least Significant Differences) ($P \leq 0.05$).

3 Results and discussion

3.1 Isolation of phosphate solubilizing bacteria

A total of 16 PSB strains showing solubilization halos of at least 15 mm diameter were isolated. From them, 5 were found in the soil from Epoisses, and 11 in the soil from La Bruyère. From the first 5 strains, 2 where first isolated in medium B, 2 in soil extract medium (C_1), and 1 strain in medium A. For the 11 strains from La Bruyère, 7 were isolated in the soil extract based medium (C_2), 2 in medium A and 2 in medium B. The counts of total bacteria for the soil from Epoisses were 15.1×10^6, 31.9×10^6 and 35.7×10^6 CFU g^{-1} of soil in media A, B and C_1 respectively. For the soil from La Bruyère total bacterial counts were 30.2×10^6, 32.0×10^6 and 29.1×10^6 CFU g^{-1} of soil in media A, B and C_2 respectively. As can be seen, the number of PSB isolated was very low compared to the total microbiota counts obtained. These results are in agreement with previous studies where the number of isolates showing a high ability to solubilize phosphate in vitro is sometimes quite low compared to the total microbiota of the soils analysed (Alexander 1977). It is remarkable that the counts of total microbiota varied with the different culture media used for the isolation, the differences being dramatic in the case of the Epoisses soil sample inoculated in medium A, in which the number of colonies isolated was half of that with the other media.

Table 1 Characteristics of the PSB strains isolated in this study from the rhizosphere of pea crops growing in two French soils

Strains	Soil	Isolation Medium	API 20NE code	TP-RAPD type	Closest related species (16S rDNA sequence)	Similarity (%)	Closest related species (APILAB database)	similarity (%)
PSB1	La Bruyère	B	0177555	I	*Pseudomonas baetica*	99.7 %	*Pseudomonas fluorescens*	99.8 %
PSB2	La Bruyère	B	0447455	II	*Pseudomonas lutea*	100 %	*Pseudomonas fluorescens*	92.1 %
PSB3	Epoisses	B	0157555	III	*Pseudomonas azotoformans*	99.6 %	*Pseudomonas fluorescens*	99.9 %
PSB4	Epoisses	B	0157555	III	*Pseudomonas azotoformans*	99.5 %	*Pseudomonas fluorescens*	99.9 %
PSB5	La Bruyère	A	0157555	III	*Pseudomonas azotoformans*	99.5 %	*Pseudomonas fluorescens*	99.9 %
PSB6	La Bruyère	A	0157555	III	*Pseudomonas azotoformans*	99.5 %	*Pseudomonas fluorescens*	99.9 %
PSB7	Epoisses	A	1457761	IV	*Bacillus toyonensis*	99.9 %	Unacceptable profile	
PSB8	Epoisses	C_1	1047557	V	*Pseudomonas jessenii*	99.8 %	*Pseudomonas fluorescens*	79.1 %
PSB9	Epoisses	C_1	1047555	VI	*Pseudomonas frederiksbergensis*	99.9 %	*Pseudomonas fluorescens*	99.1 %
PSB10	La Bruyère	C_2	1367577	VII	*Burkholderia caledonica*	99.0 %	*Burkholderia cepacia*	99.6 %
PSB11	La Bruyère	C_2	0177555	I	*Pseudomonas baetica*	99.7 %	*Pseudomonas fluorescens*	99.8 %
PSB12	La Bruyère	C_2	0667344	VIII	*Rhizobium grahamii*	98.5 %	*Rhizobium radiobacter*	96.1 %
PSB13	La Bruyère	C_2	0177555	I	*Pseudomonas baetica*	99.7 %	*Pseudomonas fluorescens*	99.8 %
PSB14	La Bruyère	C_2	0177555	I	*Pseudomonas baetica*	99.7 %	*Pseudomonas fluorescens*	99.8 %
PSB15	La Bruyère	C_2	1367577	VII	*Burkholderia caledonica*	99.0 %	*Burkholderia cepacia*	99.6 %
PSB16	La Bruyère	C_2	0667744	IX	*Rhizobium grahamii*	98.6 %	*Rhizobium radiobacter*	99.7 %

3.2 Phenotypic characterization

From all PSB strains isolated in this study only one of them, PSB7, was a Gram positive rod, and the presence of spores was observed after Gram staining. The remaining strains were Gram negative non-sporulating rods. The phenotypic characterization was performed by using the API 20NE system whose usefulness for this purpose was previously shown in different bacterial groups including Gram negative and Gram positive sporulating bacteria (Rivas et al. 2007). Nevertheless, as expected, the Gram positive sporulated strain PSB7 showed an API 20NE profile (1457765) that was not coincident with any of the species held in the APILAB database. Although this system is inadequate for identification of most rhizospheric soil bacteria (Peix et al. 2003), some rhizospheric species are included in the APILAB database and so, in the case of Gram negative strains, we compared the codes obtained with those of Apiweb. The strains *P. fluorescens* DSM 50090^T (code 1147555), *B. cepacia* LMG 1222^T (code 1067577) and *R. radiobacter* ATCC 19358^T (code 1467744) used as positive controls were correctly identified using this system. Biocoding for the API 20NE tests resulted in 0177555 for the strains PSB1, PSB11, PSB13 and PSB14, 0157555 for the strains PSB3, PSB4, PSB5 and PSB6, 1047557 for strain PSB8 and 0447455 for strain PSB2. All these biocodes were related to that of *P. fluorescens* in the APILAB database with similarities ranging from 79.1 to 99.9 % (see Table 1). The code 1367577 obtained for strains PSB10 and PSB15 showed 99.6 % similarity with *B. cepacia* and the codes 0667344 and 0667744 from strains PSB12 and PSB16, respectively, match with that of *R. radiobacter* with 96.1 % and 99.7 % similarity, respectively. In none of these cases was a good identification obtained at species level, but that at genus level was correct.

3.3 TP-RAPD pattern analysis

Figure 1 and Table 1 shows that the strains isolated in this study displayed 9 different TP-RAPD patterns. Profiles II, IV, V, VI, VIII and IX were shown by just one strain (PSB2, PSB7, PSB8, PSB9, PSB12 and PSB16, respectively). Profile I was presented by 4 strains (PSB1, PSB11, PSB13, PSB14), as was profile III (PSB3, PSB4, PSB5 and PSB6). Finally, two strains presented profile VII (PSB10 and PSB15). These

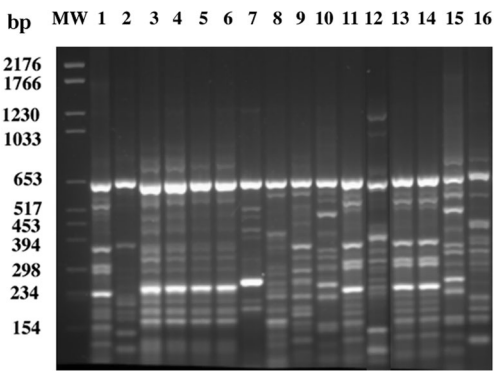

Fig. 1 Two Primers-Random Amplified Polymorphic DNA (TP-RAPD) profiles of the PSB strains isolated in this study, PSB1 to PSB16 (Lanes 1 to 16, respectively). MW: Standard VI (Roche)

results showed that in spite of the low number of bacteria able to solubilize phosphate present in the pea rhizospheres analysed, they were genetically diverse. Only the strains with the TP-RAPD type III pattern were present in both soils analysed, and they were isolated using two different culture media. The remaining strains were mainly isolated using media containing soil extracts confirming that the culture medium is crucial when isolating bacteria from soil. This results in a different composition and number of bacteria, for which the concept of "culturable bacteria" is dynamic, being a variable number depending on the medium used, as has been already reported by other authors (Lilley et al. 1996; Joseph et al. 2003). Taking into account that TP-RAPD patterns differ in different species (Rivas et al. 2001), they are a good tool for grouping bacteria in order to select representative strains for 16S rRNA gene sequencing because the species presenting the same TP-RAPD pattern have identical 16S rRNA genes (Valverde et al. 2006).

3.4 16S rRNA gene sequence analysis

The current bacterial taxonomy is based on the 16S rRNA gene which is currently sequenced in all type strains of all described species (Woese 2000; Yarza et al. 2013). Over the last decade a new database, named EzTaxon, including only these sequences has been developed in order to avoid bacterial mis-identifications that are very frequent in other databases (Kim et al. 2012). According to the results of this database most strains isolated in this study belong to the Phylum Proteobacteria and concretely to the genera *Pseudomonas*, *Burkholderia* and *Rhizobium*, and only one strain belongs to the Phylum Firmicutes, specifically to genus *Bacillus* (Table 1). As can be seen in Table 1, most of the strains belong to the genus *Pseudomonas*. According to these results, a similar genotypic diversity of PSB was found in the two soils analysed and the strains isolated belong to genera considered among the best phosphate

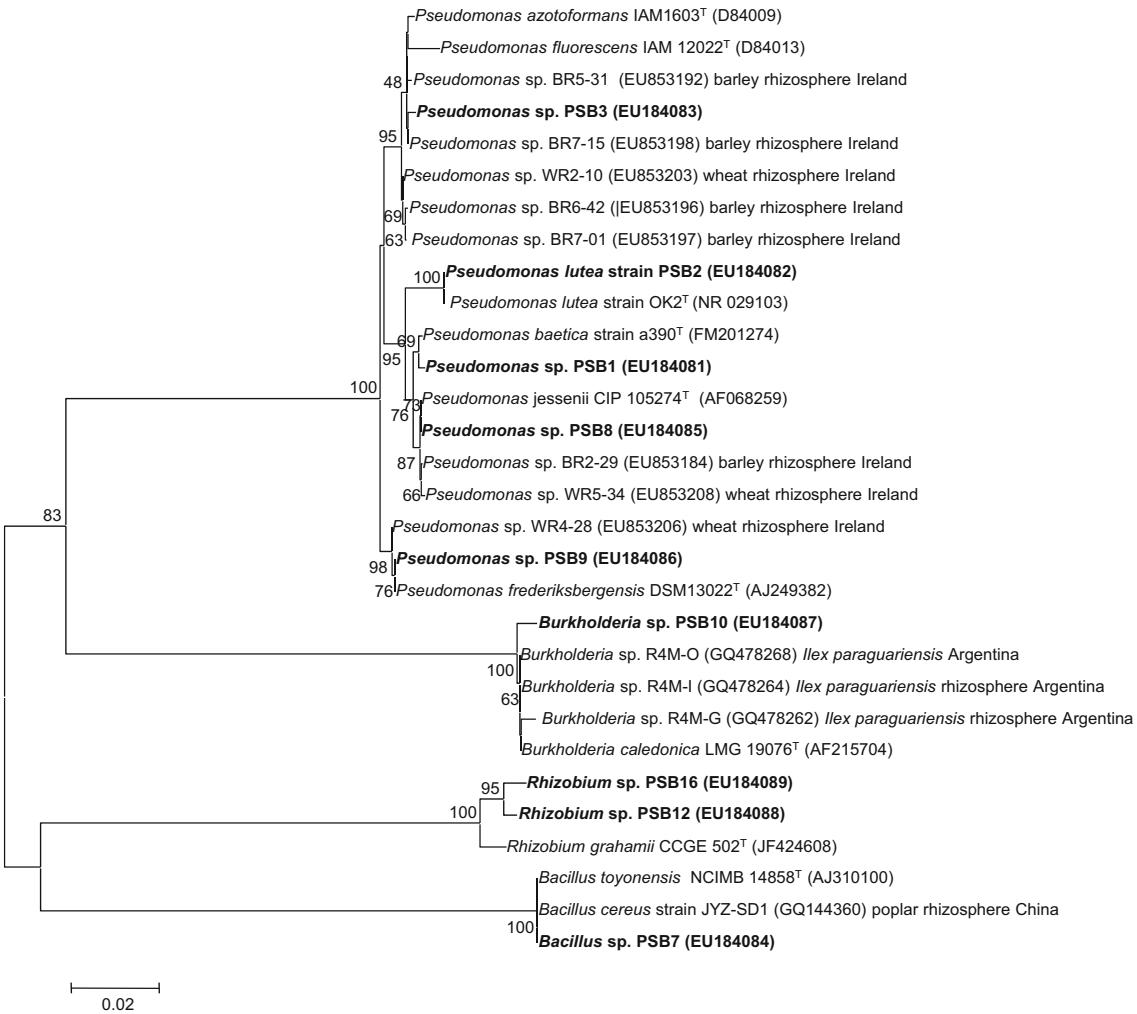

Fig. 2 Neighbour-joining phylogenetic rooted tree based on 16S rRNA sequences of representative strains from each TP-RAPD group and related PSB species isolated in other plant rhizospheres. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt

Table 2 Comparison of tricalcium phosphate solubilization by the PSB strains in carbon sources. % indicates the percentage of P released from tricalcium phosphate

Carbon source		PSB1	PSB2	PSB3	PSB4	PSB5	PSB6	PSB7	PSB8	PSB9	PSB10	PSB11	PSB12	PSB13	PSB14	PSB15	PSB16	negative control
Glucose	mg P/l	991.5	694.8	521.9	683.5	532.1	564.4	45.5	665.8	904.4	895.4	1084.4	441.9	1040.4	957.3	802.9	315.3	37.7
	%	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
P released from tricalcium phosphate in comparison with the control containing glucose																		
Galactose (%)		42.0	56.8	70.0	59.2	78.4	73.8	87.4	60.3	52.1	49.5	44.4	17.5	38.8	43.8	62.1	45.2	104.8
Fructose (%)		24.7	14.1	12.2	9.8	10.7	11.2	78.7	10.6	20.3	9.1	19.7	90.0	20.6	23.6	10.3	12.4	96.06

solubilizers in the pseudomonads, bacilli and rhizobia groups (Peix et al. 2003; Peix et al. 2004; Chung et al. 2005; Islam et al. 2007; Naik et al. 2008; Browne et al. 2009; Oliveira et al. 2009; Peix et al. 2009; Selvakumar et al. 2009; Collavino et al. 2010; Castagno et al. 2011; Liu et al. 2011; Narveer et al. 2014; Xiang et al. 2011; Kumar et al. 2012b; Ndungu-Magiroi et al. 2012; Liu et al. 2014; Ruangsanka 2014).

Figure 2 shows a phylogenetic tree including the strains from this study and some closely related species of PSB found in other plant rhizospheres. Four distant phylogenetic groups corresponding to the genera *Pseudomonas*, *Burkholderia*, *Bacillus* and *Rhizobium* were obtained.

In the phylogenetic group of genus *Bacillus*, strain PSB7 was closely related to the type strain of *B. toyonensis* CNCM I-1012^T and to one strain isolated from poplar rhizosphere (Liu et al. 2011).

Strain PSB10 belongs to the phylogenetic group corresponding to *Burkholderia* together with the type strain of *B. caledonica* LMG 19076^T and several PSB strains isolated from the rhizosphere of *Ilex paraguariensis* in Argentina (Collavino et al. 2010).

Among the pseudomonads, the 16S rRNA gene of strain PSB1 clustered with the type strain of *P. baetica* a390^T. This cluster is close to another one containing the strain PSB8, the type strain of *P. jessenii* CIP 105274^T and two PSB strains isolated from wheat (*Triticum aestivum*.) and barley (*Hordeum vulgare*) rhizospheres in Ireland (Browne et al. 2009). The strain PSB3 was closely related to the type strain *P. azotoformans* IAM 1603^T and several PSB strains isolated from wheat and barley rhizosphere in Ireland (Browne et al. 2009). The 16S rRNA gene sequence of strain PSB2 was identical to *P. lutea* OK2^T a PSB species isolated from the rhizosphere of grasses in a Spanish soil (Peix et al. 2004) and they were related to the previous cluster. The strain PSB9 was closely related to the type strain of *P. frederiksbergensis* JAJ28^T and to two PSB strains isolated from wheat and barley rhizosphere in Ireland (Browne et al. 2009). Interestingly the strain PSB3 clustered with strains forming a superior phosphate solubilizing phylogenetic group defined by Browne et al. (2009) that contains the type strain of *P. fluorescens* and other related species (Fig. 2).

The strains PSB12 and PSB16 formed a cluster together with the type strain of a recently described species of *Rhizobium*, *R. grahamii* CCGE 502^T. There are no PSB strains of *Rhizobium* closely related to our strains. Although *Mesorhizobium* strains are amongst the best phosphate solubilizing rhizobia (Peix et al. 2001; Rivas et al. 2006), surprisingly the *Rhizobium* strains isolated in this study solubilized phosphate in amounts similar to those found with *Pseudomonas* species.

Table 3 Number of nodules, shoot dry weight, and N and P content in pea plants after inoculation with phosphate solubilizing bacteria (PSB)

Treatment	Number of nodules per plant	Shoot dry weight per plant (g)	%N	N (mg plant^{-1})	%P	P (mg plant^{-1})
PSB 12	35 (±3.63) b	0.35 (±0.01) a	4.26 (±0.22) a	14.87 (±0.47) a	0.37 (±0.03) a	1.30 (±0.08) a
PSB 16	40 (±4.07) b	0.38 (±0.03) a	3.67 (±0.15) b	14.09 (±1.56) ab	0.34 (±0.02) a	1.30 (±0.13) a
R. pisi DSM 30132^{T}	48 (±4.37) a	0.25 (±0.04) b	4.31 (±0.13) a	10.87 (±1.51) bc	0.38 (±0.01) a	0.97 (±0.16) ab
Control	0 (±0.00) c	0.24 (±0.03) b	3.27 (±0.14) b	7.80 (±0.56) c	0.36 (±0.01) a	0.86 n±0.06) b

The mean values followed by the same letter did not significantly differ at $p < 0.05$

The results of the present study showed that the PSB found in the rhizosphere of pea in the two soils studied were genotypically and phenotypically diverse and confirmed previous studies showing that *Pseudomonas*, *Burkholderia* and *Bacillus* are the most active P solubilizers. However our results showed that rhizospheric *Rhizobium* strains can also be good solubilizers, which contrasts with results that have previously been reported for rhizobial strains isolated from legume nodules that showed a weaker P solubilization than *Pseudomonas* (Abril et al. 2007; Palomo et al. 2007). Although some different species of PSB were found in the two studied soils highlighting the need for further studies in different geographical locations, the most significant result of this study is that some phylogenetic groups of PSB are widely distributed in plant rhizospheres in different countries and continents. This is particularly interesting in the case of strains from the phylogenetic group of *P. fluorescens* which includes PSB strains with high ability to solubilize phosphate, indicating that they may be used as biofertilizers in many different soil types.

3.5 Effect of carbon sources on phosphate solubilization

It has been previously shown that carbon sources affect bacterial phosphate solubilization (Hameeda et al. 2006). Glucose and galactose have been reported to be very good carbon sources for phosphate solubilisation in different bacteria (Mardad et al. 2014), whereas fructose showed variable results depending on the bacteria tested (Park et al. 2009; Mardad et al. 2014). Therefore, in this study we analysed the effect of these three carbon sources on the ability of the isolated PSB strains to solubilize phosphate. The results showed that glucose was the best carbon source for phosphate solubilization with all the strains tested (Table 2), confirming the results of other authors (Relwani et al. 2008), and variable results were obtained for the other two carbon sources depending on the bacterial strain tested. Interestingly, all the *Pseudomonas* strains isolated in this study showed very low percentages of phosphate solubilisation in the presence of fructose with respect to the values obtained in the glucose-based medium, which is in agreement with the results previously found for a strain of *Pseudomonas fluorescens* (Park et al. 2009).

3.6 Effect of *Rhizobium* strains on growth and nodulation of pea

The results of the inoculation of the *Rhizobium* strains PSB12 and PSB16 on pea plants showed that both strains formed effective nodules. Although both induced significantly fewer nodules than the reference strain *R. pisi* DSM 30132^{T}, their inoculation resulted in plants with significantly higher shoot dry weights (Table 3). The plants inoculated with strain PSB12 and the reference *R. pisi* strain have significantly higher N concentrations than those inoculated with the strain PSB16, whereas no significant differences were found in the P concentrations among treatments. Nevertheless, the total amounts of these two elements were higher in pea plants inoculated with the strains PSB12 and PSB16 than in those inoculated with the reference strain. These results are in agreement with those previously found for other phosphate solubilizing rhizobia which enhanced the growth of their hosts (Peix et al. 2001; Marciano Marra et al. 2012), and suggest that *Rhizobium* sp. strains PSB12 and PSB16 have potential to be good biofertilizers for pea since increases ranging from 30 to 50 % were found in shoot dry weight and total content of P and N with respect to those inoculated with the reference strain.

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