Mechanism of acid tolerance in a rhizobium strain isolated from *Pueraria lobata* (Willd.) Ohwi

Zhang Lei, Gu Jian-ping, Wei Shi-qing, Zhou Ze-yang, Zhang Chao, and Yu Yongxiong

Abstract: The *Rhizobium* sp. strain PR389 was isolated from the root nodules of *Pueraria lobata* (Willd.) Ohwi, which grows in acidic (pH 4.6) yellow soil of the Jinyun Mountains of Beibei, Chongqing, China. While rhizobia generally have a pH range of 6.5–7.5 for optimum growth, strain PR389 grew in a liquid yeast extract – mannitol agar medium at pH 4.6, as well as in a pH 4.1 soil suspension, suggesting acid tolerance in this specific strain of rhizobium . However, at pH 4.6, the lag phase before vigorous growth was 40 h compared with 4 h under neutral conditions (pH 7.0). For PR389, the generation time after the lag phase remained the same at different pH levels despite the different durations of the lag phase. Except in the pH 4.4 treatment, the pH of the culturing media increased from 4.6, 4.8, 5.0, and 5.5 to neutral and slightly alkaline after 70 h of culture. Chloramphenicol was added to determine if protein production was involved in the increasing pH process. Chloramphenicol significantly inhibited PR389 growth under acid stress but had little effect under neutral conditions. Proton flux measured during a short acid shock (pH 3.8) revealed that this strain has an intrinsic ability to prevent H^+ from entering cells when compared with acid-sensitive rhizobia. We propose that the mechanism for acid tolerance in PR389 involves both intracellular and extracellular processes. When the extracellular pH is lower than pH 4.4, the cell membrane blocks hydrogen from entering the cell. When the pH exceeds 4.4, the rhizobium strain has the ability to raise the extracellular pH, thereby, potentially decreasing the toxicity of aluminum in acid soil.

Key words: rhizobium, *Pueraria lobata* (kudzu), screening, acid tolerance mechanism, intracellular and extracellular process.

Résumé : La souche de *Rhizobium* sp. PR389 a été isolée de nodules racinaires de *Pueraria lobata* (Willd.) Ohwi, qui croissait sur un sol jaune acide (pH 4,6) dans les montagnes Jinyun de Beibei, Chongqing, Chine. Alors que la croissance optimale des rhizobiums se déroule dans un spectre de pH de 6,5 à 7,5, PR389 croissait dans le milieu liquide YMA à pH 4,6 ainsi que dans une suspension de sol à pH 4,1, suggérant que cette souche spécifique de *Rhizobium* soit tolérante au milieu acide. À pH 4,6 cependant, la phase de latence qui précède la croissance vigoureuse était de 40 h, comparativement à 4 h en condition neutre (pH 7,0). Le temps de génération après la phase de latence de PR389 demeurait le même à différentes valeurs de pH malgré la différence des temps de latence. À l'exception du pH 4,4, le pH du milieu de culture augmentait de 4,6, 4,8, 5,0 et 5,5 à pH neutre ou légèrement alcalin après 70 h de culture. Le chloramphénicol a été ajouté afin de déterminer si la production de protéines était impliquée dans le processus d'augmentation du pH. Cet ajout a inhibé de façon significative la croissance de PR389 en condition acide, mais avait peu d'effet à pH neutre. Le flux de protons mesuré lors d'un choc acide de courte durée (pH 3,8) a révélé que cette souche possédait la capacité intrinsèque d'empêcher l'entrée de H^+ dans les cellules comparativement aux rhizobiums sensibles à l'acidité. Nous proposons que le mécanisme responsable de la tolérance au milieu acide chez PR389 implique des processus tant intracellulaires qu'extracellulaires. Lorsque le pH extracellulaire est inférieur à 4,4, la membrane cellulaire bloque l'entrée d'hydrogène dans la cellule. Lorsque le pH est plus grand que 4,4, la souche de rhizobium possède la capacité d'augmenter le pH extracellulaire et ce faisant, diminue potentiellement la toxicité de l'aluminium du sol acide.

Mots-clés : rhizobium, *Pueraria lobata* (kudzu), criblage, mécanisme de tolérance à l'acidité, processus intracellulaire et extracellulaire.

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Introduction

pH tolerance has been observed in cells of many microbes, such as *Escherichia coli*, lactic acid bacteria, and *Rhizobium* species. Several mechanisms for adapting to acidic environments are involved, including the use of proton pump systems such as F_1F_0 ATPase (Papadimitriou et al. 2007) or glutamic acid decarboxylase (Gandhi and Chikindas 2007) to increase the internal pH; the involvement of regulators such as the two-component signal transduction systems (Ventura et al. 2009); cell envelope alterations, such as that of lysylphosphatidylglycerol (Reeve et al. 2006) and alanylphosphatidylglycerol (Klein et al. 2009), which protect cells by regulating the cell's architecture, composition, stability, and activity. Furthermore the acid tolerance in *Sinorhizhobium meliloti* involves the sigma factor RpoH1, which leads to the expression of a series of proteins (Lucena et al. 2010). The biological membrane, which has a low proton conductance, is key regulating acid stress by excluding protons from low external pH environments.

Legumes, during N_2 fixation, take up an excess of cations and as a result excrete protons and organic acids to maintain an ionic balance. Depending on the legume species under cultivation (Tang 1998), decreases of pH have been observed in the rhizosphere by up to 2 units below that of the surrounding soils (Dakora and Phillips 2002; Cummings et al. 2006). However, rhizobia are commonly observed to be acid sensitive (Watkin et al. 2003; Slattery et al. 2004). Therefore, acidity has the potential to be an important stress factor for the bacteria that inhabit N_2-fixing legume rhizospheres, and many leguminous plants would benefit by associating with an acid-tolerant rhizobium species.

Acid tolerance has been reported in rhizobia from different plants, such as subterranean clover (*Trifolium subterraneum* L.) (Watkin et al. 2003), orange wattle (*Acacia saligna*) (Marsudi et al. 1999), and alfalfa (*Medicago* spp.) (Draghi et al. 2010). In our study, *Pueraria lobata* (Willd.) Ohwi, commonly known as kudzu, was chosen as the plant host for studying acid tolerance in rhizobia. Kudzu, a perennial deciduous liana legume, is broadly distributed geographically. It produces a deep root system and flourishing rootstocks in very poor and often acidic soils. Kudzu is planted in many regions throughout the world to prevent soil erosion (Wang 1998; Everest et al. 1999); however, in July of 1997, kudzu was declared a federal noxious weed by the United States Congress (Blaustein 2001). Multiple methods of eradicating kudzu involving herbicidal, chemical, and mechanical treatments are ongoing in the United States. Despite the current concern for the rapid spread of kudzu (Forseth and Teramura 1987), there are few studies on the root nodule bacteria associated with this invasive species. We investigated the possible relationship between the ability of kudzu to spread rapidly and the tolerance of its rhizobia to acid.

A rhizobium strain from the root nodules of kudzu growing in acid soils was screened, and its growth was examined under acidic conditions. Mechanisms of acid tolerance were investigated with respect to its ability to survive in acidic conditions.

Materials and methods

Isolation, purification, and identification of rhizobia strains

Three to five large ripe nodules were taken from the roots of kudzu growing in acidic soils (pH 4.6) of Jinyun Mountains at Beibei, Chongqing (altitude 650 m above sea level). Nodules were washed and immersed in 95% ethanol for 30 s. Then they were immersed in 0.1% $HgCl_2$ solution for 5 min to sterilize the nodules' surface. Following this treatment, they were washed with aseptic water 5–6 times to remove the $HgCl_2$. Sterilized flat-headed forceps were used to crush the nodules. A small amount of tissue or sap from the nodules was taken and inoculated onto a slant of yeast extract – mannitol agar (YMA, pH 7; see description in the following section) and incubated at a constant temperature of 28 °C (Vincent 1970).

A test tube was filled with aseptic water and glass beads, and a loop of the rhizobium colony grown on the slant of the YMA tube was transferred into the test tube and shaken to provide a homogeneous bacterial suspension. The suspension was then spread onto a plate of acidic Congo red (0.04 $g \cdot L^{-1}$) YMA medium (pH 4.6) and was incubated at a constant temperature of 25–28 °C (Lindström et al. 1985; Ausmees et al. 1999). The transparent, bulging colonies that did not absorb Congo red were chosen for microscopic examination. The same procedure was repeated until pure colonies of rhizobia strains were isolated. The colonies were stored in YMA for further identification.

Three steps were utilized to identify the rhizobia strains. First, we isolated visible patches of rhizobia and inoculated them in a nutrient broth (NB) (pH 7.0) and a YMA liquid medium and cultured them at 28 °C for 24–48 h. The isolates with clear NB and cloudy YMA liquid at the end of culture period needed a further check with a microscope and were compared with cells in both NB and YMA, since rhizobia are inhibited by high levels of amino acids (Jordan and Coulter 1965). When dyed by carbolfuchsin, very few rhizobium cells in NB were swollen and distorted compared with those in YMA, which were clouded by neat, short stick-shaped cells with nondyed parts, representing poly-(β)-hydroxybutyrate from the YMA. Isolates from these screening procedures were back-inoculated into kudzu plants. The plant seeds were germinated and planted in a filter paper folded in a circle in a tube of 100 mL of nitrogen-free nutrient solution with a pH of 7.0. Everything in the tube had been autoclaved before the kudzu seedlings were planted. There were two buds in each tube and five tubes for each strain. After inoculating the buds with the isolates, the tubes were covered with a cotton lid and wrapped in paper to maintain darkness for root growth inside the tubes while aboveground parts were exposed to light. The tubes were maintained in a phytotron at 25–28 °C under a 12–14 h photoperiod for 20 days. Nodules were removed when ripe and isolated again following the same procedures for purification and identification (but not back-inoculation). Through this path, the rhizobia isolated from *Pueraria* species strains PR386, PR387, PR388, PR389, and PR390 were identified, while the rhizobia of strain PR21 (an acid-sensitive strain from *P. lobata*) were isolated and kept by the Centre of Microbiology, Southwest University, Chongqing, China, since 2006.

Medium

Nutrient broth was a premade nutrient broth from Difco.

Yeast extract – mannitol agar (YMA) medium contained the following (per litre): 5 g yeast powder, 10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl (Vincent 1970), 17 g agar. In liquid YM there was no agar. During isolation, 10 mL of 0.4% Congo red was added to clearly distinguish colonies of rhizobium strains from others (Bastarrachea et al. 1988). To adjust the solution pH of the media, we used 0.1 mol·L^{-1} HCl and 0.1 mol·L^{-1} NaOH.

The nitrogen-limited culture solution used for back inoculation contained the following (per litre): 0.03 g $Ca(NO_3)_2$, 0.06 g $MgSO_4 \cdot 7H_2O$, 0.46 g $CaSO_4$, 0.136 g K_2HPO_4, 0.075 g KCl, 0.075 g $FeC_6H_6O_7$, and 1 mL of microelement balanced solution was added to 1 L of the above medium (Ahmed and Evans 1960). The microelement balanced solution contained the following (per litre): 2.86 g H_2BO_3, 1.81 g $MgSO_4$, 0.22 g $ZnSO_4$, 0.8 g $CuSO_4 \cdot 5H_2O$, and 0.02 g H_2MoO_4.

Acidic soil suspensions

Red soil samples (Y2) from Yuanmou County of Yunnan province, China, had a pH of 4.7, and yellow soil samples (C1, C5, C6, X3, and X4,) from Beibei, Chongqing, China, had pH values of 4.78, 4.20, 4.10, 4.60, and 4.50, respectively. Individual soil samples were mixed with water at a ratio of 1:5, and a soil soluble aluminum (Al) concentration was determined according to the methods of Barnes (1975) and Li et al. (2005). These soil suspensions were used as culture solutions for studying survival and multiplication of the PR389 in acidic soils with Al stress.

Pueraria lobata pot experiment in acidic soil (pH 4.6) with rhizobium inoculants

Greenhouse inoculation trials of five different *P. lobata* rhizobium isolations, PR386–PR390, were carried out by back inoculating to *P. lobata* in pots in combination with a control treatment without inoculation, resulting in six treatments in total, with each as an experimental treatment factor. Each treatment consisted of three pots. Pots were filled with 2 kg of acidic soil (pH 4.6) from the yellow soil zone in Jinyun Mountains (altitude 650 m above sea level) at Beibei, Chongqing, China. The top 2 cm of soil was removed from the sample site, and the soil was mined to a depth of 20 cm and passed through a 6.5 mm screen. Seeds of kudzu were surface sterilized in 2% sodium hypochlorite for 2 min, rinsed, and planted in sterile vermiculite. Planting began when the root radicles were 2 cm long. Seven planting holes were made in the wet soil to a depth of 2–3 cm; 1 mL of a turbid YM broth culture of the related isolations was placed in each planting hole. One millilitre of untreated YM broth was placed in holes for the control treatment pots. Radicles were placed in the planting holes. Plants were thinned to five per pot 1 week after planting (Singleton and Tavares 1986). Pots were arranged in the greenhouse in a randomized complete block design and supplied with nitrogen-free nutrient solution at 25–28 °C under a 12–14 h photoperiod (Gibson 1971) for 21 days. Nodulation rates (the number of plants developing nodules at 21 days postinoculation versus the number of plants inoculated by the same isolation), the 2 h air-dried mass of each plant plus its root, the mass of the nodules and the number of nodules per plant were measured when harvested.

To select the acid-tolerant rhizobial strains of *P. lobata*, data derived from the pot experiment were statistically compared between treatments with different inoculants. As shown below, strain PR389 was selected to be an acid-tolerant rhizobial strain from the other four stains and was chosen for further studies of acid tolerance.

Mechanism of acid tolerance of PR389

Growth of PR389 under batch cultures of different pH regimes solutions

After growing at 28 °C for 24 h, the isolate of PR389 was inoculated in YM solutions with pH values of 4.4, 4.6, 4.8, 5.0, 5.5, and 7.0. There were three flasks of 350 mL of YM culture solution for each pH level. After the inoculation, the flasks were shaker-cultured at 28 °C at 100 r·min^{-1}. Light absorbance of the culture solution was determined at 30 min intervals with a 722 photometer at a wavelength of 600 nm. The number of rhizobium cells was determined by plating at 2 h intervals starting from the inoculation. Generation times were calculated with eq. 1, where N_0 represents the number of bacteria at the commencement of the logarithmic phase, N_t is the number of bacteria in the logarithmic phase at time t, and G is the generation time (Mohamed et al. 2000). The growth curve was plotted using Microsoft Excel 7.0.

$$[1] \qquad G = \frac{t}{3.3(\lg N_t - \lg N_0)}$$

Growth of PR389 in chloramphenicol solutions

PR389 was cultured in a neutral culture solution (pH 7.0) to the mid-exponential phase ($OD_{600} = 1$) and inoculated (5% *v*/*v*) into four different culture solutions: (*i*) a neutral culture solution with 10 μg·L^{-1} chloramphenicol (NC+), (*ii*) a neutral culture solution (pH 7.0) without chloramphenicol (NC–), (*iii*) an acidic culture solution (pH 3.8) with 10 μg·L^{-1} chloramphenicol (AC+), and (*iv*) an acidic culture solution (pH 3.8) without chloramphenicol (AC–). The solutions were held in 300 mL flasks, replicated three times, and shaker-cultured at 28 ± 1 °C and 100 r·min^{-1} for 330 min. Living bacteria counts (CFU·mL^{-1}) were recorded every 30 min by plate-counting.

Proton flux assay during acid shock

Proton flux in cells is the movement of protons across the cell membrane, and thus, its value reflects the permeability of the cell membrane. The following proton flux assay was performed according to O'Hara and Glenn (1994).

For acid-shocked and non-acid-induced treatments, activated *Rhizobium* sp. strains PR389 and PR21 were separately shaker-cultured in a neutral culture solution (pH 7.0) for 36 h to the mid-exponential phase of the growth curve, followed by centrifugation and washing. Each strain had three sterilized tubes, and every tube contained 1–3 g (fresh mass) of living cells transferred in 50 mL of 0.1 mol·L^{-1} HCl solution to prepare the cell suspensions. The suspensions were stirred for ≥2 min in a water bath (28 ± 1 °C) until their pH was stable. Drops of 50 mmol·L^{-1} HCl were added to give a final pH of 3.4 ± 0.1. The pH of the suspensions was recorded at

Table 1. Performance of *Pueraria lobata* back-inoculated by different rhizobium strains.

Rhizobium strain	Average ADM of root per plant (g)	Average ADM of plant biomass per plant (g)	Nodulation rate (%)	Average nodule no. per plant	ADM of nodules per plant (g)
PR389	0.6730±0.0380a	2.3286±0.3900a	81.82a	3	0.0471a
PR390	0.6224±0.0920a	1.5143±0.5310b	58.33b	1	0.0286b
PR387	0.4873±0.0680a	1.4840±0.4740b	60.00b	1	0.0300b
PR386	0.4623±0.0540ab	1.4601±0.3700b	62.50b	1	0.0298b
PR388	0.4411±0.0520ab	1.2918±0.3440b	77.78a	3	0.0341b
Control[a]	0.0976±0.0140b	0.3518±0.0670c	0.00c	0	0.0000c

Note: ADM represents a 2 h air-dried mass after harvest. Each value is the average of 15 plants. Within a column, numbers followed by different letters are significantly different between treatments ($P < 0.01$).

[a]For the control treatment, 1 mL of untreated yeast mannitol broth was added to the planting holes before radicles were planted.

30 s intervals for 15 min. For acid-induced treatments, cell suspensions were prepared similarly, but instead of being stirred for ≥2 min, the cell suspensions were shaker-cultured for 1 h in a mild acid (pH 5.2) solution, then drops of 50 mmol·L^{-1} HCl were added to yield a final pH of 3.4 ± 0.1. The initial drop in pH (typically 0.5 pH units) was reversed as protons flowed across the membrane into the cytoplasm. The pH changes from these procedures were converted into proton flux (nmol·mg^{-1}·min^{-1}) by eq. 2.

$$[2] \qquad \text{proton flux} = \frac{\text{antilog (pH2)} - \text{antilog (pH1)}}{\Delta t \times m} \times 1000$$

where the proton flux is the proton value (nmol) passing the membrane of 1 mg of fresh cell biomass in 1 min, pH1 is the pH value at the start of the measurement period, pH2 is the pH value at the end of the period, Δt is the length (min) of the measurement period, and m is the mass (mg) of fresh cells of PR389. The plate-counting method was used to assay the percent survival of cells of both PR389 and PR21 after the acid induction and acid shock treatments. This method indirectly reflects the variation in permeability to protons among the different types of cell membranes and, hence, reflects the ability of the membrane to block H^+ moving towards the external pH.

Growth of PR389 under different pH regimes in soils suspensions

PR389 was cultured in a neutral culture solution to the mid-exponential phase and inoculated at a rate of 5% into the soil suspensions with different pH values and Al concentrations (see previous section — Acidic soil suspension). The strain in all treatments was shaker-cultured at 28 ± 1 °C and at 100 r·min^{-1} for 4 weeks, and the number of living bacteria (CFU/mL) was recorded by plate-counting every 30 min.

Data analysis

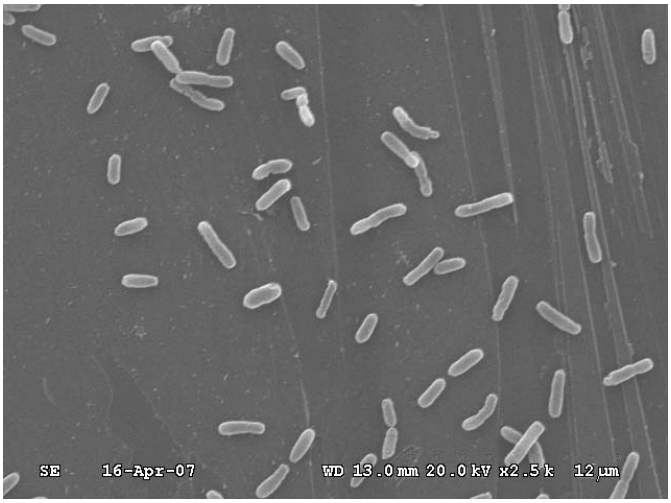
Analysis of variance (ANOVA) for the pot experiment, which includes six experimental factors — the control and five rhizobial strains of kudzu, PR386–PR390 — was performed by the software DPS version 3.01, a statistical package developed at Zhejiang University, China, and by SPSS version 19.0 (SPSS, Inc., Chicago, Illinois, USA).

Results

Morphological characterization of the rhizobium strains

Five strains, PR386, PR387, PR388, PR389, and PR390, from nodules of kudzu grew rapidly (within 3 days) on the acidic plates (pH 4.6). The colonies were translucent, lustrous, and round with a smooth margin. Microscopically, their cells were short and rod-shaped (0.63–0.71 μm by 1.24–1.84 μm) (Fig. 1) and were gram negative, having conspicuous ringed segments. All five isolations had little growth in the NB medium. If the cells did grow, they were swollen and distorted. After 20 days, PR386, PR387, PR388, PR389, and PR390 all formed nodules on kudzu in tubes of neutral N-free nutrient solution, verifying their nature of being rhizobial strains of kudzu.

Fig. 1. Cells of *Rhizobium* sp. strain PR389 from nodules of *Pueraria lobata*, as viewed under a transmission electron microscope.



In the acidic soil (pH 4.6) pot experiment, plant biomass, including nodule mass, of plants inoculated with PR389 was significantly higher than the biomasses of PR390, PR387, PR386, and PR388, as were the nodulation rates and nodule numbers for this treatment compared with most of the other strains ($P < 0.01$) (Table 1). PR389 had the best performance with a root and plant air-dried mass of 0.67 g and 2.33 g, which was 570% and 566% higher than the control, respectively ($P < 0.01$) (Table 1). The nodulation rate of PR389 was 81.82%, with three nodules per plant and a 0.0471 g air-dried mass of nodules per plant on average.

The differences in the average dry mass of *P. lobata* across the treatments were 14.084 and 6.101, respectively, which was highly significant ($F > F_{0.01}$), while the biomass of the plants in all the inoculated treatments was significantly higher than that of the control ($F > F_{0.01}$) (Table 2).

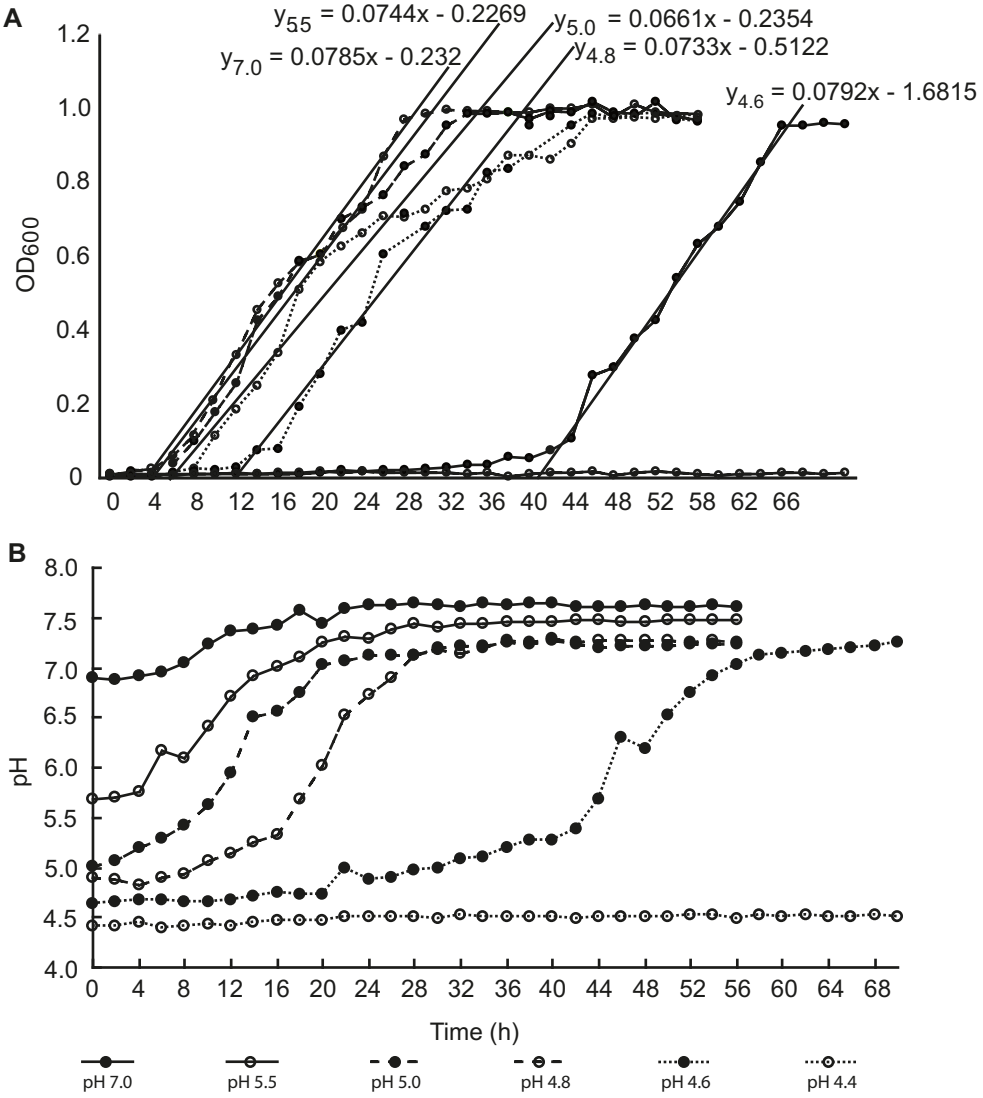
Table 2. Analysis of variance of the biomass of *Pueraria lobata* inoculated by different rhizobium strains.

	Source of variation	SS	df	Average variance	F^a	$F_{0.01}$
ADM of plant biomass (g)	Between treatments	5.9887	5	1.1977	14.084**	3.45
	Within treatment	1.0205	12	0.0850		
	Total variation	7.0093	17			
ADM of roots (g)	Between treatments	0.6124	5	0.1225	6.101**	
	Within treatment	0.2409	12	0.0201		
	Total variation	0.8533	17			

Note: ADM, air-dried mass 2 h after harvest. The result was analysed by DPS version 3.01, a statistical package developed at Zhejiang University, China.

[a]The double asterisks (**) indicate a significant difference at the related Source of variation. In the two cases in this table, source of variation between treatments are significantly different.

Fig. 2. Growth curves of *Rhizobium* sp. strain PR389 in acidic and neutral environments. (A) Growth curves of PR389 in solutions with different original pH levels. (B) The curves of pH in solutions of PR389 with different original pH levels.

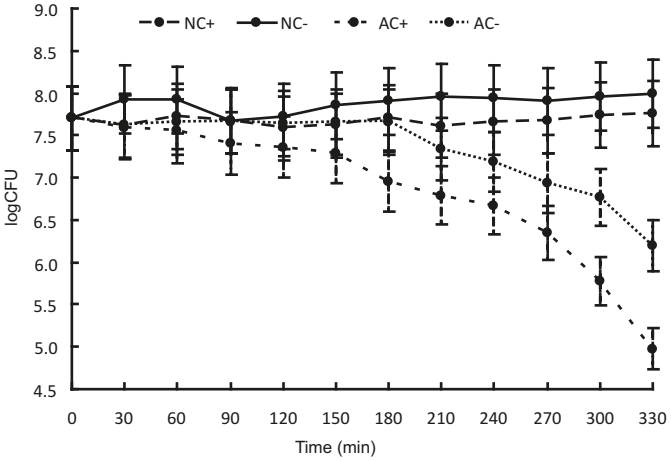


Experiments on the mechanism of acid tolerance of PR389

Growth and pH curves of PR389 under batch acidic cultural solutions

The growth curves of PR389 are presented in Fig. 2A. The lag phase of PR389 in the neutral culture solution (pH 7.0) was 4 h, after which it entered the logarithmic growth phase lasting for about 20 h. In contrast, before entering the logarithmic growth phase, PR389 experienced a lag phase of around 6, 8, 12, and 40 h under pH 5.5, 5.0, 4.8, and 4.6, respectively. This lag phase varied with the pH of the liquid medium, and there was no growth observed in the pH 4.4

Fig. 3. Acid tolerance of *Rhizobium* sp. strain PR389 in media with or without chloramphenicol. Treatments: NC+, neutral medium with chloramphenicol; NC–, neutral medium without chloramphenicol; AC+, acidic medium (pH 3.8) with chloramphenicol; AC–, acidic medium (pH 3.8) without chloramphenicol.



medium. We were not able to measure the pH and optical density (OD) for the pH 7.0, 5.5, 5.0, and 4.8 treatments after 56 h. However, a similar experiment with PR389 showed that the pH and OD after 56 h of culture were stable up to 72 h.

PR389 had growth curves with similar slopes during the logarithmic growth phase regardless of the original pH in the solutions after different lag phases (Fig. 2A). The generation time of PR389 under pH 7.0 was 1.74 h, while it was 1.84, 2.06, 1.86, and 1.72 h under pH conditions of 5.5, 5.0, 4.8, and 4.6, respectively.

Figure 2B shows that pH values gradually increased to neutral levels (pH 7 to 7.5). The pH stopped increasing at the neutral pH range, favouring the growth of PR389.

The significant influence of chloramphenicol on the acid tolerance of PR389

PR389 growth curves in Fig. 3 show that the neutral YM medium was always favorable for PR389 growth. Within 330 min, the growth curves with and without chloramphenicol in neutral conditions rose, although very little, i.e., the logCFU of NC^- rose from 7.70 to 7.99, and NC^+ from 7.70 to 7.75. No significant differences were observed between growth curves of NC^+ and NC^-. On the other hand, the growth curves of AC^+ (acidic medium supplemented with chloramphenicol) and AC^- (acidic medium without chloramphenicol) demonstrated that cell growth of PR389 declined after 180 min of culturing. The viable cell count for the AC^- treatment remained the same during the first 165 min, and the logCFU dropped from 7.92 to 6.20 after this point. For the AC^+ treatment, the cells started dying from the beginning and the logCFU dropped from 7.70 to 4.97 within 330 min. The number of viable cells at the 330th min in AC^+ was only 0.19% of that at the start, while in AC^- it was 3.1%.

Comparison of acid-induced stress on strains of acid-tolerant PR389 and acid-sensitive PR21

Performance of PR389 after acid shock was compared with that of an acid-sensitive rhizobium strain, PR21, isolated from root nodules of *P. lobata.* As is shown in Fig. 4A, the pH of the culture suspension of the acid-sensitive strain PR21 changed considerably within 14 min after the addition of HCl, regardless of whether or not the strain had been acid-induced. However, the acid-induced treatment of PR21 experienced a smaller change in pH (0.34) than did the PR21 noninduction treatment (0.7). For PR389, little variation in pH resulted from the addition of HCl into the media within this 14 min period regardless of whether or not it had been acid-induced. In the induction treatment of PR389, the pH of the medium ranged from 3.41 to 3.43, whereas the initial pH of the medium was 3.39 and it remained at 3.39 in the non-induction treatment. The two curves for PR389, induced and noninduced, were virtually parallel to each other.

Figure 4B reveals that PR21 differed between induced and noninduced treatments by proton flux. The maximum proton flux (36.68 nmol $H^+ \cdot mg^{-1} \cdot min^{-1}$) in PR21 in the noninduced treatment occurred after 4 min, suggesting that a large number of protons were entering the cells through the membrane. At the 5th minute, proton flux of noninduced PR21 was much smaller, implying equilibrium was reached between the cell and its environment. In contrast, the proton flux of the acid-induced PR21 remained around 10.0 nmol $H^+ \cdot mg^{-1} \cdot min^{-1}$, indicating that H^+ migration into cells was limited.

Figure 4C displays a significant difference ($P \leq 0.05$) between the survival rates of cells of PR389 and PR21 after the acid shock. The survival rate of PR389 did not change significantly ($P \geq 0.05$) in the acid-induction treatment in comparison with the noninduction treatment, showing that this treatment had no substantial effect on its acid tolerance. In contrast, the survival rate of PR21 increased from 0.25% in the noninduction treatment to 53.33% in the induction treatment, showing the obvious function of mild acid induction against H^+ toxicity for the acid-sensitive strain.

Adjustment of PR389 to acid soil suspensions

After being cultured for 28 days in an acidic soil, the survival and growth of PR389 cells in treatments did not show significant differences ($P \geq 0.05$), although Al concentrations varied from 1.59 to 13.97 $mmol \cdot L^{-1}$ (Table 3). The largest difference between the original and terminal pH happened in soil sample C6. Except for soil sample X4, the pH of soil suspensions rose slightly to >4.5, at which point Al is in a nontoxic insoluble form (Macdonald and Martin 1988).

Figure 5 shows that the most rapid growth of PR389 occurred in suspension C6 (which had the lowest original pH of 4.1) after being cultured in soil suspensions for 4 weeks. Nevertheless, no significant difference ($P \geq 0.05$) was noticed among the soil suspensions regarding the survival and growth rate of PR389, and the quantity of PR389 cells in all suspensions remained above 10^9 $CFU \cdot mL^{-1}$ at the end of the culture period.

Discussion

Mechanisms of the acid tolerance of *Rhizobium* sp. strain PR389

Pot experiments in which PR389 formed nodules on the roots of kudzu statistically more efficiently than the other isolations in this study (PR386, PR387, PR388, and PR390) indicate that PR389 is a competitive rhizobium strain of kudzu plants in an acidic environment. Compared with other

Fig. 4. Comparison of *Rhizobium* sp. strain PR389 and an acid-sensitive rhizobium strain (PR21) treated with acute acid shock (pH 3.8). (A) pH values of PR389 and PR21 in medium of pH 3.4 ± 0.1 in noninduced treatments and PR389 and PR21 in pH 3.4 ± 0.1 medium after being induced by a pH 5.2 medium (induced treatments). (B) Proton flux accumulation (nmol $H^+ \cdot mg^{-1} \cdot min^{-1}$) at different time intervals for PR389 and PR21 in noninduced treatments and induced treatments. (C) The survival rate (%) for the noninduced treatment for PR389 and PR21 and the induced treatments of PR389 and PR21.

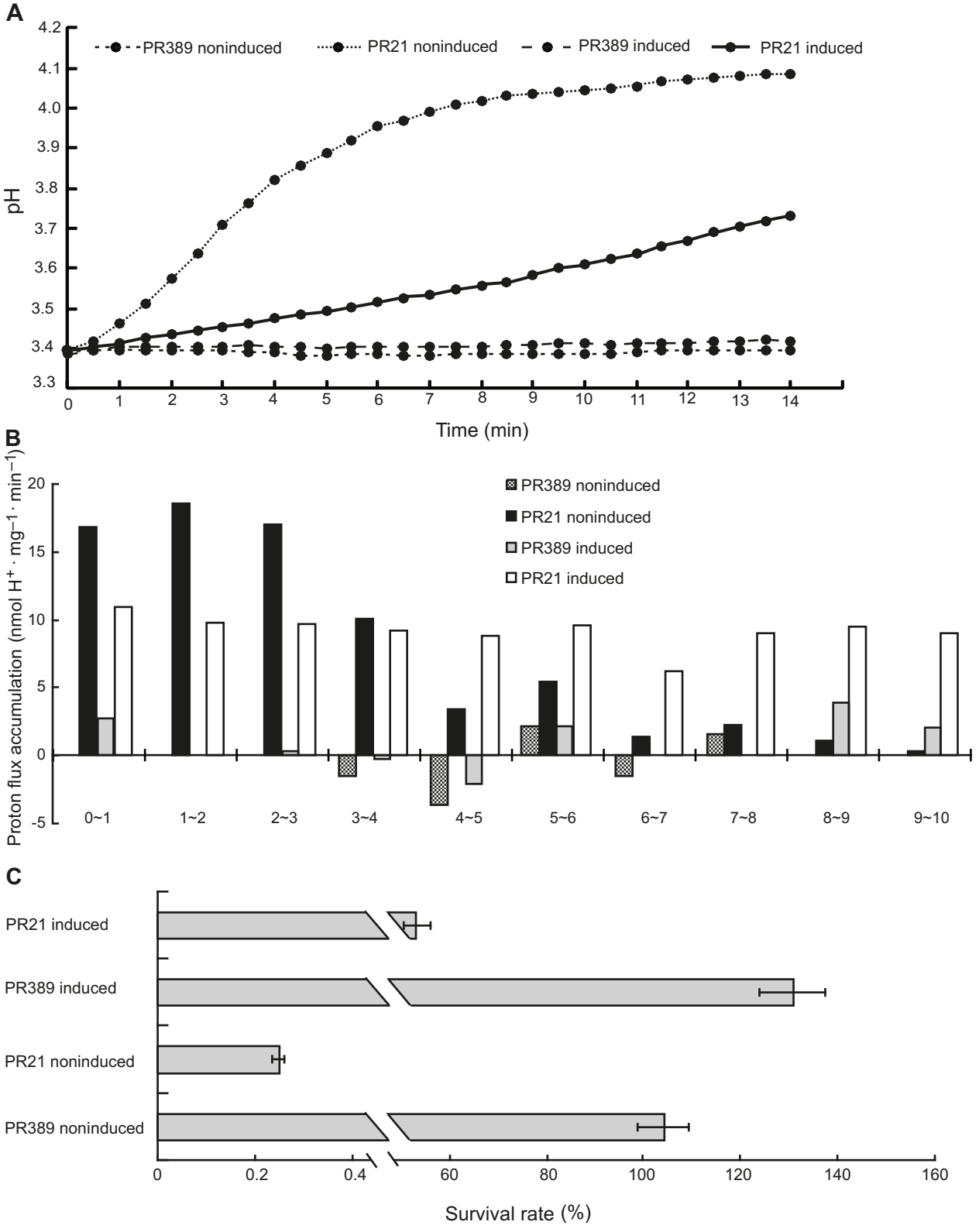
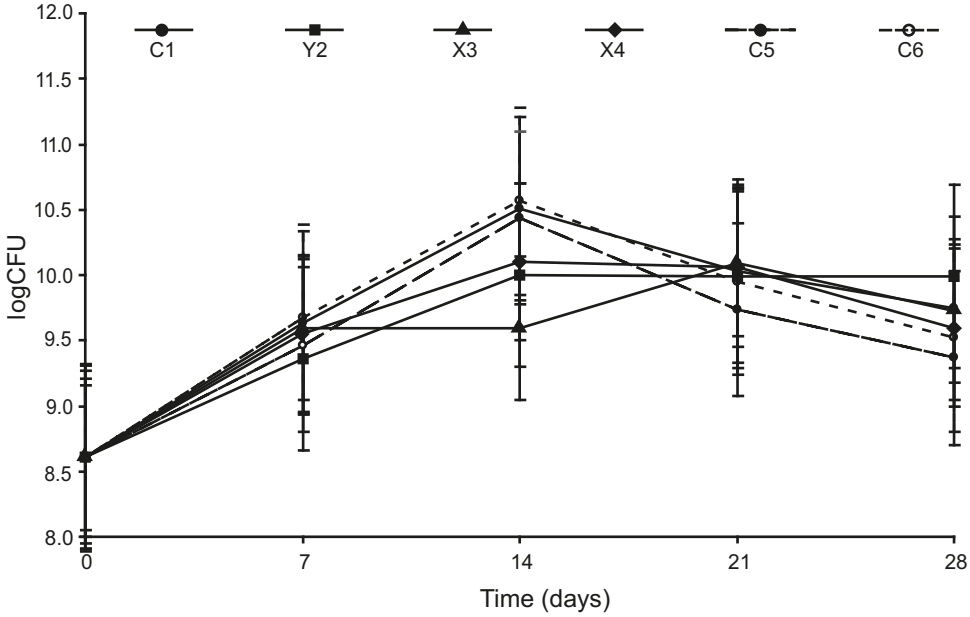


Table 3. The pH changes of *Rhizobium* sp. strain PR389 in acid soil suspensions 28 days after culturing.

Soil sample code	pH of original soil suspending liquids	Concn. of acid soluble Al in suspension ($mmol \cdot L^{-1}$)	pH of soil suspending liquids after 28 days of culture	$P \geq 0.05$	$P \geq 0.01$
Y2	4.70	1.59	4.75	a	A
C1	4.78	3.00	4.92	a	A
C5	4.20	1.82	4.55	a	A
C6	4.10	4.57	4.58	a	A
X3	4.60	13.97	4.67	a	A
X4	4.50	6.74	4.32	a	A

Note: *P* values with the same letter within a column indicate that the treatments are not significantly different.

Fig. 5. Growth curves of *Rhizobium* sp. strain PR389 in acid soil suspensions with different acidities and Al^{3+} concentrations for a 28 day culture period.



rhizobium species where acid tolerance has been studied, e.g., *Rhizobium leguminosarum* bv. *trifolii* (Richardson and Simpson 1989; Watkin et al. 2003), *Rhizobium tropici* (Vinuesa et al. 2003), *Bradyrhizobium japonicum* (O'Hara and Glenn 1994), *R. leguminosarum* bv. *viceae* (Howieson and Ewing 1986), *Rhizobium meliloti* (O'Hara et al. 1989; Tiwari et al. 1996), and *Sinorhizobium meliloti* (Dilworth et al. 1999; Draghi et al. 2010), PR389 shares the similarity that its generation time remained in similar range between neutral and acidic cultural conditions but that it had a broader range of acid tolerance than the others. PR389 survived in both pH 4.1 soil liquid suspension and pH 4.6 artificial liquid media, the lowest pH so far observed in any rhizobium strain.

During batch cultures ranging from neutral to acidic conditions, PR389 reduced extracellular acidity along with increased growth if the original solution's pH was >4.4, implying an acid tolerance mechanism with the ability to hold H^+ outside the cell. PR389 under the intense acid shock (pH 3.4 ± 0.1) confirmed this ability. The almost zero proton flux of the PR389 cells in both induced and noninduced treatments suggests that a PR389 cell, or possibly the membrane of the cell, has a defense mechanism that blocks the entry of H^+ into the cell during acid shock, while the induction of pH 5.2 helped PR21 to cope with acid stress by reducing the rate of H^+ penetration but not enough to completely prevent the entrance of free H ions. This mechanism of blocking H^+ used by PR389's membrane is probably shared by six acid-tolerant strains of *R. meliloti*, WSM419, WSM533, WSM539, WSM540, WSM852, and WSM870, which maintain an alkaline intracellular pH when the external pH declines from 7.2 to 5.6 (O'Hara et al. 1989).

According to the recent research, the ability of holding H^+ outside might be attributed to lysylphosphatidylglycerol or alanylphosphatidylglycerol, or other congeners possibly existing in the inter membrane in PR389, which may function to diminish the permeability of the lipid bilayer to protons, as observed in *R. tropici* (Vinuesa et al. 2003), *S. meliloti* (Reeve et al. 2006), and *Pseudomonas aeruginosa* (Klein et al. 2009). However, unlike these three strains, where acid induction is needed to maintain acid tolerance, the ability of PR389 to hold excessive H^+ outside the cell does no't need to be induced by weak acidity. For the same reason, this mechanism is also different from the acid tolerance mechanism demonstrated previously in *Streptococcus mutans*, *Enterococcus hirae* (Belli and Marquis 1991), *Salmonella typhimurium* (Foster 1993), and *R. leguminosarum* bv. *trifolii* (Chen et al. 1993).

Also during a long-term batch culture, PR389 changed the acidity of the media from as low as pH 4.6 to above 7.5

within 70 h, demonstrating another acid-tolerance mechanism in PR389 accumulating the excretion of alkaline substances into the extracellular environment to decrease the acidity. This type of response to acid stress has not been observed in any other rhizobium strains other than *R. trifolii* strain, WU95, which changed the pH of its liquid artificial media from 4.7 to 5.17, 5.10 to 5.59, and 6.2 to 6.58 within 72 h, when the original pHs were 4.7, 5.1, and 6.2, respectively (Richardson and Simpson 1989).

The slight rise in growth of PR389 in the NC^+ medium (with chloramphenicol in neutral conditions) and NC^- medium (without chloramphenicol in neutral conditions) is consistent with the data depicted in Fig. 2A, where the growth curve for the neutral media starts to rise after a 4 h lag phase. The lack of a significant difference between the growth curves for NC^+ and NC^- suggests that chloramphenicol does not affect the growth of PR389 in neutral solutions. On the other hand, the growth curves for AC^+ (acidic medium supplemented with chloramphenicol) and AC^- (acidic medium without chloramphenicol) demonstrated that the addition of chloramphenicol significantly decreased the survival of PR389 in acute acidic conditions (pH 3.8), suggesting that de novo protein synthesis related to chloramphenicol is related to the acid tolerance of PR389. Here, protein synthesis could be started by the sigma factor RpoH1, or instead of basing the acid tolerance on a few specific genes, it could involve whole sets of genes associated with various cellular functions, as in the case of *S. meliloti* 1021 under acid stress (Hellweg et al. 2009). Further research needs to specially address which genes are critical for PR389 to survive acidic environments, including its survival in acute acidity, such as pH 3.8, and at its lethal pH 3.3 (data not shown).

The mechanism of PR389 resisting Al toxicity in an acidic soil is also related to its ability to regulate the pH of its microenvironment, which leads to less soluble Al, namely the less toxic monomeric Al^{3+}, which exists in acidic soil with pHs lower than 4.5. Obviously, the rise of pH in soil medium of PR389 was much less than in the synthetic medium (shown in Fig. 2B), which should attribute to the complexity of pH composition in soil than in a synthetic solution. The complicated structure and interactions among organic and inorganic substances in soil makes soil suspension a large buffer to any pH changes. For instance, by adding acid at rate of 4 kmol $H^+ \cdot ha^{-1} \cdot year^{-1}$, soil pH would only decline by one unit in the upper 30 cm within 30 years for sandy loam soils and within 120 years for clay soils (Helyar et al. 1990). In our experiment, changes in pH values ranging from 0.05 to 0.48 among different soil suspensions actually show the ability of PR389 to raise the acidity of its surroundings, including soils.

There are two essential questions that need to be further addressed. What kind of alkali does PR389 produce to raise the external pH? and What are the mechanisms by which PR389 cells sense the pH change in the extracellular environment and stop the pH from going beyond 7.0 in the artificial medium? The two common ways for oral bacteria to generate an alkali, specifically ammonia, involve urease and arginine deiminase systems where cells increase their tolerance of acidic conditions by producing NH_3, which combines with protons in the cytoplasm to produce NH_4^+ and raises their internal pH (Cotter and Hill 2003). The arginine deiminase system has been identified in a variety of bacteria, including mycoplasmas, halobacteria, *Pseudomonas* sp., *Bacillus* spp., and a number of lactic acid and dental bacteria, catabolising arginine to ornithine, ammonia, and CO_2 (Sheng et al. 2010). These observations provide insights for tracking alkali production by PR389.

Potential application of PR389

This research shows the necessity of understanding the relationship of kudzu, a successful and damaging invasive species, to the tolerance of its rhizobium to better manage the invasiveness of kudzu. Given its large biomass and fast growth in the forests, rights-of-way, roadsides, and non-crop lands, nitrogen-fixing bacteria in kudzu's root system are essential for providing nitrogen to the plant. The tolerance of the rhizobium to acidic, alkaline, and barren soils raises the potential for it to survive and flourish and, hence, allows kudzu to grow in poor quality soils. Chemical and mechanical methods to eradicate kudzu have been applied for decades, yet kudzu is still spreading in the United States and other nations. It is time to consider new methods of control by understanding kudzu's dependence on its rhizobia. On the other hand, because of the acid tolerance of the rhizobium of kudzu, planting kudzu is still a good choice for preventing soil erosion in acidic soils in China and Japan where the land is intensely used, yet acid rain remains a serious concern.

Research into the acidity and the related Al concentrations in areas with acidic soils, which represents 21% of the Chinese territory mainly distributed in south China, concluded that mobile Al^{3+} concentrations in these soils changed from 2.6–4.4 to 23.0–27.0 $mmol \cdot kg^{-1}$, with an average range of 5.1–10.3 $mmol \cdot kg^{-1}$ (Liao and Jiang 2000). These acidic soils are toxic to microbes and plants in varying degrees (Li et al. 2009). PR389 and similar strains could be used to prevent Al toxicity to plants by changing the acidity of the microenvironments in the rhizosphere.

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