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Full Length Research Paper

Isolation and characterization of rock phosphate solubilizing actinobacteria from a Togolese phosphate mine

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A phosphate mine from Togo, an original biotope rich in insoluble rock phosphate (RP), was explored for the presence of RP solubilizing actinobacteria. One hundred and fifty actinobacteria isolates originating from this mine was tested for their ability to grow on a synthetic minimum medium (SMM) containing insoluble RP as unique phosphate source. Only 29 isolates (19%) were able to weather RP in SMM medium. Five 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. Four of these strains were shown to belong to the genus *Micromonospora* and one, to the genus *Streptomyces.*

Key words: Actinobacteria, isolation, characterization, Togolese phosphate mine, rock phosphate solubilization.

INTRODUCTION

Phosphorus is one of the major nutrients limiting plant growth. In contrast, P is required for growth and development of plants. It is also involved in photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration (Fernández et al., 2007). However, many agricultural soils worldwide are P deficient (Arcand and Schneider, 2006) and, therefore require P to replenish the P-demand by crop plants.

Worldwide, 5.7 billion hectares contain too little available P for sustaining optimal crop production (Hinsinger, 2001), and P-ion concentration in most soils varies from 0.1 to 10 µM (Raghothama, 1999). Suboptimal levels of P can, however lead to 5 to 15% loss in the yield of plants (Hinsinger, 2001). In order to compensate for this natural P-deficiency, expensive chemical phosphate fertilizers are applied in agriculture to improve crop yield (Gyaneshwar et al., 2002). The repeated and injudicious applications of these fertilizers, however lead to (1) the loss of soil fertility, (2) disturbance to microbial diversity and their associated metabolic activities, (3) reduced yield of agronomic crops, and (4) a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils or with Ca in calcareous soils (Toro, 2007) before plant roots have had a chance to absorb it. Moreover, the chemical fertilizer industry is now considered as extremely polluting

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Abbreviations: RP, Rock phosphate; **SMM,** synthetic minimum medium.

(Shigaki et al., 2006; Vassilev et al., 2006) by accelerating eutrophication of fresh waters and polluting ground waters used for drinking (Shigaki et al., 2006). This has led to the search for environment-friendly and economically feasible alternative strategies for improving crop production in low or P-deficient soils (Macias et al., 2003). In many countries, ground rock phosphate (RP) reduced to fine particles was used in traditional agriculture.

The RP of Hahotoé (Togo) is a fluoroapatite with a large reserves and an interesting P content (27% P) (Tchangbedji et al., 2003). RP is a natural, cheap and clean compound but unfortunately it is a poor P fertilizer since its solubilization is too slow to satisfy plant needs (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). Occurrence, importance and use of phosphate solubilizing microorganisms (PSM) in various ecological niches have been documented as an alternative to chemical phosphatic fertilizers and provides the available forms of P to plants (Bojinova et al., 2008; Oliveira et al., 2008).

Actinobacteria are among the most fascinating (PSM). Their developmental life cycle with its morphological and physiological differentiation and their ability to develop in extremely different soils (Jiang et al., 2005; Pathom-Aree et al., 2006), and the rich repertoire of secondary metabolites (anti-fungi, insecticides, anthelminthics, phytohormone-like compounds, etc.) that could benefit plant growth (Mba, 1997; Jain and Jain, 2007; Hamdali et al., 2008a,b,c) have resulted in a large research community studying these microbes. However, for action-mycete PSM from Togo, the presence, the taxonomic groups and the mechanisms involved in the RP weathering processes are not well elucidated. Hahotoé-Kpogamé phosphate mine from Togo 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, actinobacteria able to release soluble phosphate from RP. The preliminary solubilization mechanism and taxonomic characterization of the most efficient solubilizing isolates were investigated.

MATERIALS AND METHODS

Collection of rock phosphate samples from the mine

Rock phosphate samples were collected in April 2009 from the extracted rock phosphate stockpiles of Hahotoé - Kpogamé phosphate mine from Togo (6°2123 N; 1°2341 E) (Figure 1). Three 25 m × 25 m areas were sampled at the site, representing three replicates. The distance between the replicates was approximately 10 m. From this replicate area, a composite RP sample was taken, consisting of 10 core samples of 500 g wet weight and 4 cm in diameter collected from 0 to 10 cm depth after removing 3 cm surface residues. The soil samples from the 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 Hahotoé - Kpogamé phosphate mine (RP^H) was determined using fluorescence RX (Stereoscan 260, Cambridge, England) and consisted of Ca, 63.72%: P, 26.91; Si, 3.55; Fe, 2.12; Al, 2.01; Na, 0.56; S, 0.23; Mg, 0.15; Cl, 0.12; Sr, 0.09; Te, 0.09; K, 0.07; Ti, 0.07; Mn, 0.05; Y, 0.04; Zn, 0.04; La, 0.03; Cu, 0.015; V, 0.012 and Zr, 0.005% (Tchangbedji et al., 2003).

Isolation of total bacteria and actinobacteria

2 g (wet weight) of the soil sample was resuspended in 18 ml of sterile physiological serum (9 g/L, NaCl), homogenized and sonicated according to Ouhdouch et al. (2001). 0.1 ml of various dilutions of the treated sample was plated in triplicate on the surface of nutrient agar (Difco, USA) and of a solid medium prepared with RP soil extract as described in Barakate et al. (2002) with glycerol (5 g/L) and agar (15 g/L) being added to this extract. The pH was adjusted to 7 and the medium was sterilized at 121°C for 20 min. This medium was supplemented with 40 mg/ml actidione and 10 mg/ml 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 actinobacteria. Actinobacteria 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 actinobacteria to total bacteria in the site. All values are means of three replicates plates from the RP sample.

Screening for actinobacteria able to use rock phosphate as sole phosphate source

Selection of actinobacteria able to use RP as sole phosphate source was carried out by plating 100 colonies (from the investigated mine) on the synthetic minimum medium (SMM, Hamdali et al., 2008a) containing 10 g/L glucose, 2 g/L NaNO3, 0.5 g/L MgSO4·7H2O, 0.5 g/L KCl, 0.01 g/L FeSO4·7H2O and RP^H (0.5 g/L) as sole phosphate source or on the SMM containing soluble K_2HPO_4 (0.5 g/L) or no phosphate source. Spores of the actinomycete isolates able to show the most active growth on SMM containing RP^H as sole phosphate source were stored in 20% (w/v) sterile glycerol at -20°C.

Estimation of the ability of the selected actinobacteria to release soluble phosphate from RP

Three culture replicates were inoculated with 10^6 spores /ml of each actinomycete isolate and grown for 5 days at 28°C on a rotary shaker (180 g/min) in 250 ml Erlenmeyer flasks containing 50 ml of liquid SMM medium with 0.5 g/L RP^H. The cultures were centrifuged at 10,000 × g for 10 min and the pH of the supernatant was measured every day. 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 non-inoculated flasks incubated under the same conditions.

Test of siderophore excretion

In order to determine whether siderophores were present in the

Figure 1. Geographical overview of Togo and localization of the mine site.

culture supernatants of the five selected actinomycete isolates, they were grown for 5 days under the conditions described above. The supernatants were centrifuged at 10,000 × g/min and concentrated 10-fold by evaporation using a speed vac concentrator (Appligene, France). Twenty microliter of the concentrated supernatants were 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 4 or 6 mg/ml 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

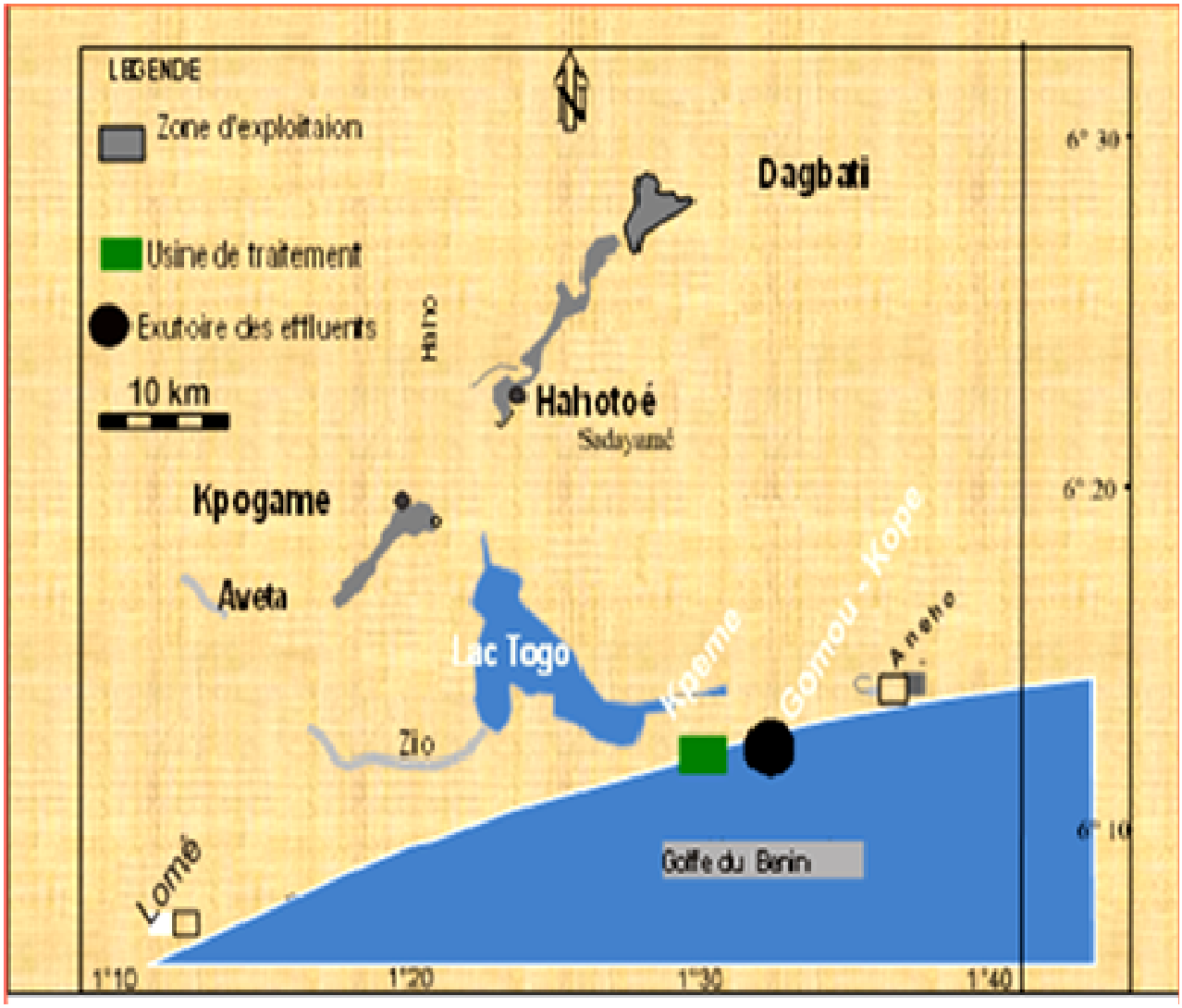

Figure 1. Contd

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.

Taxonomic characterization of selected RP solublizing actinomycete isolates

The morphological, cultural, physiological and biochemical characteristics of the selected isolates were evaluated as described in the International Streptomyces Project (ISP) (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 to 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 17 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). Accordingly, the selected isolates were placed in genera and taxonomic groups on the basis of the different morphological characteristics and chemical compositions of cells.

RESULTS

Isolation of actinobacteria able to use rock phosphate as sole phosphate source

The distribution of total bacteria and actinobacteria in the RP soil extract collected from the Hahotoé - Kpogamé phosphate mine is shown in Table 1. Total bacteria represent 94.3×10^5 cfu/g in this mine site.

Of a total, 150 actinobacteria isolates with different morphological characteristics selected from the RP^H soil, only 29 could use RP^H when plated on the solid SMM medium containing RP^H as unique phosphate source

Table 1. Distribution of total bacteria and actinobacteria in rock phosphate sample and the percentage of actinobacteria in rock P soil extract of hahotoé- Kpogamé (Togo).

Parameter	RP^H	ANOVA
Total bacteria (× 10^5 cfu/g)	94.3	P = 0.07
Actinobacteria (× 10^5 cfu/g)[a]	14.5[a]	P < 0.001
Percentage of actinobacteria in total bacteria	15.4[a]	

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

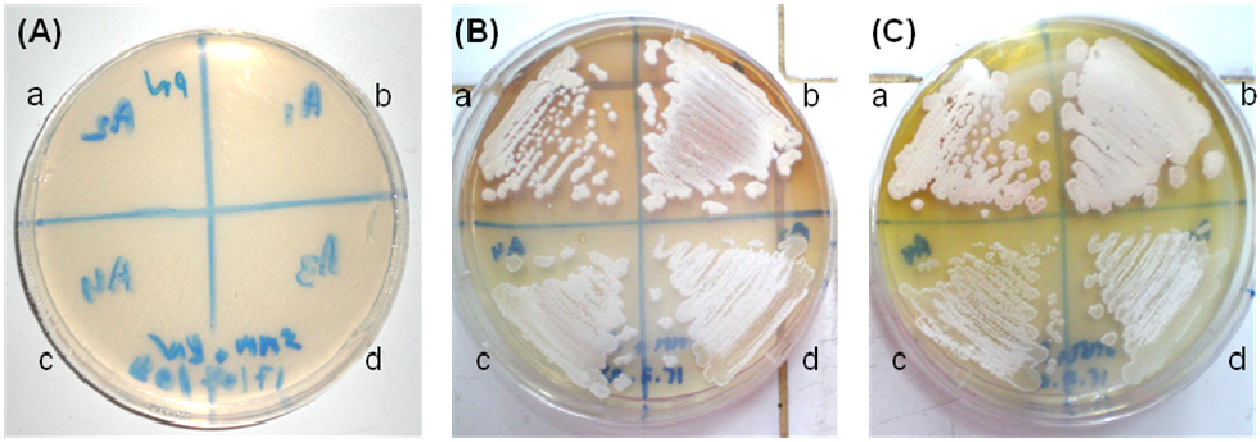

Figure 2. Sceening procedure to isolate strains able to grow on SMM medium with RP^H as sole phosphate source. (A) SMM without K_2HPO_4, (B) SMM with 0.5 g/L K_2HPO_4, and (C) SMM with 0.5g/L RP^H. The strains a, b, c, d, f is able to use RP^H as sole phosphate source.

(Figure 2). Of these 29 isolates, the 5 which showed the most active growth on SMM medium containing RP^H as sole
phosphate source were selected for more extensive study.

Abilities of the selected isolates to release soluble phosphate from RP

The five selected actinobacteria strains showed different abilities to release soluble phosphate from RP^H (Figure 3). Phosphate release ranged from 4.38 to 25.87 µg/ml. AT12 was the most efficient strain releasing 25.87 µg/ml soluble P in the growth medium (Figure 3). AT26 and AT2 released 9.11 and 9.09 µg/ml phosphate, whereas AT3 and AT4 are likely able to release a very small fraction of soluble P in the growth medium with only 6.38 and 4.38 µg/ml phosphate, respectively (Figure 3).

Investigation of the solubilization mechanism

No acidification of the growth medium was observed for any of the strains; even an important alkalinization of the medium was noticeable toward the end of the growth (Figure 4), suggesting that the process of solubilization did not involve the excretion of organic acids. The AT12, AT26 and AT2 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 (Figure 5), whereas the AT3 and AT4 strains excreted very little of these substances. It is noteworthy that these two strains were the least efficient RP solubilizers whereas AT12 was the most efficient RP solubilizing strain (Figure 3).

Taxonomical characterization of the selected isolates

The five selected strains were tested for taxonomic diversity using morphological, cultural, physiological and biochemical criteria as well as others features (Table 2). Morphology of the actinobacteria colonies was determined on the media used for their isolation. The aerial and substrate mycelium color was determined on media ISP2, 3, 4 and 6 (Table 2). The five strains showed different abilities to assimilate 17 carbon sources tested. All strains were able to use citrate, sucrose, fructose, glucose, mannitol, lactose, melibiose, inositol, xylose, D-raffinose and glycerol as sole carbon sources, whereas these strains, except AT4, were not able to use sorbitol and arabinose (Table 2). Maltose, mannose, galactose

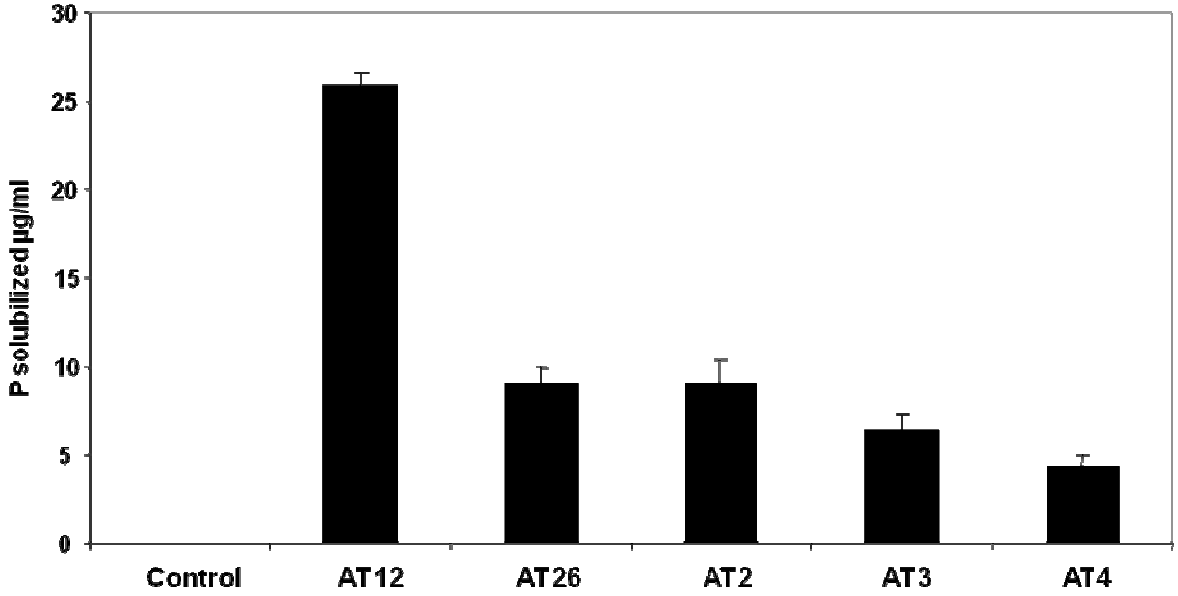

Figure 3. Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures of the five selected isolates grown for five days in SMM containing 0.5 g/L RPH and in the medium of the non-inoculated flasks incubated under the same conditions (control). Error bars represent standard deviations of the mean values of the results of three independent culture replicates.

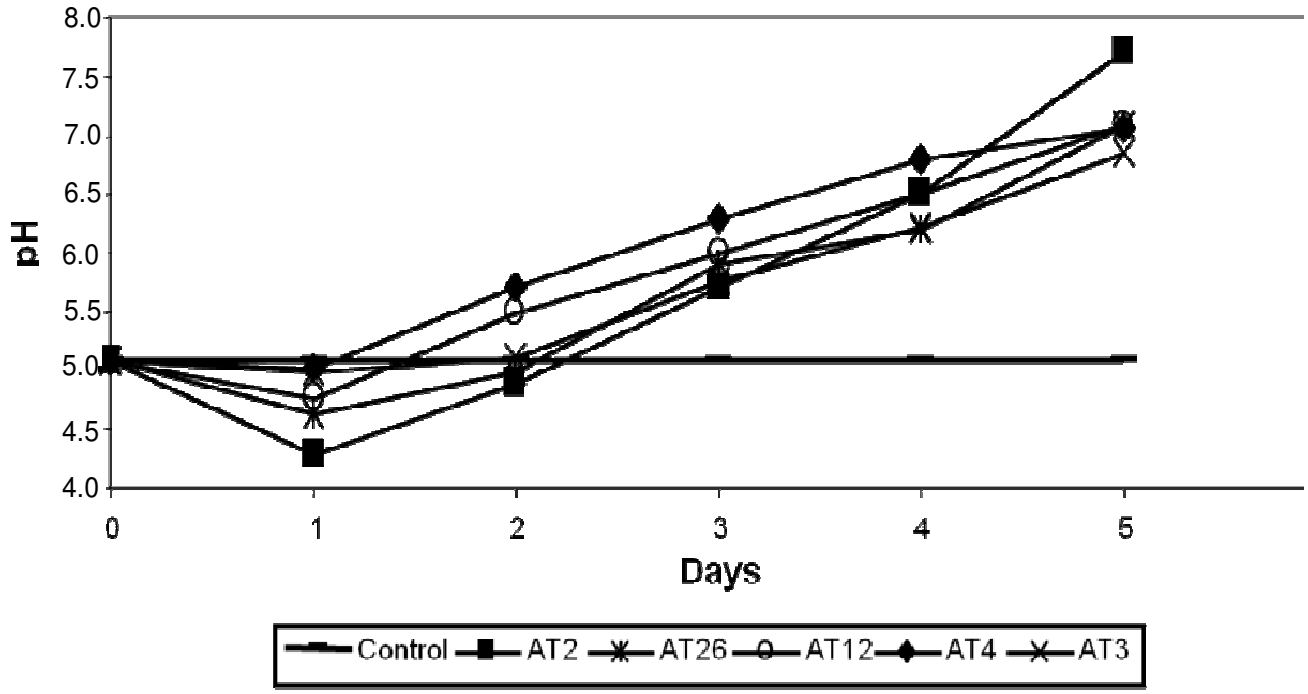

Figure 4. Evolution of pH of the culture supernatant of the five selected rock phosphate solubilizing Actinomycete strains grown in SMM containing 0.5 g/L RP^H and in the non-inoculated control incubated under the same conditions.

and rhamnose were not used by AT26 and AT4 (Table 2). All strains were sensitive to Novobiocine (30 mg), Polymyxin B, (300 U), Cefalotin (30 mg), gentamycine (10 mg) and bacitracine (10 U) (Table 2). Strains AT26, AT2 and AT4 were resistant to sulfamides but AT12 and AT3 were sensitive. The analysis of cellular constituents of the five isolates revealed the presence of the D-diaminopimelic acid (DAP) isomer except for the isolate

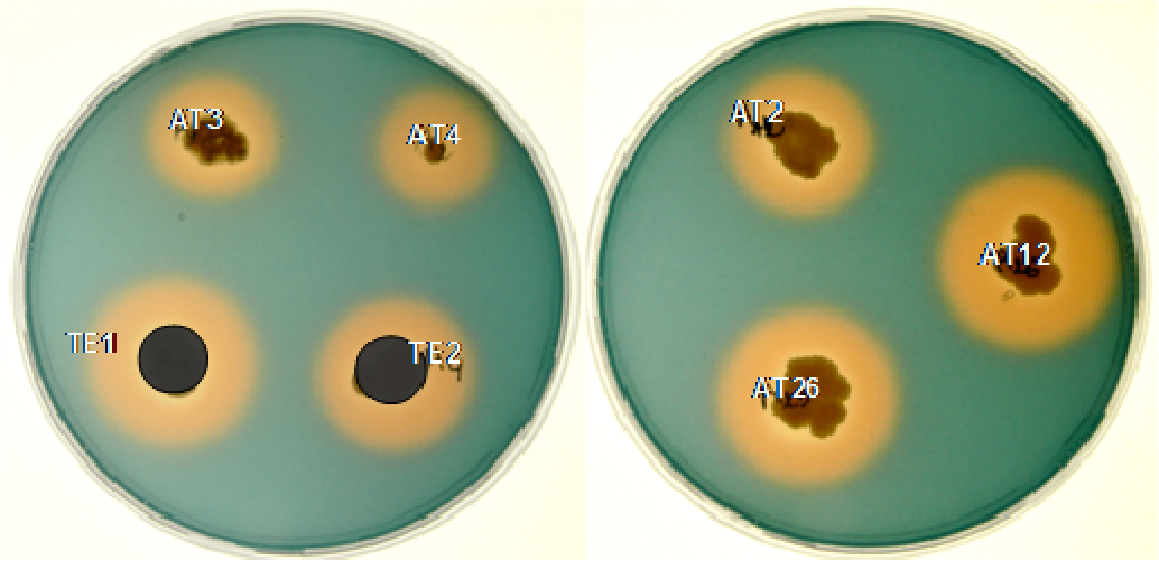

Figure 5: Cellulose disks impregnated with 20 µl of solution of Desferrioxamine B at TE1 and TE2 concentration (4 and 6 mg/ml respectively) or with 20 µl of the concentrated culture supernatants of cultures of the five selected isolates grown for 5 days in liquid SMM containing 0.5 g/L RPH and deposited on the surface of a CAS–blue agar plate.

AT2 which had LL-DAP (Table 2). Four of the selected isolates were shown to belong to the genus of *Micromonospora* (AT12, AT26, AT3 and AT4 isolates), and one to the genus *Streptomyces* (AT2 isolate).

DISCUSSION

This study demonstrates the presence of actinobacteria able to solubilize insoluble Rock Phosphate in the Hahotoé-Kapogamé rock phosphate mine studied. The proportion of actinobacteria growing on insoluble RP^H, and thus likely to be able to solubilize insoluble RP^H, was approximately 19%. Similar results were reported in previous studies on phosphate solubilizing bacteria from different phosphate mines. For instance, Hamdali et al. (2008a) showed that the abundance of actinobacteria solublizing Moroccan rock phosphate was approximately 18%. Similarly, Ben Farhat et al. (2009) demonstrated that phosphate solubilizing bacteria (mainly *Serratia marcescens*) from the Tunisian phosphate mine of Gafsa represented 18.6% of total bacteria. Reyes et al. (2006) have found that phosphate solubilizing bacteria (mainly *Azotobacter* sp.) from the Monte-Fresco RP mine in Venezuela represented 19% 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 represented 7.52 to 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 five most fast growing actinobacteria isolates on SMM containing RP^H as sole phosphate source, AT12 was the most powerful phosphate solubilizers in SMM broth (25.87 µg/ml soluble P). This strain have a solubilizing activity comparable to that reported for *Streptomyces griseus*-like from Moroccan rock phosphate mine in liquid SMM medium (Hamdali et al., 2008a ,b, 2009) and for *Xanthomonas maltophilia* and *Bacillus thuriengiensis* from Carolina rock phosphate in liquid PYD medium (De Freitas et al., 1997). Furthermore, AT12 strain had a significant better ability to solubilize RP^H than AT4 strain 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 suggested 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) (Welch et al., 2002; Hamdali et al. 2008a, b, 2009), and thus increase phosphate solubilization. None of the selected isolates was surrounded by a clear halo, characterizing microorganisms producing organic acids on the classical Pikovskaya (Pikovskaya, 1948), and NBRIP media (Nautiyal, 1999). None of the following acids (oxalic, citric, DL malic, succinic nor fumaric acids) was found in the culture filtrates of the five strains studied, using TLC

Table 2. Biochemical and morphological characteristics of the five active isolates.

Characteristics	AT12	AT26	AT2	AT3	AT4
ISP3	+++	+++	++	++	++
ISP4	+++	+++	-	++	++
ISP6	+	+	-	+	+
Aerial spore mass	grey	white	grey	white	white
Aerial mycelium	-	-	+	-	-
Colony reverse	grey	cream	grey	cream	cream
Soluble pigment	-	-	Yellow	-	-
DAP- isomer	DL	DL	LL	DL	DL
Gram staining	+	+	+	+	+
Tyrosin hydrolysis	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
C. source utilization					
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	+
Glucose	+	+	+	+	+
Glycerol	+	+	+	+	+
Maltose	+	-	+	+	-
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Mannose	+	-	+	+	-
Citrate	+	+	+	+	+
Mannitol	+	+	+	+	+
Galactose	+	-	+	+	-
Inositol	+	+	+	+	+
Rhamnose	+	-	+	+	-
Xylose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
Sorbitol	-	-	+	-	-
Arabinose	-	-	+	-	-
Novobiocine (30 mg)	S	S	S	S	S
Polymyxin B (300 U)	S	S	S	S	S
Cefalotin (30 mg)	S	S	S	S	S
Gentamycine (10 mg)	S	S	S	S	S
Bacitracine (10 U)	S	S	S	S	S
Sulfamides (250 mg)	S	R	R	S	R

+ = Tested positive/utilized as substrate; - = tested negative/not utilized as substrate. R: resistant, S: sensitive.

chromatography (detection limit of 10 mg/ml) (data not shown). Our results show that the most effective RP^H solubilizing strains do not produce organic acids but excrete chelator substances as revealed by the blue CAS–agar test (Schwyn and Neilands, 1987). Since Hahotoé RP was mainly a phosphate fluoroapatite, these substances were likely to be strong chelators. Chelators were also known to be involved in the RP solubilization process of actinobacteria (Hamdali et al. 2008a, b, 2009), and of other microorganisms such as *Aspergillus niger, Enterobacter* sp. and *Erwinia* sp. (Abd-Alla and Omar, 2001; Zhao et al., 2002).

The taxonomic study showed that all selected isolates belong to the *Micromonospora* genus except one to the genus of *Streptomyces*. Although, they belong to the same genus, the isolates showed different RP solubilization capabilities. The fine taxonomic characterisation as well as isolation, purification and structural elucidation of siderophore produced are under investigation. The results of this screening are expected to lead to the formulation of novel bio-phosphate fertilizers constituted by the association of pulverized RP and spores of the green house powerful selected strains.

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