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Evidence for gluconic acid production by *Enterobacter intermedium* as an efficient strategy to avoid protozoan grazing

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ABSTRACT

The effect of pure gluconic acid and of gluconic-acid-producing bacteria on the activity of three protozoan species, *Colpoda steinii* (a ciliate), *Vahlkampfia* sp. (an amoeba) and *Neobodo designis* (a flagellate), was determined *in vitro* and in soil microcosms. Pure gluconic acid was shown to mediate disappearance of active cells, due to encystment and/or death of protozoa, at 0.15 mM in saline medium. Similarly, the presence of gluconic acid inhibited excystment of the three protozoa tested. *Enterobacter intermedium* 60-2G (Wt), a gluconic acid-producing rhizobacterium, elicited the same effects on protozoa when co-cultured in the presence of 5 g L^{-1} glucose. However, the effect was not observed when glucose was omitted from the medium. Similarly, a *pqqA* isogenic mutant strain, unable to produce gluconic acid from glucose, exhibited a reduced effect on protozoan activity. Rhizosphere-microcosm studies performed with wheat (*Triticum aestivum* L.) confirmed the reduced ability of the *pqqA* mutant to limit protozoa reproduction compared to the Wt strain. Since the sodium salt of gluconic acid did not cause any significant stress to protozoa and considering that addition of 50 mM Tris–Cl (pH 7.2) abolished the deleterious effect of gluconic acid, acidification of the medium appeared as the key factor that induced encystment/death of protozoa. We propose that production and excretion of gluconic acid should be considered an efficient mechanism evolved by bacteria to escape, tolerate or defend themselves against protozoan grazing in rhizosphere environments.

1. Introduction

Gluconic-acid-producing bacteria are widespread in soil environments. These bacteria have been thoroughly studied mainly because they participate actively in mobilizing phosphorus (P) between sparingly soluble mineral phosphates and the soil solution, thus enhancing the fertility of P-deficient soils (Rodríguez and Fraga, 1999). The metabolic and genetic basis of this so-called MPS$^+$ phenotype (for Mineral Phosphate Solubilization) has been identified as the direct oxidative pathway of glucose (Goldstein, 1995, 2007). Enzymes of this pathway (*e.g.* membrane dehydrogenases) are oriented to the outer face of the cytoplasmic membrane, and thus are capable of oxidizing their substrates in the periplasmic space. Consequently, the organic acids produced through the direct oxidative pathway of glucose (gluconic and 2-ketogluconic) may diffuse freely through outer membrane porins, acidifying the external milieu.

Having considered that for many bacterial species there is no obvious bioenergetic role for this pathway (an example of dissimilatory bypass), it has been suggested that the direct oxidative pathway of glucose might play an ecophysiological role in the rhizosphere (Goldstein, 1995). In such a context, bacteria would trade soluble P (mobilized from the insoluble pool by actively oxidizing glucose to gluconic acid in the periplasm) for carbon compounds excreted by plants through their roots (rhizodeposits). Nevertheless, from an evolutionary perspective, a benefit in the form of increased exudation would be too unspecific to select for bacterial traits which benefit plants upon which bacteria depend (Denison et al., 2003; Kiers and Denison, 2008). On the other hand, these rhizodeposits would also benefit potential competitors.

Consequently, we hypothesized that there should exist a direct feedback between gluconic acid production/excretion and bacterial

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fitness improvement. One possibility is that either glucose oxidation or gluconic acid production/excretion might displace competitors. Indeed, it has been shown that several gluconic acid-producing (MPS^+) bacterial species inhibit the growth of both bacterial and fungal phytopathogens, thus rendering them biocontrol agents (Vassilev et al., 2006). Furthermore, it has been shown that gluconic acid produced by *Pseudomonas* species has a strong antifungal effect against *Gaeumannomyces graminis* var. tritici (Ggt), the etiologic agent of the take-all disease of wheat (Kaur et al., 2006).

Another possibility is that gluconic acid production and excretion might deter natural predators. In fact, predation is increasingly being recognized as an important selective force that determines traits relevant for bacterial survival and evolution, in addition to competition for nutrients and other limiting resources (Matz and Kjelleberg, 2005; Jousset et al., 2008). The predatory activities of soil protozoa are believed to have selected for bacterial phenotypes that are grazing-resistant (Matz et al., 2002; Matz and Jürgens, 2003). Among the strategies that bacteria evolved to face, avoid, and/or escape protozoan predation, the production of bioactive (toxic) metabolites is of paramount importance (Jousset et al., 2006, 2008).

We hypothesized that gluconic acid production and excretion by rhizospheric bacteria would elicit a deleterious effect on protozoan grazers either by inducing their encystment/lysis, by inhibiting their excystment or both. Therefore, the main objective of the present study was to examine the consequences of co-culturing *Enterobacter intermedium* 60-2G, a gluconic acid-producing bacterium, with active forms of three well known protozoan grazers, *in vitro* as well as in soil- and rhizosphere-microcosms.

2. Materials and methods

2.1. Organisms and culture conditions

The three soil protozoa and the bacterial strains used in this study are listed in Table 1. *Escherichia coli* and *E. intermedium* were grown on Luria-Bertani (LB) agar medium or LB broth with continuous agitation at 37 °C and 30 °C respectively. Appropriate filter-sterilized antibiotics were added to *E. intermedium* cultures (*pqqA* mutant and complemented *pqqA* mutant) at the following concentrations: kanamycin 50 μg mL^{-1}, tetracycline 25 μg mL^{-1}. For some experiments, a chemically-defined medium, National Botanical Research Institute's phosphate growth medium (NBRIP medium) [$MgCl_2 \cdot 6H_2O$ (5 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.25 g L^{-1}), KCl (0.2 g L^{-1}), $(NH_4)_2SO_4$ (0.1 g L^{-1})] (Nautiyal, 1999) was used, amended with 5 g L^{-1} glucose and 10 mM K_2HPO_4.

As protozoan predators we used *Neobodo designis* (a flagellate), *Colpoda steinii* (a ciliate) and *Vahlkampfia* sp. (an amoeba). The protozoa were fed with *E. coli* DH5α in Neff's modified amoeba saline (AS) (Page, 1988). Protozoa culture stocks were kept encysted at 4 °C on a suspension of *E. coli* DH5α cells containing 10^3–10^4 CFU mL^{-1} in AS. The protozoa were reactivated by co-culturing for several days, at 18–20 °C in the dark, in fresh suspensions of *E. coli* DH5α containing an initial density of 10^7–10^8 CFU mL^{-1}. For all experiments, active protozoa were taken from 3 to 5-day-old co-cultures.

2.2. Quantification of acid production

The total amount of acid produced in NBRIP minimal medium by stationary cultures of *E. intermedium* 60-2G and its mutant derivative strains was estimated using titration according to Schleissner et al. (1997). For this, *E. intermedium* strains were grown overnight with shaking at 30 °C, in 20 mL LB containing the respective antibiotics. Bacterial cells were pelleted in a bench centrifuge at 10,000 × *g* for 5 min, washed twice with NBRIP salts and resuspended in 100 mL NBRIP. This suspension was incubated on a shaker at 25 °C. Aliquots of 5 mL of each culture were collected after 8 h and 24 h growth and cells pelleted. After measuring the corresponding pH, the supernatant was titrated with 0.1 N sodium hydroxide (NaOH) using phenolphthalein as an indicator. The titration was repeated three times from each one of three replicate experiments and the amount of acid produced calculated accordingly. Gluconic acid was estimated from the same samples with the D-gluconic acid/D-glucono-D-lactone assay kit (R-Biopharm AG, Darmstadt) following the manufacturer's instructions.

Additionally, acid production by bacteria was estimated indirectly by monitoring the solubilization of $Ca_3(PO_4)_2$ in NBRIP plates. For this, 10 μL of overnight-grown cultures were spotted on top of NBRIP plates. The plates were incubated at 30 °C for 48 h and solubilization halos were photographically recorded.

2.3. Co-cultivation of active protozoa and bacteria

A series of batch culture experiments were performed i) *in vitro*, ii) in soil microcosms (see below), and iii) in rhizosphere-microcosms (see below) to examine the population response of the protozoa to the presence of *E. intermedium* 60-2G (wild-type strain), the *pqqA* mutant, the complemented *pqqA* mutant, and *E. coli* DH5α cells. For *in vitro* experiments bacterial cultures were grown overnight at 30 °C with continuous agitation in LB broth containing the corresponding antibiotics. Cells were harvested by centrifugation (10,000 × *g* for 5 min), washed twice with AS and resuspended in AS. Bacteria were mixed at a final density of 5 × 10^7 CFU mL^{-1} approximately with a suspension of active protozoa (5 × 10^3 cell mL^{-1}), for a total volume of 500 μL in 1.5 mL Eppendorf tubes. AS medium was either supplemented, or not, with glucose (range 0.6–10 g L^{-1}). On the other hand, the same experiments were conducted by supplementing the AS medium

Table 1
Bacterial strains and protozoa used in this study.

Strain	Description	Reference or source
Bacteria		
E. coli DH5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	
E. intermedium 60-2G	Wild-type strain isolated from grass rhizosphere in Korea. This strain exhibits MPS and PGPR activities. GA^+	Kim *et al.* (2002)
E. intermedium pqqA	Insertion mutant of strain 60-2G. Kanamycin resistant gene from the pRL648 plasmid inserted at *pqqA* gene of strain 60-2G. Kan^r, GA^-	Han *et al.* (2008)
E. intermedium pqqA(p46HB5)	*pqqA* mutant strain transformed with plasmid p46HB5 harboring *pqqA-E* genes of strain 60-2G. Kan^r, Tet^r, GA^+	Han *et al.* (2008)
Protozoa		
Vahlkampfia sp. And12	Unidentified lobose amoeba isolated from a pristine soil in Jaén, Spain; GenBank accession no. AY965862	Lara *et al.* (2007)
N. designis And31	Flagellate isolated from a pristine soil in Jaén, Spain; GenBank accession no. AY965872	Lara *et al.* (2007)
C. steinii Sp1	Ciliate isolated from a polycyclic aromatic hydrocarbon-contaminated soil in Jaén, Spain; GenBank accession no. DO388599	E. Lara (EPFL, Switzerland)