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Evidence for mutualism between a plant growing in a phosphate-limited desert environment and a mineral phosphate solubilizing (MPS) rhizobacterium

Alan H. Goldstein [a,b,*], Kate Braverman [a], Nelson Osorio [b]

[a] *Division of Biology, Alfred University, Alfred, NY 14802, USA*

[b] *Department of Biology and Microbiology, California State University at Los Angeles, Los Angeles, CA 90042, USA*

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Abstract

Alkaline desert soils are high in insoluble calcium phosphates but deficient in soluble orthophosphate (Pi) essential for plant growth. In this extreme environment, one adaptive strategy could involve specific associations between plant roots and mineral phosphate solubilizing (MPS) bacteria. The most efficient MPS phenotype in Gram-negative bacteria results from extracellular oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase. A unique bacterial population isolated from the roots of *Helianthus annus jaegeri* growing at the edge of an alkaline dry lake in the Mojave Desert showed no MPS activity and no gluconic acid production. Addition of a concentrated solution containing material washed from the roots to these bacteria in culture resulted in production of high levels of gluconic acid. This effect was mimicked by addition of the essential glucose dehydrogenase redox cofactor 2,7,9-tricarboxyl-1H-pyrrolo[2,3]-quinoline-4,5-dione (PQQ) but the bioactive component was not PQQ. DNA hybridization data confirmed that this soil bacterium carried a gene with homology to the *Escherichia coli* quinoprotein glucose dehydrogenase. These data suggest that expression of the direct oxidation pathway in this bacterium may be regulated by signaling between the bacteria and the plant root. The resultant acidification of the rhizosphere may play a role in nutrient availability and/or other ecophysiological parameters essential for the survival of this desert plant. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

For over a century agricultural microbiologists and microbial ecologists have been interested in the ability of some bacteria to dissolve poorly soluble mineral phosphates such as bone meal or hydroxyapatite (reviewed in [1,2]). We have termed this phenotype mineral phosphate solubilizing (MPS). The ability of MPS bacteria to release soluble orthophosphate (Pi) from rock phosphate ore makes this phenotype of great potential importance for the development of ecorational phosphate fertilizer technologies for agriculture [2,3]. Likewise, microbial ecologists have long been interested in the possible role of

* Corresponding author. Division of Biology, Alfred University, Alfred, NY 14802, USA. Tel.: +1 (607) 871-2645; Fax: +1 (607) 871-2359; E-mail: fgoldste@bigvax.alfred.edu

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MPS bacteria in phosphate cycling in natural ecosystems, especially arid and semiarid soils where most of the mineral phosphate is not bioavailable being precipitated in poorly soluble calcium phosphate materials such as hydroxyapatite [4,5].

Certain strains of Gram-negative bacteria are highly efficacious at dissolving calcium phosphates (e.g. fluroapatite) whose water solubility is extremely low. We have shown that this phenotype is the result of gluconic and 2-ketogluconic acid production via the direct oxidation pathway [6]. The enzymes for this pathway are located on the outer face of the cytoplasmic membrane. The first step in this pathway is the oxidation of glucose to gluconic acid by glucose dehydrogenase (GDH). GDH is a member of the largest group of quinoproteins, those that require the redox cofactor 2,7,9-tricarboxyl-1H-pyrrolo[2,3]-quinoline-4,5-dione (PQQ) [7]. Gluconic acid is often further oxidized to 2-ketogluconic acid, one of the strongest naturally occurring organic acids ($pK_a \sim 2.6$). Because this pathway operates in the periplasmic space, the extracellular environment can become highly acidic. MPS^+ bacteria using the direct oxidation pathway can release significant amounts of Pi from calcium phosphates via this acidification of their extracellular environment [3,6,9].

While many workers have reported enhanced levels of MPS bacteria in the rhizosphere vs. bulk soil (reviewed in [1]) the role of these bacteria in plant growth and biomass accumulation remains a topic of debate [4]. Recently, we have shown that MPS bacteria isolated from the roots of two desert plants both carried a gene with homology to the *Escherichia coli* GDH gene (*gcd*) whereas an MPS^- isolate did not [5].

We are interested in exploring the possibility that MPS bacteria play a role in Pi cycling in microbial ecosystems occurring in natural soils. It is reasonable to propose that the surface of plant roots (rhizosphere) is one niche where sufficient supplies of C might exist to support this type of organic acid-driven Pi solubilization. This report utilizes a stress physiology paradigm. The alkaline desert soil chosen for this study had an undetectably low level of soluble Pi but a very high level of mineral (calcium) phosphate. Under these soil conditions, the rapid biomass accumulation shown by the annual plant *Helianthus annus jaegeri* presumably required highly efficient Pi solubilization and/or acquisition mechanisms which may include one or more unique rhizosphere populations of Pi solubilizing microorganisms.

2. Materials and methods

2.1. Sample collection and Pi determination

Plant root and soil samples were collected on 24 August, 1994 at the California State University Desert Studies Center located in the Soda Springs (Zzyzx) region of the California Mojave National Scenic Area. Samples were collected approximately 3 km north of the California State University field station along the western edge of Soda Dry Lake (35° 10′ north, 116° 7′ west). Soda Dry Lake is the sink of the Mojave River and occasionally receives its flood waters. Much of the sediments of Soda Dry Lake are saturated with ground water and develop an efflorescence of alkaline salts from evaporation (typical of such ‘wet’ playas), making most of its surface inhospitable to vascular plants. Plant growth occurs only at the extreme outer edge of the lake.

For both root and plant-free soil samples, the visible efflorescence of alkaline surface salts was removed to a depth of approximately 2 cm. Root samples were taken from the last plant community before reaching the interior plant-free zone. At this site, plant growth consisted entirely of a mixed community of *Helianthus annus jaegeri* (annual sunflower) growing among *Distichlis spicata stricta* (salt grass). Roots of five *Helianthus annus jaegeri* plants were dug to a depth of 15 cm. The roots were taken by loosening the soil around the root and then gently removing the plant material from the ground. The roots were shaken to remove loose soil, this rhizosphere soil was retained for pH measurement. The roots were placed in a sterile paper bag and put on ice. A soil sample was taken at a distance of 1 m further into the center of the dry lake, i.e. inside the plant-free zone. A bulked sample was collected from a depth of 2 cm to 15 cm. There was no visible root material in this sample. The soil was bagged and placed on ice.

All samples were held at 4°C for approximately 24 h before further treatment. After washing (described below), a root dry weight of 7.81 g was de-

termined by placing the roots at 140°C for 24 h. Plant-free soil used for mineral nutrient analysis was air dried for 1 week and passed through a 200 mesh filter. To determine water soluble Pi, 50 g of soil was shaken with 150 ml of water at 200 rpm for 24 h. The soil was removed by low speed centrifugation. The supernatant was 0.2 micron filtered and 10 ml of this solution was concentrated 10× via Savant speed-vac and used to determine water soluble Pi via the method of Bencini et al. [8]. A dry soil sample was sent to the J.R. Simplot Inc. Fertilizer Division R and D Facility (Pocatello Idaho) for analysis of total P and total Ca.

2.2. Isolation of rhizobacteria

All samples were handled in the bioguard hood using sterile technique. Roots were rinsed briefly with sterile deionized water water and then placed in a 500 ml Erlenmeyer flask and shaken in 200 ml of sterile DI water at 50 rpm for 1 h to further remove soil. The roots were transferred to 200 ml of water and shaken at 200 rpm for 24 h. This solution (intensive wash solution) was decanted and microbial populations estimated via serial dilution plating on glucose minimal hydroxyapatite indicator medium as previously described [6,9]. A soil sample from the plant-free zone (10 g) was also shaken with 200 ml of water at 200 rpm for 24 h, the soil was pelleted by low speed centrifugation (200 rpm, 5 min) and the supernatant used for dilution plating.

2.3. pH measurements

The pH of rhizosphere soil and soil from the plant-free zone was measured by placing a pH electrode into a saturated paste made by mixing soil with DI water. The pH of the intensive wash solution was measured directly.

2.4. Concentration of the intensive root wash solution

This solution was filtered through a 0.2 micron filter and concentrated to 1/10 volume via the Savant speed-vac. This material was divided into two 10 ml aliquots. One (untreated) was placed directly into −80°C. The other (treated) was subjected to proteinase K digestion (10 μg ml^{-1}, 37°C, 30 min), followed by boiling for 5 min and then placed at −80°C.

2.5. Assay of the mineral phosphate solubilizing (MPS) phenotype and gluconic acid production

The MPS phenotype was assayed visually by production of clearing zones on glucose minimal hydroxyapatite indicator medium as previously described [6,9]. Gluconic acid production was assayed enzymatically as previously described [9].

2.6. Bacterial growth studies

Liquid culture studies were conducted as previously described with slight modifications [6,9]. Cultures of *E. coli* strain HB101 (grown at 37°C) or the strain isolated from the plant roots and designated as AG205 (grown at 30°C) were grown overnight in rich medium (LB medium) [6]. 0.1 ml was then inoculated into 50 ml of 1% glucose minimal medium made up in water or 45 ml water plus 5 ml concentrated intensive root wash solution (treated or untreated). PQQ (generously provided by Professor J.A. Duine, Delft Technical University) was added to a final concentration of 10 nM.

2.7. DNA hybridization experiments

DNA was extracted from the rhizobacterial isolate and characterized via Southern hybridization as previously described [5].

3. Results and discussion

Dilution plating of the intensive root wash solution on LB medium resulted in growth of microbial colonies of only one apparent morphotype. This microbial population was present at approximately 2.7×10^8 cfu per gram of dry root. Because root samples from five plants were bulked, only a single set of dilution plates were made. The population was calculated from both the 10^4 and 10^6 dilution plates which gave similar results. Ten of the 104 colonies on the 10^4 dilution plate were streaked to single colony and tested by Gram-staining. All ten isolates were Gram-negative rods. One of these isolates was

designated as AG205. This culture was sent to ATCC for identification and was typed via fatty acid profile (FAME) as *Enterobacter cloacae*.

No growth was observed on the dilution plates made from the plant-free soil wash solution. The pH of a saturated paste of bulk soil measured 9.97. The pH of a saturated paste of rhizosphere soil measured 10.01. The pH of the intensive wash solution measured 6.45 (vs. 7.3 for the DI water control). No Pi was detected in the water extracted soil samples. Analysis of bulk soil showed 0.08% P and 3.4% Ca by mass.

We and others have used dissolution of hydroxyapatite on indicator plates as a preliminary screening system for the assay of the MPS phenotype (reviewed in [1]). We have further shown that, in Gram-negative bacteria, highly efficacious hydroxyapatite solubilization corresponds to gluconic and 2-ketogluconic acid production via the direct oxidation pathway. Depending on growth conditions, an efficient MPS^{+} bacterium such as *Pseudomonas cepacia* or *Erwinia herbicola* can oxidize 50% or more of the glucose present in a 1% glucose minimal medium (1% glucose = 50 mM). AG205 showed no significant MPS phenotype on hydroxyapatite plates and, as expected, did not produce a detectable amount of gluconic acid when grown on 1% glucose minimal medium. Addition of either 10 nM PQQ or 10% concentrated intensive wash solution resulted in production of high levels of gluconic acid (Table 1).

One can bioassay for the presence of PQQ using *E. coli* K-12 as a sensor system. *E. coli* K-12 and its derivatives (including HB101) constitutively expresses the *gcd* gene which codes for the glucose dehydrogenase apoenzyme (apoGDH). However, K-12 does not synthesize PQQ ([6] and references therein). Therefore, in the absence of exogenous PQQ, apoGDH is non-functional. With the addition of exogenous PQQ, K-12 produces detectable amounts of gluconic acid [9]. Effective binding of exogenous PQQ is presumably simplified by the location of the apoenzyme on the outer face of the cytoplasmic membrane.

As shown in Table 1, *E. coli* HB101 did not produce gluconic acid when grown with 10% intensive wash solution whereas AG205 produced 23 mM gluconic acid under these conditions. Therefore, the unknown bioactive component is apparently not PQQ. Since AG205 showed similar gluconic acid production levels in the presence of both the unknown bioactive component and 10 nM PQQ, it is reasonable to propose that the bioactive component of the concentrated wash solution functioned via induction of PQQ biosynthesis or release of some endogenous pool of preexisting PQQ. The same effect was observed if the concentrated wash solution was sequentially exposed to proteinase K and boiling for 5 min.

Fig. 1 shows that AG205 does, in fact, carry a gene with homology to the highly conserved region of the quinoprotein glucose dehydrogenase gene *gcd* of *E. coli*. As discussed by Goldstein [5], this highly conserved region has been shown to be present in all nine quinoprotein dehydrogenase genes cloned and sequenced to date.

A bioenergetic role for the direct oxidation pathway has been shown for some bacterial genera [10], however in other bacteria it has not been possible to attribute an essential physiological role for this pathway giving rise to the term 'dissimilatory bypass'. For example, a *P. cepacia* mutant lacking glucose dehydrogenase activity was isolated by Lessie et al. [11]. This mutant grew as well as the wild-type with glucose as the carbon source. Other bacterial species have been identified that incompletely oxidize glucose to gluconic or 2-ketogluconic acid in the periplasmic space but fail to transport and assimilate the oxidized substrate ([4] and references therein).

We have suggested that acidification of a microniche might be an effective competitive strategy in some ecosystems [4,5]. The work presented here sug-

Table 1
Production of gluconic acid by AG205 or *E. coli* HB101 grown in 1% glucose minimal medium

Bacterium	Blank	+10 nM PQQ	Intensive wash solution (untreated)	Intensive wash solution (treated)
E. coli HB101	undetected	2.7 mM, S.D. = 0.5 mM	undetected	undetected
AG205	undetected	18.0 mM, S.D. = 0.7 mM	22.2 mM, S.D. = 1.1 mM	23.1 mM, S.D. = 1.3 mM

The detection limit of the enzyme assay system was ~0.25 mM. All data represent the mean of at least three independent experiments. The blank contains the appropriate bacterial strain but no additions of PQQ or intensive wash solution.

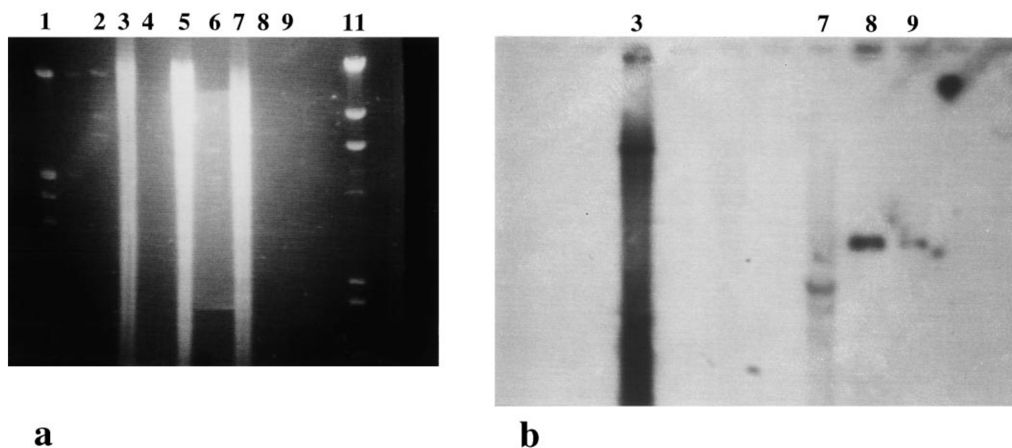

Fig. 1. Southern blot of isolated DNA from AG205, *E. coli* HB101, and *P. cepacia*. The *P. cepacia* DNA is included as a negative control since, to date, we have not been able to use any part of the *E. coli gcd* gene as a heterologous probe to identify the quinoprotein GDH gene in *P. cepacia* even though this organism produces gluconic acid via the direct oxidation pathway. a shows the ethidium bromide-stained gel and b shows the corresponding Southern blot.

Lane #	Sample
1	λ *Hin*dIII markers (0.5 μg)
2	λ *Hin*dIII markers adjusted to give fragment numbers ∼equal to single-gene copy numbers expected for 10 μg of fully digested *E. coli* DNA
3	10 μg *Bam*HI-digested *E. coli* HB101 DNA
4	Blank
5	10 μg *Bam*HI-digested *P. cepacia* DNA
6	Blank
7	10 μg *Bam*HI-digested AG205 DNA
8	*Bam*HI-digested pGP478 carrying the *E. coli gcd* gene; adjusted to give ∼equal single-gene copy numbers expected for 10 μg of fully digested *E. coli* DNA
9	*Bam*HI-digested pGP478 carrying the *E. coli gcd* gene; adjusted to give ∼equal single-gene copy numbers expected for 1 μg of fully digested *E. coli* DNA
10	Blank
11	λ *Hin*dIII markers (0.5 μg)

gests that this strategy may involve some form of signaling between the plant and its associated rhizobacteria. There are, of course, alternative explanations for our data. However, since the bioactive agent mimics PQQ, the results presented here suggest regulation or induction of PQQ biosynthesis. Induction of PQQ biosynthesis is clearly an effective mechanism for regulation of the direct oxidation pathway. The probability that this is a specific rhizosphere phenomena is strengthened by the fact that no equivalent bacteria were isolated from plant-free soil 1 m away. It is interesting to note that recent work in the corresponding author's lab has shown that two of four rhizobacterial isolates from India selected for extremely high MPS capacity were typed as *E. cloacae* by ATCC (manuscript in preparation).

According to Hausenbuiller [12] the continental United States may be conveniently divided into four categories with respect to the P content in the surface foot of soil. Our measured value of 0.08% P puts this Mojave Desert soil in the highest category (0.066–0.130). As expected, given a pH of 10.0 and the relatively high levels of Ca, there was no detectable Pi. In this ecosystem it is reasonable to propose that, during a period of rapid plant biomass accumulation, specific stimulation of MPS^+ bacteria in the rhizosphere could provide a competitive advantage by increasing available Pi. Acidification of the root surface may have additional benefits with respect to plant growth in this highly alkaline soil.

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