Physiological studies and comparative analysis of rock phosphate solubilization abilities of Actinomycetales originating from Moroccan phosphate mines and of *Streptomyces lividans*

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A B S T R A C T

Actinomycete strains originating from Moroccan phosphate mines (MPM) were selected for their ability to use the insoluble ground hydroxyapatite called rock phosphate (RP), present in their biotope, as sole phosphate (P) source. Physiological studies carried out with these strains and with the reference strains, *Streptomyces lividans* and *Streptomyces griseus*, demonstrated that all strains were able to grown in a synthetic minimal medium (SMM) containing either soluble (SP) or insoluble (RP) phosphate as sole P source. The MPM strains and *S. griseus* took up glucose much more actively and exhaustively than *S. lividans*, constituting more abundant glycogen reserves than the latter. All strains took up soluble P at comparable rates, storing it as polyphosphates. In SMM + RP, a sharp increase in the concentration of soluble P was detected in the culture broths of all MPM strains and *S. griseus*, at stationary phase, but not in that of *S. lividans*. The P peak detected in the supernatant of these strains correlated with the successive appearance of two compounds absorbing at 320 nm and 430 nm, respectively. These compounds are thought to be strong ion chelators involved in the destruction of the hydroxyapatite structure leading to soluble P release. The good growth of *S. lividans* in SMM + RP indicated that this strain was also able to release P from RP but consumed it as soon as it was released, unlike the other strains. Our study is expected to lead to the development of a novel type of slow release bio-phosphate fertilizer constituted by the association of the MPM strains and ground RP. This novel product would precisely supply plant needs and thus limit the pollution of the environment.

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1. Introduction

Readily available phosphorus (P) is often the nutritional element limiting plant growth in many agricultural soils worldwide (Vassilev et al., 2006b). Even if an ordinary soil contains approximately 0.4–1.2 g of P per kg of soil (Fernández and Novo, 1988), 95–98% of this P is present in the form of insoluble metallic chelates that cannot be utilized by plants (Goldstein, 1994; Reddy et al., 2002). In consequence, most agricultural soils have to be supplemented with large quantities of soluble P fertilizers (Arcand and Schneider, 2006). Nowadays, the application and the production of these fertilizers which involves the use of sulfuric acid are considered as extremely polluting (Shigaki et al., 2006; Vassilev et al., 2006a). In fact, most of the supplementary soluble P added is either adsorbed by the various constituents of the soil (clay, carbonates, oxides, etc…) or precipitated as insoluble metallic chelates or rapidly washed away by rain water, accelerating the eutrophication of fresh water and polluting ground water (Shigaki et al., 2006). Consequently, very little of the applied P is available for plants, making repeated and thus polluting application necessary (Abd-Alla, 1994).

Innovative strategies are now required to reduce the polluting and expensive application of chemical P fertilizers in agricultural practice. In many countries, ground rock phosphate (RP) reduced to fine particles was used in traditional agriculture. Morocco possesses the largest RP reserves worldwide. The Moroccan RP is a hydroxyapatite that was shown to be constituted by 56.53% O, 16.35% Ca, 9.37% P, 2.42% F, 2.03% Al, 1.94% Mg, 1.81% Na, 0.77% S, 0.60% Fe, 0.12% Sn (Hamdali et al., 2008a). 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 order for RP to become an efficient P fertilizer, innovative methods must be found to free the P from its strong

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ionic interactions with the calcium or the other ions in the RP. Several recent scientific reports show that some soil borne microorganisms, including *Aspergillus niger* (Abd-Alla and Omar, 2001), *Arthrobacter* sp., *Enterobacter* sp. and *Erwinia* sp. (Zhao et al., 2002), etc., are indeed able to promote solubilization of RP and to increase crop yields (Rudresh et al., 2005; Antoun and Babana, 2006; Ouahmane et al., 2007). The solubilization process achieved by these phosphate solubilizing microorganisms (PSM), involves the excretion of either organic acids (Gyaneshwar et al., 2002) or of substances with strong chelating power (Abd-Alla and Omar, 2001; Zhao et al., 2002). In both cases, these substances destroy the strong ionic interactions linking the phosphate to its metallic/ionic partners.

Among these PSM, Actinomycetes (Mba, 1997; Hamdali et al., 2008a,b,c) are of special interest since, besides their ability to solubilize insoluble phosphate, these filamentous and sporulating bacteria are often able to colonize plant tissue and are natural producers of antibiotics or anti-fungi that could protect the plants against various phytopathogens (Ikeda, 2003; Jain and Jain, 2007). In order to increase the probability of identifying Actinomycetes especially efficient in solubilizing RP, we searched a specific biotope rich in insoluble RP, the Moroccan phosphate mines (Hamdali et al., 2008a). The most efficient RP solubilizing Actinomycete isolates were shown to belong to the *Streptomyces* and *Micromonospora* genera (Hamdali et al., 2008a). The MPM strains were shown to promote good wheat plant growth as well as to protect the plant against the devastating effects of a soilborne phytopathogenic fungus (*Pythium ultimum*), at least in greenhouse conditions (Hamdali et al., 2008b,c). The efficiency of the RP solubilization process relies on complex and specific nutritional and physiological growth conditions of the PSM (Hameeda et al., 2006). In order to get a better understanding of the physiological context triggering the RP solubilization process in the life cycle of our Actinomycete strains, the MPM strains as well as *Streptomyces lividans* TK24 (Hopwood et al., 1983; a reference strain used in many laboratories) and *Streptomyces griseus* M1323 (related to a strain found in the MPM), were grown in a synthetic minimal liquid medium containing either soluble phosphate (K_2HPO_4, P proficiency) or RP (P limitation) as unique P source. Several parameters were assessed during growth allowing physiological comparisons of the different strains. These included the monitoring of biomass yields, of glucose and phosphate consumption as well as the accumulation of intracellular storage polymers such as glycogen and polyphosphates. The absorption of supernatants at 320 nm and 430 nm was also assessed since a λ scan of these supernatants revealed the appearance of substances absorbing at these wavelengths.

2. Materials and methods

2.1. Actinomycete strains and culture conditions

The six Actinomycete strains used in this study are *S. lividans* TK24 (Hopwood et al., 1983), *S. griseus* M1323 (IPC Paris, France) and four strains previously isolated from the Moroccan phosphate mines and possessing RP solubilization abilities (Hamdali et al., 2008a,b,c). These strains were previously identified as related to *Streptomyces* species (BH$_7$ related to *S. griseus* was renamed SG^{MPM}, BH$_2$ related to *S. cavourensis* was renamed SC and BH$_3$ related to *Streptomyces* B11 was renamed SB11) and *Micromonospora* species (KH$_7$ related to *Micromonospora aurantiaca* was renamed MA^{MPM}) (Hamdali et al., 2008a,b,c).

Spores of these strains, stored in 20% sterile glycerol at $-20\,^{\circ}$C, were used to inoculate (at 10^6 cfu ml^{-1}) in 250-ml Erlenmeyer flasks, 60 ml cultures of liquid synthetic minimal medium (Hamdali et al., 2008a). This medium was supplemented or not

with 0.5 g l^{-1} RP (approximately 2.2 mM phosphorus) or with 0.5 g l^{-1} soluble K_2HPO_4 (3H$_2$O) (2.2 mM phosphorus) as sole phosphate source. Cultures were grown in triplicate for 7 days at 28 °C under constant agitation on a rotary shaker at 180 g min^{-1}.

2.2. Estimation of biomass yields and of glucose and phosphate consumption

Samples of 2 ml of each culture were collected every 6 h during 7 days than filtered through a 0.45 μm-pore-size filter (Supor-450; Pall Corporation) and washed twice with distilled water. The mycelium was re-suspended in 500 μl of phosphate buffer (0.5 mM K_2HPO_4/KH_2PO_4, pH 7.0, EDTA, 5 mM, pH 8.0) and broken in the presence of glass beads (106 μm diameter, v/v of biomass) using a FastPrep® FP220A instrument (Qbiogene, France), twice for 45 s at a speed of 6.5. The lysate was centrifuged at $8000 \times g$ for 5 min to remove cell debris. Growth (biomass) was estimated by protein quantification using the Bradford reagent (Bio-Rad). A standard curve was established with bovine serum albumin solutions (5–55 μg ml^{-1}) measuring absorbance at 595 nm using a Spectronic Genesys 2 spectrophotometer.

The concentration of glucose and phosphate were assayed in the filtrates of culture supernatants. The final values are the arithmetic mean of triplicate cultures. The pH and the absorbance (at 320 nm and 430 nm, respectively) of the supernatant were measured every 6 h during incubation. The concentration of glucose was determined with the enzymatic kit from Sigma–Aldrich. A standard curve was established with a solution of glucose (0.05–5 mg l^{-1}). Similar measures were carried out in the non-inoculated flasks incubated in the same conditions. The concentration of phosphate (P) was determined with the PiBlue Phosphate Assay Kit from Gentaur. The concentration of the green complex formed between orthophosphate and malachite green dye was measured at 620 nm. A standard curve was established with a solution of potassium phosphate (0.4–50 μM K_2HPO_4).

2.3. Quantification of the total intracellular glucose content: free glucose and glucose stored as glycogen

In order to determine the total intracellular glucose content that we assume was mainly stored as glycogen, 0.4 g mycelial samples were collected after 40 h of culture, re-suspended in 5 ml of 0.6 N HCl then autoclaved at 121 °C for 60 min (Bond et al., 1995). After cooling at room temperature, the pH was adjusted to 7 with 0.6 M NaOH. In these conditions, glycogen was hydrolyzed to glucose units. Total glucose concentration was then measured as glucose unit equivalents with a glucose detection kit (Sigma–Aldrich).

2.4. Quantification of total intracellular phosphate: free phosphate and phosphate stored as polyphosphate

In this study a slightly modified version of the Kornberg's method was used for the determination of total polyphosphate (PolyP) (Kumble and Kornberg, 1995). 0.2 g of mycelium from 22 h, 36 h, 54 h, 74 h, 96 h and 110 h grown cultures of the different Actinomycete strains in SMM liquid medium containing either RP (2.2 mM, 0.5 g l^{-1}) or K_2HPO_4 (3H$_2$O) (2.2 mM, 0.5 g l^{-1}) as sole P source (Hamdali et al., 2008a) were collected by filtration, washed with distilled water and re-suspended in 750 μl of lysis buffer STE (10 mM Tris–HCl at pH 8.0, 1 mM EDTA, 6 mM MgCl$_2$ and 250 mM sucrose). The cells were broken with a Fast Prep apparatus as described previously. The resulting homogenate was incubated with 750 μg ml^{-1} of the protease from *S. griseus* (Sigma) at 37 °C for 2 h. The sample was extracted with phenol/chloroform (1:1, w/v equilibrated with Tris–HCl, pH 7.5) and centrifuged at $14,000 \times g$ for