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Screening for rock phosphate solubilizing Actinomycetes from Moroccan phosphate mines

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ABSTRACT

Three Moroccan phosphate mines, an original biotope rich in insoluble rock phosphate (RP), were explored for the presence of RP solubilizing Actinobacteria. Three hundred actinomycete isolates originating from these mines were tested for their ability to grow on a synthetic minimum medium (SMM) containing insoluble RP as unique phosphate source. Only 55 isolates (18.3%) were able to weather RP in SMM medium. Eight isolates showed the most active growth and solubilization capability. These isolates were shown to be able to solubilize RP in liquid cultures. The study of mechanisms involved in these weathering processes indicated that the isolates produce siderophores but not organic acids. Seven of these strains were shown to belong to the genus *Streptomyces* and one, to the genus *Micromonospora*. This study is expected to lead to the formulation of novel bio-phosphate fertilizers constituted by the association of pulverized RP and spores of the *ad hoc* actinomycete strains.

1. Introduction

Soil phosphorus (P) content is generally about 400–1200 mg kg^{-1} (Fernández and Novo, 1988) but most of this P is present as insoluble metallic complexes (with iron, aluminium, silicium, etc.) in acidic soil (Whitelaw, 2000) or calcium carbonate in alkaline soil (Gyaneshwar et al., 2002). In consequence, only a small fraction of P is available for plant growth (1 mg kg^{-1} or less) (Goldstein, 1994). P deficiency is limiting crop production in many agricultural soils worldwide (Arcand and Schneider, 2006). In order to compensate for this natural poverty in phosphate, expensive chemical phosphate fertilizers are used in agriculture to improve crop yield (Gyaneshwar et al., 2002). However, the chemical fertilizer industry is now considered as extremely polluting (Shigaki et al., 2006; Vassilev et al., 2006). Furthermore, amended soluble P is often rapidly washed away, accelerating eutrophication of fresh waters and polluting ground waters used for drinking (Shigaki et al., 2006). The development of sustainable agriculture requires a strong reduction in agrochemical inputs and their replacement by more ecological, efficient and cheap natural products (Macias et al., 2003). For instance, the poorly

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soluble rock phosphate (RP, a hydroxyapatite) was traditionally used in agriculture as a natural slow release phosphate fertilizer (Zapata and Zaharah, 2002). In the fields, this rather insoluble substrate is likely to be made soluble by the action of microorganisms able to promote the release of soluble inorganic phosphate from it (Arcand and Schneider, 2006). The literature describes these phosphate-solubilizing microorganisms (PSM) as belonging to the genera *Aspergillus*, *Penicillium*, *Thrichoderma*, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Erwinia* and *Actinomycetes* (Mba, 1997; Rodriguez and Fraga, 1999; Rudresh et al., 2005).

Among these PSM, Actinomycetes are of special interest since these filamentous sporulating bacteria are able to develop in extremely different soils (Jiang et al., 2005; Pathom-Aree et al., 2006) and produce various substances (anti-fungi, insecticides, anthelminthics, phytohormone-like compounds etc.) that could benefit plant growth (Fabre et al., 1988; Manulis et al., 1994; Ikeda, 2003; Jain and Jain, 2007). However, for actinomycete PSM, the taxonomic groups and the mechanisms involved in these RP weathering processes are not well elucidated. Moroccan phosphate mines constitute an unexplored ecological niche likely to shelter a population of microorganisms especially well equipped to solubilize insoluble RP. The aim of this study was to isolate, from this peculiar biotope, Actinomycetes able to release soluble phosphate from RP. The solubilization mechanism used by these bacteria was investigated and taxonomic characterization of the most efficient solubilizing isolates was achieved.

2. Materials and methods

2.1. Collection of rock phosphate samples from the mines

Rock phosphate samples were collected in March 2004 from the extracted rock phosphate stockpiles (Operated by 'Office Chérifien des Phosphates', OCP Morocco) of three different Moroccan phosphate mining centres: Benguerir (RP^B), Khouribga (RP^K) and Youssoufia (RP^Y). Three 25 m × 25 m areas were sampled at each site, representing three replicates. The distance between the replicates was approximately 10 m. From each replicate area, a composite RP sample was taken, consisting of 10 core samples 500 g wet weight 4 cm diameter collected from 0 to 10 cm depth after removing 3 cm surface residues. The soil samples from each replicate area were then homogenized by mixing, sieved (<2 mm) and placed in a sterile tightly closed polyethylene bag. The samples were stored at 4 °C and processed within 48 h.

The mineral composition of the Rock Phosphate originating from the Khouribga phosphate mine (RP^K) was determined using scanning electron microscopy (Stereoscan 260, Cambridge, England) and consisted of O, 56.53%; F, 2.42%; Na, 1.81%; Mg, 1.94%; Al, 2.03%; P, 9.37%; S, 0.77%; Sn, 0.12%; Ca, 16.35%; Fe, 0.60%.

2.2. Isolation of total bacteria and Actinomycetes

Two gram (wet weight) of each soil sample were resuspended in 18 ml of sterile physiological serum (9 g l^{-1}, NaCl), homogenized and sonicated according to Ouhdouch et al. (2001). 0.1 ml of various dilutions of the treated samples was plated in triplicate on the surface of nutrient agar (Difco, USA) and of a solid medium prepared with RP soil extracts as described in Barakate et al. (2002) with glycerol (5 g l^{-1}) and agar (15 g l^{-1}) being added to these extracts. The pH was adjusted to 7 and the medium was sterilized at 121 °C for 20 min. This medium was supplemented with 40 μg ml^{-1} actidione and 10 μg ml^{-1} nalidixic acid, growth inhibitors of fungi and Gram negative bacteria, respectively. After plating, the agar plates were incubated for 21 days at 28 °C in order to allow growth of the slow growing Actinomycetes. Actinomycetes were recognized on the basis of morphological features following the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966).

The statistical analysis of total bacteria and actinomycete strains distribution was carried out using ANOVA and the Newman–Keuls test was used to compare the average abundance and percentage contribution of the Actinomycetes to total bacteria in the three sites. All values are means of three replicates plates from the same RP samples.

2.3. Screening for Actinomycetes able to use rock phosphate as sole phosphate source

Selection of Actinomycetes able to use RP as sole phosphate source was carried out by plating 300 colonies (100 colonies from each investigated mine) on the synthetic minimum medium (SMM) containing 10 g l^{-1} glucose, 2 g l^{-1} $NaNO_3$, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1} KCl, 0.01 g l^{-1} $FeSO_4{\cdot}7H_2O$ and RP^K (0.5 g l^{-1}, containing approximately 2.2 mM phosphorus) as sole phosphate source or on the SMM containing soluble K_2HPO_4 (0.5 g l^{-1}, 4.38 mM) or no phosphate source. Spores of the actinomycete isolates able to show the most active growth on SMM containing RP^K as sole phosphate source were stored in 20% (w/v) sterile glycerol at −20 °C.

2.4. Estimation of the ability of the selected Actinomycetes to release soluble phosphate from RP

Three culture replicates were inoculated with 10^6 spores ml^{-1} of each actinomycete isolate and grown for 5 days at 28 °C on a rotary shaker (180 g min^{-1}) in 250 ml Erlenmeyer flasks containing 50 ml of liquid SMM medium with 0.5 g l^{-1} RP^K. Cultures were centrifuged at 10,000 × *g* for 10 min and the pH of the supernatant was measured every day (Fig. 3). The supernatant was analyzed for P_2O_5 content by the chlorostannous reduced molybdo-phosphoric acid blue colour method (Olsen and Sommers, 1982). Similar measures were carried out in cultures of the control strain *Streptomyces griseus* M1323 (IPC Paris, France) and *Streptomyces lividans* TK24 (Hopwood et al., 1983) and in non-inoculated flasks incubated under the same conditions.

2.5. Test of siderophore excretion

In order to determine whether siderophores were present in the culture supernatants of the eight selected actinomycete isolates and of *S. griseus* M1323 and *S. lividans* TK24, they were grown for 5 days under the conditions described above.

The supernatants were centrifuged at 10,000 × *g* min^{-1}, filtered and concentrated 10-fold by evaporation using a speed vac concentrator (Appligene, France). Twenty microliter of the concentrated filtrates were deposited on sterile cellulose disks (5 mm diameter) placed aseptically on blue CAS–agar plates as described by Schwyn and Neilands (1987) and incubated at 30 °C for 3 days. Disks impregnated with 2, 4, 6, 8 or 10 μg ml^{-1} Desferrioxamine B (Sigma–Aldrich, Germany), a well known siderophore (Tunca et al., 2007), placed aseptically on blue CAS–agar plates and incubated under the same conditions, were used as positive controls. The disks impregnated with solutions containing siderophore were surrounded by a zone of colour change (blue to yellow-orange) of the CAS that was due to iron chelation (Schwyn and Neilands, 1987). The size of the zones and the intensity of the colour change were estimated and compared to the controls.

2.6. Morphological, physiological and chemotaxonomic characterization of the selected strains

The morphological, cultural, physiological and biochemical characteristics of the selected isolates were evaluated as described in the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Cultural characteristics were observed on yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3) and inorganic salts–starch agar (ISP4) media at 30 °C for 7–21 days and the colour series were determined according to the system proposed by Nonomura (1974). The assimilation of carbohydrates was studied by using the medium ISP9, containing 16 different carbohydrates at a concentration of 1% (w/v) as sole carbon source. The chemical analyses of the diaminopimelic acid isomer were performed as described by Becker et al. (1964). Spore chain morphology and spore shapes were observed on the same media using light microscopy.

2.7. Amplification and sequencing of the 16S rDNA of the selected strains

The purified siderophore producing isolates were grown for 2 days at 28 °C with agitation in 500 ml flasks containing 100 ml of Hickey–Tresner medium containing 1 g l^{-1} yeast extract, 1 g l^{-1} beef extract, 2 g l^{-1} NZamine A, 10 g l^{-1} Dextrin, 20 mg l^{-1} $CoCl_2 \cdot 6H_2O$ (Hopwood et al., 1985). Biomass was harvested by centrifugation (8000 × *g* for 10 min) and washed twice with double-distilled water. 200 mg of mycelia was used for DNA extraction as described by Liu et al. (2000). The 16S rDNA was amplified using the PCR method with *Taq* DNA polymerase (Qiagen, USA) and universal primers PA (5′-AGAGTTTGATCCTGGCTCAG-3′) and PH (5′- AAGGAGGTGATCCAGCCGCA- 3′). Amplification was carried out in 50 μl reaction mixture containing 1.5 U of AmpliTaq Gold *Taq* polymerase (Applied Biosystems), 10 μl of 5× AmpliTaq Gold reaction buffer (Applied Biosystems), 2.5 mM of each dNTP, 1 μM of each primer and 100 ng of genomic DNA. Reaction conditions were: 97 °C for 4 min, (97 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s) × 35 cycles followed by incubation at 72 °C for 10 min. The amplified products were visualized on a 0.8% (w/v) agarose gel stained with ethidium bromide. Sequencing reactions were performed by Macrogen (Seoul, Korea). The sequences obtained were compared for similarity with sequences present in the genomic database banks, using the 'NCBI Blast' program available at the ncbi-nlm-nih.gov Web site.

3. Results

3.1. Isolation of Actinomycetes able to use rock phosphate as sole phosphate source

The distribution of total bacteria and Actinomycetes in the RP soil extracts collected from the three sites is shown in Table 1. Total bacteria were slightly more abundant in the Benguerir soil than in the Youssoufia and Khouribga soils, while Actinomycetes were significantly more abundant in the Benguerir than in the Youssoufia and Khouribga RP soils. Of the 300 actinomycete isolates with different morphological characteristics selected from the three RP soils, only 55 could use RP when plated on the solid SMM medium containing RP^K as unique phosphate source (Fig. 1). Thirty-four of the 55 originated from Benguerir, 11 from Youssoufia and 8 from Khouribga. Of these 55 isolates, the 8 which showed the most active growth on SMM medium containing RP^K as sole phosphate source were selected for more extensive study. Two of the eight were from Youssoufia (YH_1 and YH_3), five from Benguerir (BH_1, BH_2, BH_3, BH_5 and BH_7) and one (KH_7) from Khouribga.

3.2. Abilities of the selected isolates to release soluble phosphate from RP

The eight selected actinomycete strains showed different abilities to release soluble phosphate from RP (Fig. 2). Phosphate release ranged from 8.34 to 29.67 μg ml^{-1}. BH_7 and BH_2 were the most efficient strains releasing 29.67 and 21.43 μg ml^{-1} soluble P in the growth medium, respectively

Table 1 – Distribution of total bacteria and Actinomycetes in rock phosphate samples and percentage of Actinomycetes in Rock P soil extracts

	RP^K: Khouribga	RP^Y: Youssoufia	RP^B: Benguerir	ANOVA
Total bacteria (×10^5 cfu g^{-1})	42.4	55.3	67.3	$p = 0.07$
Actinomycetes (×10^5 cfu g^{-1})[a]	0.82a	1.77a	8.47b	$p < 0.001$
Percentage of Actinomycetes in total bacteria[a]	2.07a	3.47a	12.59b	

Different letters indicate significant differences at $p < 0.01$.
[a] Newman–Keuls *t*-test was used to compare mean percentages and Actinomycetes density.

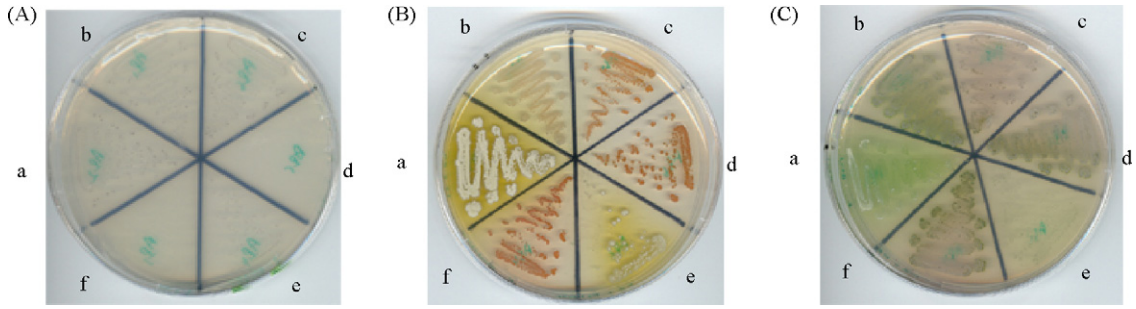

Fig. 1 – Screening procedure to isolate strains able to grow on SMM medium with RP^K as sole phosphate source. (A) SMM without K_2HPO_4, (B) SMM with 4.38 mM K_2HPO_4, and (C) SMM with 2.2 mM RP^K. The strains a, b, c, d, f are able to use RP^K as sole phosphate source whereas strain e is not.

(Fig. 2). *S. griseus* M1323 released 19.67 μg ml^{-1} phosphate whereas *S. lividans* TK24 likely consumed the free phosphate spontaneously released from RP (4.97 μg ml^{-1} in the control non-inoculated flask) since the free phosphate concentration in the presence of *S. lividans* fell to 0.197 μg ml^{-1} (Fig. 2).

3.3. *Investigation of the solubilization mechanism*

No acidification of the growth medium was observed for any of strains; even a slight alkalinization of the medium was noticeable toward the end of growth (Fig. 3) suggesting that the process of solubilization did not involve the excretion of organic acids. None of the isolates was surrounded by a clear halo, characterizing microorganisms producing organic acids, on the classical Pikovskaya (Pikovskaya, 1948) and NBRIP media (Nautiyal, 1999) and none of the following acids (oxalic, citric, DL-malic, succinic nor fumaric acids) was found in the culture filtrates of the eight strains studied, using TLC chromatography (detection limit of 10 μg ml^{-1}) (data not shown).

The BH_7, BH_2, KH_7 and YH_1 strains were the most efficient producers of siderophores as judged by the size of the zone and the intensity of the colour change of the CAS–agar (Fig. 4) whereas the control strain *S. lividans* TK24 and the strain BH_5 excreted very little, if any, of these substances. It is noteworthy that these two strains are the least efficient RP solubilizers whereas BH_7 is the most efficient RP solubilizing strain (Fig. 2).

3.4. *Taxonomical characterization of the selected isolates*

The eight strains showed different abilities to assimilate 16 carbon sources tested. All strains were able to use citrate, sucrose, fructose, glucose, maltose, mannitol, mannose, lactose and glycerol as sole carbon sources whereas inositol, D-raffinose, sorbitol, rhamnose, arabinose, galactose, and xylose were not used by YH_3, BH_3, BH_5 and BH_1 (Table 2). All strains were sensitive to Novobiocine (30 μg) and Polymyxin B (300 U) (Table 2). Strains YH_1, YH_3, BH_3, BH_5, BH_1 and BH_7 were

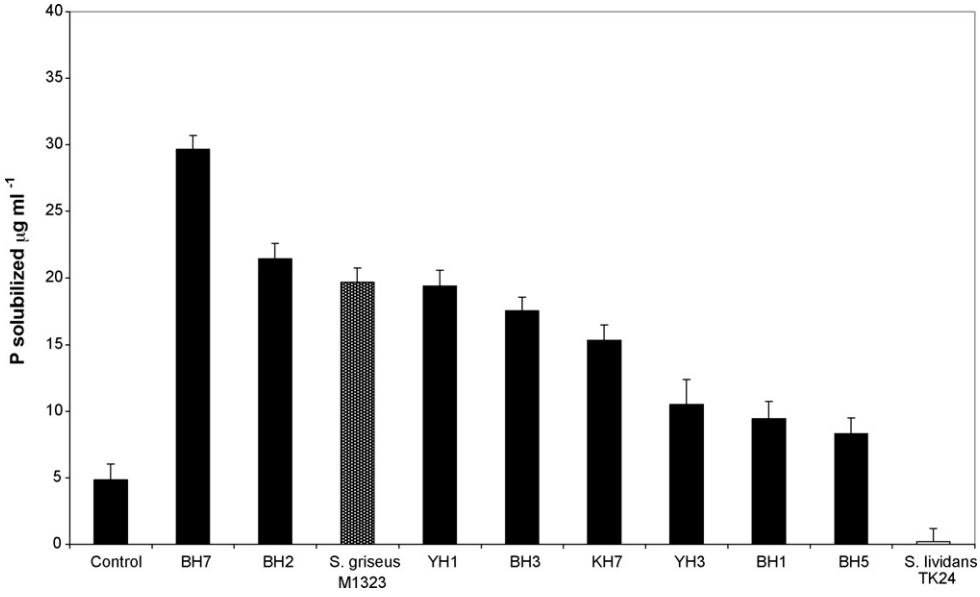

Fig. 2 – Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures of the eight selected isolates and of the control strains (*S. griseus* M1323 and *S. lividans* TK24) grown for five days in SMM containing 0.5 g l^{-1} RP^K and in the medium of the non-inoculated flasks incubated under the same conditions (control). Error bars represent standard deviations of the mean values of the results of three independent culture replicates.

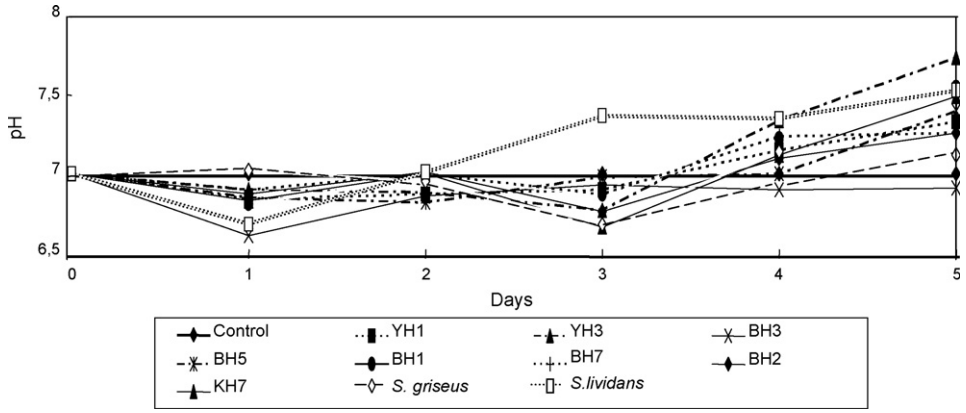

Fig. 3 – Evolution of the pH of the culture supernatant of the eight selected rock phosphate solubilizing Actinomycete strains, in the control strains (*S. griseus* M1323 and *S. lividans* TK24) grown in SMM containing 0.5 g l^{-1} RP^K and in the non-inoculated control incubated under the same conditions.

resistant to sulfamides but BH_2 and KH_7 were sensitive. Strains YH_3, BH_3 and BH_5 were resistant to gentamycine (10 μg) and bacitracine (10 U) whereas YH_1, BH_1, BH_7, BH_2 and KH_7 were sensitive to these antibiotics. Only strains BH_2 and YH_3 were resistant to cefalotin (30 μg).

The analysis of cellular constituents of the eight isolates revealed the presence of the L- diaminopimelic acid (DAP) isomer except for the isolate KH_7 which had DL-DAP (Table 2). Seven of the selected isolates were shown to belong to the genus of *Streptomyces* and one to the genus *Micromonospora*.

The sequencing of the 16S RNA in these strains (Table 3) confirmed this classification. BH_3 and BH_5 isolates exhibited 98% and 94% sequence identity to *Streptomyces* sp. B11, respectively. YH_1 and BH_7 isolates exhibited 96% and 98% sequence identity to *S. griseus*, respectively. BH_2 exhibited 97% identity to *Streptomyces cavourensis* and KH_7 exhibited 97% identity to *Micromonospora aurantiaca*.

4. Discussion

Our study demonstrated the presence of Actinomycetes able to solubilize insoluble Rock Phosphate in the three Moroccan rock phosphate mines studied. The greater abundance of these strains in the Benguerir mine might simply be due to a higher content of organic matter in the Benguerir RP than in the Khouribga and Youssoufia RP samples, since organic matter is likely to promote better development of all bacterial strains including Actinomycetes. The proportion of Actinomycetes able to grow on insoluble RP, and thus likely to be able

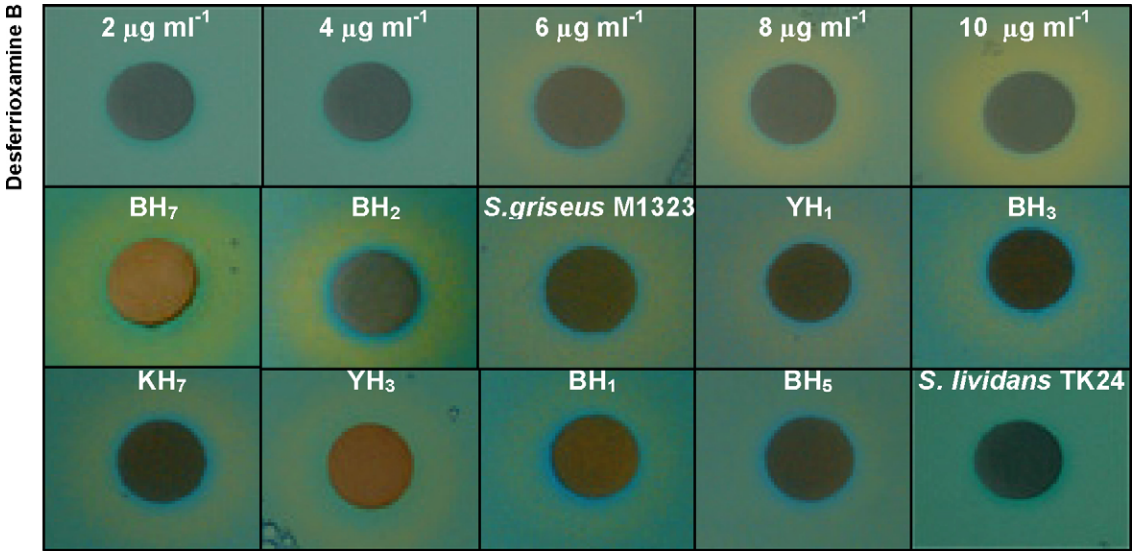

Fig. 4 – Cellulose disks impregnated with 20 μl of solution of Desferrioxamine B at different concentration or with 20 μl of 10-fold concentrated culture supernatants of cultures of the eight selected isolates and of the control strains (*S. griseus* M1323 and *S. lividans* TK24) grown for 5 days in liquid SMM containing 0.5 g l^{-1} RP^K and deposited on the surface of a CAS–blue agar plate.

Table 2 – Biochemical and morphological characteristics of eight selected isolates

Characteristics	RP solubilizing actinomycete isolates							
	YH_1	YH_3	BH_3	BH_5	BH_1	BH_7	BH_2	KH_7
Origin	Youssoufia	Youssoufia	Benguerir	Benguerir	Benguerir	Benguerir	Benguerir	Khouribga
ISP3	+++	++	++	++	++	+++	+++	+++
ISP4	+++	–	–	–	+	+++	+++	+++
ISP6	+	–	–	–	–	+	+	+
Aerial spore mass	Green clear	White	White	White	White	Green	Green clear	Green
Colony reverse	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Soluble pigment	Deep green	–	–	–	–	Deep green	Deep yellow	Deep green
Spore morphology	RF	RF	RF	RF	SS	RF	RF	Single conidia
DAP-isomer	LL	LL	LL	LL	LL	LL	LL	DL
Gram staining	+	+	+	+	+	+	+	+
Tyrosin hydrolysis	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–
C. source utilization								
Sucrose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Galactose	+	–	–	–	–	+	+	+
Inositol	+	–	–	–	–	+	+	+
Rhamnose	+	–	–	–	–	+	–	+
Xylose	+	–	–	–	–	+	–	+
D-Raffinose	+	–	–	–	–	+	–	+
Sorbitol	+	–	–	–	–	+	+	–
Arabinose	+	–	–	–	–	+	–	–
Antibiotic sensibility								
Novobiocine (30 μg)	S	S	S	S	S	S	S	S
Polymyxin B (300 U)	S	S	S	S	S	S	S	S
Cefalotin (30 μg)	S	R	S	S	S	S	R	S
Gentamycine (10 μg)	S	R	R	R	S	S	S	S
Bacitracine (10 U)	S	R	R	R	S	S	S	S
Sulfamides (250 μg)	R	R	R	R	R	R	S	S

+, Tested positive/utilized as substrate; –, tested negative/not utilized as substrate. RF: rectiflexible, SS: spiral. R: resistant, S: sensitive.

to solubilize insoluble RP, was approximately 18.3%. This is unexpectedly low since most of the phosphate in this biotope is in an insoluble form. However, other bacterial species likely take part in this solubilization process which benefits the whole bacterial population. For instance, Reyes et al. (2006) studied the diversity of RP solubilizing bacteria from the Monte-Fresco Rock phosphate mine in Venezuela showing that phosphate solubilizing bacteria (mainly *Azotobacter* sp.) were significantly more abundant in the soil of the mine (19% of total bacteria) than in an unmined soil (5% of total bacteria). Similarly, Babana (2003) reported that phosphate-solubilizing bacteria (mainly *Pseudomonas* sp.) isolated from four RP soil samples of Tilemsi and Koygour in Mali represent 7.52–30.26% of total bacteria. These authors did not report the presence of Actinobacteria in their samples likely because their medium and growth conditions were not appropriate to select these slow growing bacteria.

Our study also demonstrated that the ability to solubilize RP obviously varies from strain to strain, some being much more efficient than others. Among the eight most fast growing actinomycete isolates on SMM containing RP^K as sole phosphate source, BH_7 (*S. griseus*-related) and BH_2 (*S. cavourensis*-related) are the most powerful phosphate solubilizers in SMM broth and have a solubilizing activity comparable to that reported for *Xanthomonas maltophilia* and *Bacillus thuriengiensis* from Carolina rock phosphate in liquid PYD medium (De Freitas et al., 1997). Furthermore, BH_7, the *S. griseus*-related strain, has a significantly better ability to solubilize RP than *S. griseus* M1323, suggesting a good adaptation of this strain to its ecological niche. The different aptitudes for the solubilization of RP might reflect different modes of solubilization. Reports in the literature suggest that microbial solubilization of mineral phosphate might be either due to the excretion of organic acids causing acidification of the external medium (Whitelaw, 2000) or to the excretion of chelating substances (such as siderophores) that form stable complexes with phosphorus adsorbents (aluminium, iron and calcium) (Watteau and Berthelin, 1994; Welch et al., 2002) and thus increase

Table 3 – Percentage of sequence identity to the 16S RNA sequence of other actinomycete strains

Selected isolate	% Sequence identities	Actinomycete strains
YH_1	96	*Streptomyces griseus* *Streptomyces anulatus strain* NRRL 8–2873
YH_3	98	*Candidatus Streptomyces philanthi biovar coarctatus* *Streptomyces endosymbiont of philanthus venustus*
BH_3	98	*Streptomyces* sp. B11 *Streptomyces* sp. FXJ23
BH_5	94	*Streptomyces* sp. B11 *Streptomyces* sp. FXJ23
BH_1	98	*Candidatus Streptomyces philanthi biovar basilaris* *Candidatus Streptomyces philanthi biovar coarctatus*
BH_7	98	*S. griseus* *Streptomyces anulatus strain* NRRL 8–2873 *Streptomyces sp.* YIM 80147
BH_2	97 96	*Streptomyces cavourensis* sub sp. *Washintogensis strain* NRRL 8–8030 *S. griseus* *Streptomyces* sp. MTR1 *Streptomyces fimicarius strain* ISP
KH_7	97 95	*Micromonospora aurantiaca* *Micromonospora flavogrisea* *Micromonospora echinaurantiaca*

The alignments were made with 1400–1500 bp long DNA fragments.

phosphate solubilization. We demonstrated that our most effective RP solubilizing strains do not excrete organic acids but excrete chelator substances as revealed by the blue CAS–agar test (Schwyn and Neilands, 1987). Since Khouribga RP is mainly a calcium/phosphate hydroxyapatite, these substances are likely to be strong calcium chelators. Chelators were also known to be involved in the RP solubilization process of other microorganisms such as *Aspergillus niger*, *Enterobacter* sp. and *Erwinia* sp. (Abd-Alla and Omar, 2001; Zhao et al., 2002).

5. Conclusion

The present work indicates that some Actinomycetes from Moroccan phosphate mines are indeed able to release soluble phosphate from insoluble rock phosphate. Seven of the eight most active isolates belong to the genus *Streptomyces* and one to the genus *Micromonospora*. The ability of these strains to solubilize RP is not related to the excretion of organic acids but to the likely production of a calcium chelator whose purification and structural elucidation is in process.

The results of this investigation are expected to lead to the formulation of novel bio-phosphate fertilizers constituted by the association of pulverized RP and spores of the *ad hoc* actinomycete strains that could be less polluting than the traditional chemical phosphate fertilizers.

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