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Kawaguchi, Akira
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Graduate School of Bioresource and Bioenvironmental Sciences,Kyushu Unviersity

Akazawa, Takanori
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Graduate School of Bioresource and Bioenvironmental Sciences,Kyushu Unviersity

Sakai, Masao
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Faculty of Agriculture,Kyushu University

Kanawaza, Shinjiro
Laboratory of Soil Microbiology,Division of Soil Science and Plant Production,Department of Plant Resources,Faculty of Agriculture,Kyushu University

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Role of Ca Resistance in Competitive Survival of Fluorescent Pseudomonads in Soil with High Salinity

Akira KAWAGUCHI*, Takanori AKAZAWA*, Masao SAKAI†, and Shinjiro KANAZAWA

Laboratory of Soil Microbiology, Division of Soil Science and Plant Production, Department of Plant Resources, Faculty of Agriculture,
Kyushu University, Fukuoka 812–8581, Japan
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The Ca^{2+}–resistant strain HR2–6 of *Pseudomonas putida* was isolated from spinach root grown in a greenhouse in high salinity soil. Since Ca often accumulates in greenhouse soils in Japan due to many years of Ca applications, the role of Ca^{2+} resistance in the survival of the strain was investigated. To conduct the investigation, Ca^{2+}–sensitive mutants were obtained with UV–irradiation. The Ca^{2+}–sensitive mutant CAS–1 was found to grow as well as the wild type HR2–6 in a TSB medium without added Ca^{2+}, but with 150 mM and 200 mM Ca^{2+} added, the growth of the Ca^{2+}–sensitive mutants were 7– and 56–fold lower than that of the wild type.

In sterile spinach–greenhouse soil (a non–competitive environment), populations of the Ca^{2+}–sensitive mutant and wild–type strain were similar, but in nonsterile greenhouse soil (a competitive environment), populations of the Ca^{2+}–sensitive mutant were 112–fold lower than the wild type after 50 days. These data suggest that Ca^{2+} resistance can be an important factor in the survival of *P. putida* in soil that has an accumulation of Ca.

INTRODUCTION

The successful colonization of the rhizosphere by an introduced beneficial bacteria usually requires that the bacteria not only be well adapted to the rhizosphere, but that it also have some selective advantage over the many indigenous bacteria with the potential to colonize that rhizosphere. Characteristics that may enhance the establishment and/or survival of introduced bacteria include a high growth rate relative to that of the indigenous microbial population (Bowen and Rovira, 1976), resistance to adverse environmental conditions (Polonenko *et al.*, 1981; Chan and Alexander, 1983; Loper *et al.*, 1985) or starvation (Acea *et al.*, 1988), cell motility (de Weger *et al.*,1987), the production of substances that aid in adherence to plant roots (van Peer *et al.*, 1990; Tari and Anderson, 1988), and the production of antibiotics (Mazzola *et al.*, 1992).

Intensive plant cultivation systems such as those found in greenhouses have generally resulted in soil salinization due to the high dose rates of chemical fertilizers (Souma, 1988). A high concentration of inorganic salts accumulating in the plant rhizosphere is likely to be a factor in salinity stress on both the roots and the roots–associating rhizobacteria. In a previous paper, we observed that soil salinization had induced a

* Laboratory of Soil Microbiology, Division of Soil Science and Plant Production, Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University
† Corresponding author (E–mail: msakai@agr.kyushu–u.ac.jp)

significant change in the bacterial populations, especially those of fluorescent pseudomonads, in the soil–root system (Matsuguchi and Sakai, 1995). Furthermore, our previous study suggested that among the inorganic ions accumulating in the soil, Ca^{2+} is the most critical factor for the growth of fluorescent pseudomonad isolates in the culture medium (Sakai *et al.*, 1995). Due to many years of Ca application, greenhouse soils in Japan often become accumulated with Ca.

The objective of this study was to determine whether Ca^{2+} resistance was important for the survival and competitiveness of these bacteria in Ca–accumulated soil.

MATERIALS AND METHODS

Isolation of Ca^{2+}–sensitive mutants

A parent and its mutant strain, which was Ca^{2+} sensitive, were compared for their survival in greenhouse soil with high salinity. To perform the comparison, UV–irradiated cells were screened for Ca^{2+}–sensitive mutants by replica plating on a modified tryptic soy (MTS) agar medium and MTS agar medium containing 200 mM $CaCl_2$. The MTS medium contained bacto tryptone 17 g, bacto soytone 3 g, and bacto dextrose 2.5 g per liter. The pH was adjusted to 7.3 with 1.0 M NaOH. The *Pseudomonas putida* HR2–6 was used as the Ca^{2+}–resistant wild–type strain in this study. *P. putida* HR2–6 was isolated from spinach root grown in greenhouse soil that contained high salinity (Sakai *et al.*, 1995). The Ca^{2+}–sensitive mutants were selected, and the effect (response) of Ca^{2+} on their growth was further determined. The growth responses of the strains were tested in MTS supplemented with $CaCl_2$, KCl, or NaCl as previously described (Sakai *et al.*, 1995).

Marking of *P. putida* strain with kanamycin resistant (Km^r) gene

P. putida strains consisting of the parental strain and its Ca^{2+}–sensitive mutant were marked with a Km^r gene for the nonsterile soil experiments (the competitive environment). The strains marked with a Km^r gene were prepared as follows, using a Km^r–loaded transposon vector pJFF350 (Fellay *et al.*, 1989). *E.coli* S17–1 containing the pJFF350 was used as a donor in matings with the *P. putida* strains. The donor *E.coli* harboring pJFF350 and the recipient *P. putida* cells were separately grown in LB medium and harvested in the early log phase. The cells were washed in a 10 mM phosphate buffer (pH 7.0) solution and suspended in the same buffer solution at a cell density of 10^9 CFU/mL. The *P. putida* cells were then heat–shocked for 10 min at 52 °C to improve the recovery of exconjugants. Equal volumes of each cell suspension were mixed quickly and thoroughly, and 50 μL aliquots of the mixture were spotted on LB plates, allowed to dry in the air, and then incubated overnight at 28 °C. The bacterial cells from each spot were then resuspended in the phosphate buffer solution, and the appropriate dilutions were plated on P–1 plates (Katoh and Itoh, 1983) containing kanamycin at 50 mg/L. Most of the *E. coli* cells died when grown on P–1 plates, and only the Km^r *P. putida* cells survived.

Soil preparation and bacterial inoculation

The Gray Lowland soil with high salinity used in this experiment was collected from a spinach cropping greenhouse in Fukuoka, Japan. Some of the characteristics of this soil

have been described in previous papers (Matsuguchi and Sakai, 1995; Sakai *et al.*, 1995). The soil was sieved through a 2–mm pore–size screen before being used. A portion of the soil was sterilized by autoclaving 500–mL screw–cap bottle (Corning Glass Works, Corning, N.Y.), each with 100 g of soil for 40 min. Test bacteria were grown in MTS at 28 °C to the mid–log phase. The cells were then centrifuged at 6,000×g for 7 min, and resuspended in sterile distilled water to a density of 10^9 CFU/mL. A one mL portion of the bacterial suspension was added to 100 g of soils contained in the screw–cap bottles. The bacterial suspension was mixed thoroughly in the soil. This brought the soil to a final bacterial density of 10^7 CFU per g of soil. The final water content of the soil was adjusted to 40% of the water–holding capacity. The bottles were left at 20 °C in the dark. The caps of the bottles were further sealed with Parafilm, and the water loss from samples, though minimal, was routinely checked by changes in the sample weight and adjusted with sterile distilled water when necessary.

At different time intervals, a 3–g sample was taken from each bottle, and the number of bacteria was determined by decimal dilution of the soil with sterile distilled water and plating of 0.1 mL of each soil suspension on triplicate plates of P–1 agar containing 50 μg of kanamycin per mL. After 48 h of incubation at 28 °C, the colonies on plates with 20 to 200 colonies were counted. A survival comparison of a Ca^{2+}–resistant strain, HR2–6 (Km^r), and a Ca^{2+}–sensitive mutant, CAS–1 (Km^r), were inoculated individually into spinach greenhouse soil and sterile spinach greenhouse soil to investigate the competitive effects with other microbes. Each treatment consisted of three replicates.

RESULTS

Mutant isolation and characterization

After UV mutagenesis, three out of ca 8,000 colonies of strain HR2–6 recovered on MTS agar failed to grow when transferred to MTS agar supplemented with $CaCl_2$. Strain CAS–1 was the most sensitive, since it showed the inhibition of growth at the lowest Ca^{2+} concentration.

The growth response of the Ca^{2+}–sensitive mutant CAS–1 to various concentrations of $CaCl_2$ in MTS medium is shown in Fig. 1. For the strain CAS–1, Ca^{2+} was so toxic that it completely inhibited the growth at 150 mM $CaCl_2$. However, the growth of its parental strain HR2–6 was little affected at 150 mM $CaCl_2$. The threshold of growth inhibition for strain CAS–1 by $CaCl_2$ was observed at concentrations as low as 100 mM, and a complete inhibition was observed at 150 mM or higher. In contrast, the growth of its parental strain, HR2–6, was tolerant to $CaCl_2$ even at the concentration of 200 mM. In addition, the growth of strains HR2–6 and CAS–1 showed no differences in the MTS medium with high concentrations of KCl and NaCl (Fig. 2). Since strain CAS–1 showed specific sensitivity to Ca^{2+}, it was used for the survival studies.

Survival in spinach greenhouse soil

In sterile spinach greenhouse soil (the non–competitive environment), the population levels of the Ca^{2+}–resistant strain HR2–6 and the Ca^{2+}–sensitive mutant CAS–1 showed no significant differences over a 50–day period (Fig. 3). The populations of the Ca^{2+}–resistant strain HR2–6 and the Ca^{2+}–sensitive mutant CAS–1 were 3.9×10^8 and 2.5×10^8

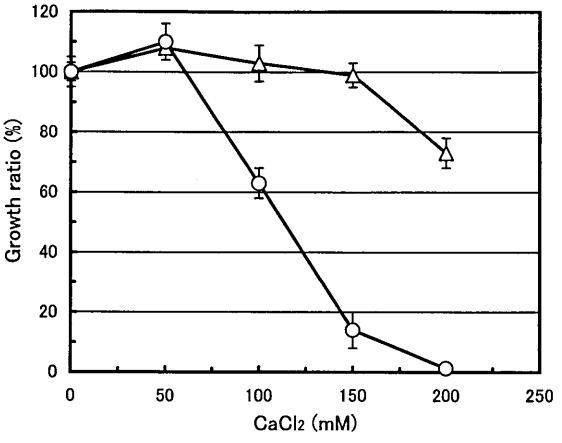

Fig. 1. The effect of different concentrations of $CaCl_2$ on the growth of *P. putida* Ca^{2+} -resistant strain HR2-6 (—△—) and Ca^{2+}-sensitive mutant CAS-1 (—○—). The optical densities of the cultures were measured after 20 h of growth. Vertical lines indicate the SE of the means of three replicates.

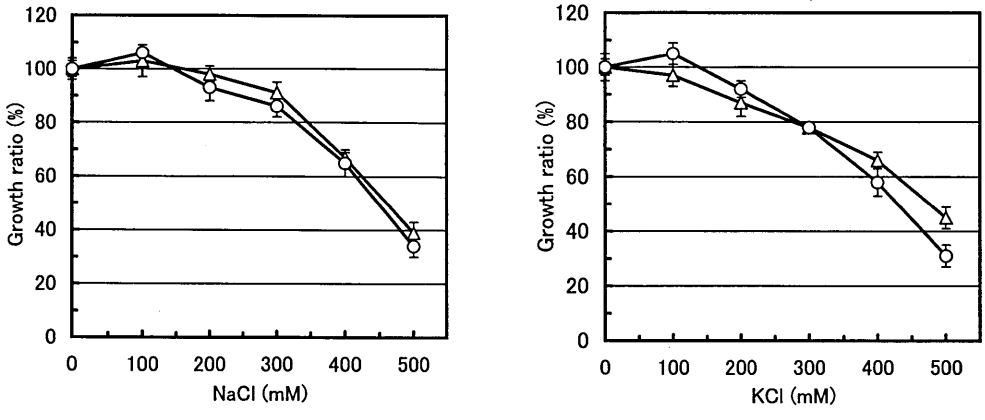

Fig. 2. The effect of different concentrations of NaCl and KCl on the growth of *P. putida* Ca^{2+}-resistant strain HR2-6 (—△—) and Ca^{2+}-sensitive mutant CAS-1 (—○—). The optical densities of the cultures were measured after 20 h of growth. Vertical lines indicate the SE of the means of three replicates.

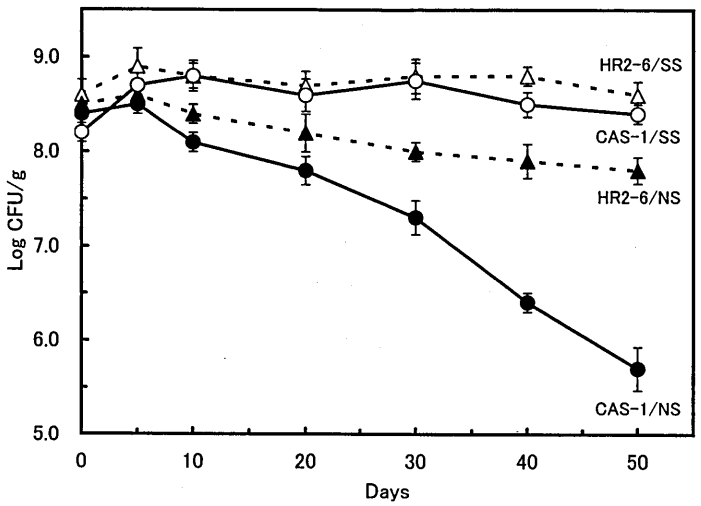

Fig. 3. Differential survival of Ca^{2+}-resistant strain HR2-6 and Ca^{2+}-sensitive strain CAS-1 of *P. putida* in sterile (SS) versus nonsterile (NS) soil from a spinach greenhouse. Strains were established in the soil at initial populations of approximately 10^8 CFU/g of soil. Vertical lines indicate the SE of the means of three replicates.

CFU/g of soil, respectively, 50 days after the bacteria were added. In the nonsterile soil (competitive environment), however, the population of the Ca^{2+}-resistant strain HR2-6 was 112-fold higher than that of the Ca^{2+}-sensitive mutant CAS-1 (Fig. 3). The populations of the Ca^{2+}-resistant strain declined less than 5-fold over a 50-day period, from 3.2×10^8 to 6.3×10^7 CFU/g of soil. However, the populations of the Ca^{2+}-sensitive mutant declined more than 500-fold during the same period.

DISCUSSION

Root colonization by introduced PGPR is important for biological control of root pathogens (Weller, 1988; O'Sullivan and O'Gara, 1992). However, the maintenance of populations and the growth of PGPR in the soil should also be important for long-term root colonization and biological control.

The population dynamics of the Ca^{2+}-resistant parental strain and its Ca^{2+}-sensitive mutant in the spinach-greenhouse soil were compared. In the sterile soil (non-competitive environment), where more pronounced differences were expected due to the influence of the high Ca concentration in the soil, the differences between strains were not very clear. The parental strain showed, though only in the nonsterile soil (competitive environment), a significantly higher final population size than that of the Ca^{2+}-sensitive mutant. These data suggest that Ca^{2+} resistance can be an important factor in the competitive survival of *Pseudomonas* strains in soil accumulated with Ca^{2+}. Calcium has been widely used as a liming (for correcting acidic soil) in agriculture for

many years, and in some areas, Ca^{2+} has accumulated to levels that are toxic to some microorganisms (Sakai *et al.*, 1995). The Ca^{2+} resistance in *P. putida* HR2–6 may have evolved to overcome the selective pressure caused by many years of Ca^{2+} applications in intensive cropping soils.

Bacterial systems for regulating inorganic–ions uptake can be important factors in competition with other microbes and in their survival when the inorganic ions are either limiting or present at toxic levels (Silver and Walderhaug, 1992). Calcium is an essential element that is also toxic at high levels (Norris *et al.*, 1991; Onek and Smith, 1992). Bacteria exposed to toxic levels of Ca^{2+} have evolved a number of mechanisms to regulate Ca^{2+} uptake and resist Ca^{2+} toxicity (Gangola and Rosen, 1987; Ivey *et al.*, 1993). Investigations of the physiological effects of high Ca^{2+} levels on fluorescent pseudomonads and the characterization of their Ca^{2+}–resistance mechanisms are in progress.

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