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Characterization of Acid-Aluminium Sensitive Mutants of Soybean Symbiont *Bradyrhizobium japonicum* Generated by Transposon Mutagenesis

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Acid-aluminium sensitive mutants of symbiotic bacterium *Bradyrhizobium japonicum* BJ11 (designated as AAS11) and KDR15 (designated as AAS15) were constructed by mini-Tn5 transposon mutagenesis to study genes involved in acid-aluminium tolerance (AAT) in *B. japonicum*. Transposon delivery was carried out through conjugation between *B. japonicum* strains as recipients and *Escherichia coli* S17-1 (ë pir) carrying pUTmini-Tn*5*Km1 as a donor strain. The result showed that frequency of transconjugation was in the range of 6.7 x 10^{-7} to 7.1 x 10^{-6} cell per recipients. AAS11 and AAS15 mutants did not grow on Ayanaba media (pH 4.5) containing 50 µM Aluminium. These mutants remained able to form root nodules of Siratro (*Macroptilium arthropurpureum*) plants revealing genes interrupted by transposon which were responsible for acid-Al tolerance did not correlate with the nodulation genes. Strains tolerant to acid-aluminium and their mutants with a wild type sensitive to acid-aluminium were characterized by accumulating phosphate and aluminium absorption. Compared to the wild type acid-aluminium tolerant *B. japonicum*, there was approximately a three- to eight-times decrease in phosphate accumulation and a five- to seven-times increase in aluminium absorption by these mutants. These results suggest that aluminium and phosphate contents in the bacterial cells may be involved in mechanisms of acid-Al tolerance of *B. japonicum* grown in acid-aluminium stress conditions.

Key words: *Bradyrhizobium japonicum*, acid-aluminium tolerance, transposon mutagenesis, aluminium absorption, phosphate intracellular

Bradyrhizobium japonicum is one of the nitrogen fixing bacteria which can symbiose with either soybean or siratro plants through root nodule formation. Most of the nitrogen source required by soybean plants can be provided by this symbiosis. However, the high solubility of aluminium in acid soils can poison root nodule bacteria (Flis *et al.* 1993). This condition will influence symbiosis between root nodule bacteria and the soybean plant. Soil acidity can induce nitrogen deficiency in soybeans by preventing root nodule formation. Low levels of phosphorus, calcium, and molybdenum, and high levels of aluminium, iron, and manganese are important factors of soil acidity and can be toxic for plants and root nodule bacteria. Soil acidity generally increases the lag time or slows the growth rate of bacteria (Keyser and Munns 1979). To date, there are several reports revealing that some nitrogen fixing bacteria can survive on media with a pH 4.5 containing 50 µM Al, 200 µM Mn, Ca 50 µM, and 5 µM $PO4^-$ (Ayanaba *et al.* 1983; Endarini *et al.* 1995; Wahyudi *et al*. 1998).

Reports on the acid-Al tolerant bradyrhizobial or rhizobial strains exerting tolerance to acid-Al are not fully understood. Acid-Al tolerance appears to be related with amounts of phosphate supplemented in the media or its availability in the soil (Flis *et al*. 1993). Cellular phosphate has been shown in many different bacteria as one of the mechanisms to counter the effects of metal toxicity (Keasling and Hupf 1996; Alvarez and Jerez 2004). Gemell *et al.* (1993) and Watkin *et al.* (1997) reported that increasing the phosphate and calcium concentration in the media at low pH will increase the growth of the root nodule bacteria, bradyrhizobial, and rhizobial strains. Increasing phosphate up to 100 µM was able to encounter toxicity of aluminium (50 µM) at pH 5.5 to *B. japonicum* in the soil (Mukherjee and Asanuma 1998).

A transposon is a DNA fragment which can transpose from one site to another site in the genome. One of the applications of the transposon is to mutate a gene (mutagenesis) and determine the physical location of genes of interest (Guilhabert *et al*. 2001). In this study, the transposon mini-Tn5Km1 (De Lorenzo *et al.* 1990) was used to generate acid-Al sensitive mutants of *B. japonicum*. This transposon has a relatively high frequency of transposition, low insertionally specificity, and can be expressed in most gram negative bacteria. Other advantages of the transposon mini-Tn5Km1 is the availability of a detailed genetic and physical map.

This study demonstrates the production of acid-aluminium sensitive mutants of *B. japonicum* generated by transposon mutagenesis as defined in root nodule formation, cellular phosphate content, and aluminium absorption in relation with genes interrupted by the transposon which are involved in acid-aluminium tolerance.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions. Two, acid-Al tolerant *B. japonicum* strains, BJ11 and KDR15 (Imas 1994), were routinely grown in yeast extract mannitol agar (YMA) (mannitol 10 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1}, NaCl 0.2 g l^{-1}, yeast extract 5 g l^{-1}) supplemented with Congo Red (CR) 0.0025% (w/v) at room temperature. Acid-Al sensitive *B. japonicum* strain (BJ13) was used as a control. *Escherichia coli* S17-1 (λ pir), which carries

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pUTmini-Tn5Km1 (de Lorenzo *et al.* 1990), was routinely grown on Luria agar (LA) (tryptone 5.0 g l^{-1}, NaCl 10 g l^{-1}, yeast extract 5.0 g l^{-1}, agar 15 g l^{-1}) supplemented with kanamycin (50 µg ml^{-1}) and ampicillin (50 µg l^{-1}) at 37 °C. The siratro plant was used for root nodulation experiments.

Antibiotic Resistance Test. Acid-Al tolerant *B. japonicum* strains BJ11 and KDR15 and *E. coli* S17-1 (λ pir) were tested for their antibiotic resistance. Four antibiotics were used in the following concentrations: Kanamycin (Km) (50 µg ml^{-1}), Rifampicin (Rif) (50 µg ml^{-1}), Ampicilin (Amp) (50 µg ml^{-1}), and Tetracyclin (Tc) (50 µg ml^{-1}). All strains were plated on suitable media supplemented with each antibiotic. This work was carried out to determine selectable markers that will be used in transposon mutagenesis experiments.

Transposon Mutagenesis and Screening of Acid-Al Sensitive Mutants. The recipients, acid-Al tolerant *B. japonicum* BJ11 and KDR15, were grown on YMA + CR (0.0025% b/v) + Rif (50 µg ml^{-1}). All cultures were incubated aerobically at room temperature agitated at 140 rpm for 60-72 h. The donor, *E. coli* S17-1 (λ pir), was grown on LB + Km (50 µg ml^{-1}) + Amp (50 µg ml^{-1}) and incubated aerobically with agitation at 140 rpm for 18-20 h at 37 °C. The transposon mini-Tn*5*Km1 carried by *E. coli* S17-1 (λ pir) was transferred to recipients by diparental mating conjugation in which the ratio of donor and recipient cells was 1:1 (~ 10^8 cells ml^{-1}). All matings were carried out on membrane filters (0.45 µm) placed on LA modified media (LA omitting NaCl 1.0 g l^{-1}) without antibiotic, and incubated for 12, 18, and 24 h at room temperature. The transconjugants were plated on YMA + CR (0.0025% w/v) + Km (50 µg ml^{-1}) + Rif (50 µg ml^{-1}). Screening of acid-aluminium sensitive mutants of *B. japonicum* was performed on Ayanaba media (pH 4.5; 50 µM Al) (Ayanaba *et al.* 1983). Colonies which grew on YMA + CR (0.0025% w/v) + Km (50 µg ml^{-1}) + Rif (50 µg ml^{-1}) but which failed to grow on Ayanaba media were choosed for further analysis.

Root Nodulation Experiment. All acid-Al sensitive mutants of *B. japonicum* and the wild type were tested for root nodulation on siratro plants. Each of these mutants were inoculated on siratro (*Macroptilium atropurpureum*) plants at a concentration of 10^6 cells ml^{-1}. The siratro was planted in 25 mm diameter x 200 mm long reaction tubes using the medium as described by Speidel and Wollum (1980) and grown in a green-house. Root nodule formation was examined from day 7 to day 30 after inoculation.

Cellular Phosphate Content. Mutants and the wild type were grown on phosphate media as described by Keyser and Munns (1979). All cultures were incubated at room temperature and agitated at 60 rpm. When cells reached a density until gave an OD620 reading of 0.7 (~10^9 cell ml^{-1}), cells were pelleted (10,000 rpm, 15 min, 4 °C) and the subsequent pellet was then incubated with 10 ml of 25% HCl for 24 h and then diluted in sterile distilled water to give a total volume of 25 ml. A 2.0 ml aliquot was then added to 2 ml HNO_3 and 1 ml molybdate-vanadate solution and incubated for 20 min. Total phosphate content was assayed spectrophotometrically using the molybdate-vanadate method at λ 420 nm (Mukherjee and Asanuma 1998). The total phosphate content on BJ13 was also measured as a control.

Analysis of Aluminium Absorption. Acid-Al sensitive mutants of *B. japonicum* and the wild type were grown on phosphate media as described by Keyser and Munns (1979). All cultures were agitated at 60 rpm and incubated at room temperature. When cells reached a density which gave an OD_{620} reading of 0.7 (ca 10^9 cells ml^{-1}), cells were pelleted (10,000 rpm, 15 min, 4 °C) and then resuspended in 50 ml sterilized 2-(N-morpholino) ethanesulfonic acid (MES, pH 5.4) buffer containing 50 µM Al for 30 h (Mukherjee and Asanuma 1998). After the relevant periods, cells were pelleted (10,000 rpm, 15 min, 4 °C). Aluminium was assayed using an Atomic Absorption Spectrophotometer at λ 303 nm. The total aluminium absorption on BJ13 was also measured as a control.

RESULTS

Antibiotic Resistance Test. All acid-Al tolerant *B. japonicum* strains showed resistance to ampicilin and rifampicin at 50 µg ml^{-1}. On the other hand, *E. coli* showed resistance to kanamycin and ampicilin at 50 µg ml^{-1} (Table 1). The ability of *E. coli* to grow on LA media supplemented by kanamycin and ampicilin was due to the fact that this bacterium carried the pUTmini-Tn5Km1 component which has gene resistance to kanamycin (Km1) and ampicilin (Amp). Therefore, Kanamycin and Rifampisin were choosen as a selectable markers of the *B. japonicum* transconjugant generated by transposon mutagenesis.

Transposon Mutagenesis. Transposon mini-Tn5Km1 was transferred to the recipient *B. japonicum* by conjugation diparental mating. This generated *B. japonicum* mutants which were resistant to kanamycin. The plasmid pUTmini-Tn5Km1 has origin of replication (*ori*) from pR6K. It can replicate only in a host providing the *pir* factor (protein initiation of replication). Transfer of Km1 into *B. japonicum* was achieved by using the *mob* gene of the plasmid RP4 which was driven by the products of the *tra* gene provided *in trans* on the *E. coli* S17-1 (λ pir) chromosome. Depending on the strains, the frequency of transconjugation varied between 6.7 x 10^{-7} to 7.1 x 10^{-6} per recipient (Table 2). The

Table 1 Growth of *Bradyrhizobium japonicum* and *Escherichia coli* on media containing antibiotics

Strain	Antibiotic (µg ml^{-1})			
	Tc 50	Rif 50	Ap 50	Km 50
BJ11	+	+	+	-
KDR15	+	+	+	-
BJ13	+	+	+	-
E.c	-	-	+	+

BJ11: *Bradyrhizobium japonicum* BJ11, KDR15: *Bradyrhizobium japonicum* KDR15, BJ13: *Bradyrhizobium japonicum* BJ13, E.c: *Escherichia coli* S17-1 (λ pir), +: grew, -: did not grow.

Table 2 Frequency of transconjugation of transposon Mini-Tn5Km1 from *E. coli* S17-1 (λ pir) to acid-Al tolerant *B. japonicum* as a function of mating time

Bacterial conjugation time (h)	Frequency of transconjugation[a] *B. japonicum* x *E. coli* S-17-1 (λ pir)	
	BJ11	KDR15
12	6.7 x 10^{-7}	3.4 x 10^{-6}
18	1.1 x 10^{-6}	5.1 x 10^{-6}
24	7.1 x 10^{-6}	6.3 x 10^{-6}

[a]Frequency of transconjugation is calculated per recipient.

highest frequency of transconjugation was obtained using a mating time as long as 24 h.

Screening of Acid-Al Sensitive Mutants and Root Nodule Formation. Acid-Al sensitive mutants are transconjugants which grow on YMA + CR (0.0025% w/v) + Km (50 µg ml^{-1}) + Rif (50 µg ml^{-1}), but which failed to grow on Ayanaba media (pH 4.5, Al 50 mM). One acid-Al sensitive mutant generated from BJ11 (designated AAS11) and one from KDR15 (designated AAS15) were obtained. All mutants and their wild type afforded the ability to form root nodules on siratros (Table 3).

Cellular Phosphate Content. Total cellular phosphate declined in acid-Al sensitive mutants of *B. japonicum* when compared to their wild type. The amounts of total cellular phosphate of AAS11 were 3X lower than its wild type, and relatively lower than BJ13. Similarly, the total cellular phosphate of AAS15 mutant was 8X lower than its wild type and 4X lower than BJ13 (Fig 1).

Aluminium Absorption Analysis. Aluminium was found in mutants (AAS11 and AAS15), the wild type acid-Al toleran BJ11 and KDR15, and the wild type acid-Al sensitive strain BJ13. However, the amounts of Al absorbed by AAS11, AAS15, and BJ13 were higher than those in the cells of the acid-Al tolerant strains. The amount of aluminium absorbed by AAS11 was 7X higher than for its wild type, whereas the mutant AAS15 absorbed Al 5X higher than its wild type. The amounts of Al were about two 2X in the cells of the BJ13 strain than in the cells of the acid-Al tolerant strains (Fig 2).

Table 3 Results of root nodulation of the siratro plant by strains of *Bradyrhizobium japonicum* (wild type and mutant)

Strain	No. nodule formed		
	Day	Number/plant	Position
Wild type			
BJ 11	15	4	3 PR/1 SR
KDR15	15	4	3 PR/1 SR
Mutan			
AAS 11	15	6	6 PR
AAS 15	13	5	5 PR

PR: Primary root, SR: Secondary root.

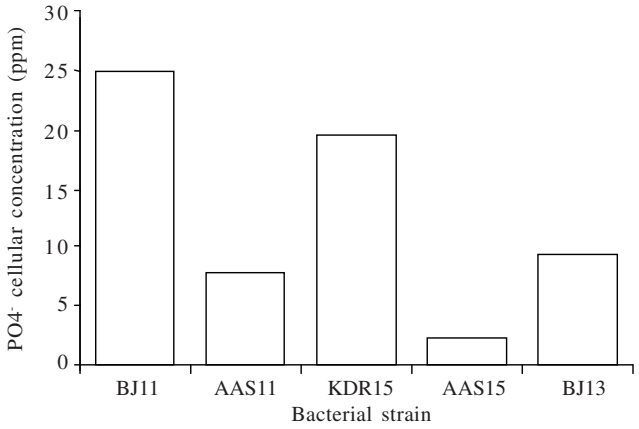

Fig 1 Cellular phosphate content of wild type acid-Al *B. japonicum* BJ11 and its mutant (AAS11), wild type *B. japonicum* KDR15 and its mutant (AAS15) and wild type acid-Al sensitive *B. japonicum* BJ13, grown on phosphate media pH 6.4.

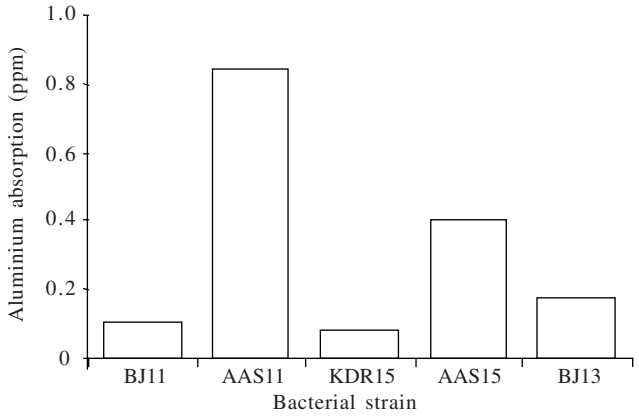

Fig 2 Absorption of aluminium by wild type acid-Al *B. japonicum* BJ11 and its mutant (AAS11), *B. japonicum* KDR15 and its mutant (AAS15) and wil type acid-Al sensitive *B. japonicum* BJ13, inoculated in MES buffer pH 5.4 containing aluminium (50 µM).

DISCUSSION

Both of the strains BJ11 and KDR15 grew large watery colonies. Fuhrmann (1990) divided *B. japonicum* into three colony types which are large watery, large mucoid, and small dry. Ayanaba *et al.* (1983) reported that colony types appeared to influence tolerance of the acid-Al stress on soybean rhizobia. Strain that formed small dry, pinpoint colonies were more sensitive to acd-Al than those which formed large and 'gummy' colonies.

The transposon mini-Tn5Km1 from plasmid pUTmini-Tn5Km1 was successfully introduced into acid-Al tolerant *B. japonicum* strains. The suicide plasmid, pUTmini-Tn5Km1, contains the transposase gene *in cis* configuration outside of the transposable elements. This prevents secondary transposition when the plasmid is lost from the cell (de Lorenzo *et al.* 1990). The highest frequency of transconjugation obtained was about 7.1×10^{-6} per recipient, with a mating time as long as 24 h at 1:1 ratio between *E. coli* S17-1 (λ pir) and *B. japonicum*. This frequency is higher than *B. japonicum* (5.7×10^{-9}) using mini-Tn5Km1 for mutagenesis with a 1:10 ratio between donor and recipients (Wahyudi *et al.* 1998). These results indicate that mating time and donor:recipient ratio are affecting conjugation efficiency. The differences in frequencies between strains may have been due to the inherent properties of the bacterial cell systems. Similar responses have also been reported by Wahyudi *et al.* (1998). DNA Polymerase I, membrane filters, and termination transcription factor can also influence transposition events (Berg 1989).

One acid-Al sensitive mutant generated from BJ11 (AAS11) and one mutant generated from KDR15 (AAS15) were obtained. The inability of mutants to grow on Ayanaba media is due to the transposon mini-Tn5 being inserted into the *B. japonicum* chromosome, especially in the genes involved in acid-Al tolerance. Transposon mutagenesis on *R. meliloti* WSM419 has also been reported by Goss *et al.* (1990). These authors observed genes that controlled acid tolerance (*act*). The disruption of *act* genes resulted in *R. meliloti* WSM419 not being able to maintain its internal pH when grown on acid media. Riccillo *et al.* (2000) reported that *gsh* gene (gene for glutamine synthetase) was presumed to play an important role for acid tolerance on *R. tropici* CIAT899. Tucker *et al.* (2002) reported that acid conditions

will induce genes encoding for glutamate decarboxylase in *E. coli* MG1655.

Both strains of *B. japonicum* wild types, BJ11 and KDR15, and their mutants, AAS11 and AAS15, were able to perform root nodulation on siratro plant. The ability of *B. japonicum* mutants to form root nodules on these plants indicates that the genes for nodulation (*nod*) have not been affected by inactivation of the genes involved in acid-Al tolerance in *B. japonicum*.

The amounts of total cellular phosphate were larger in the acid-Al tolerant strains when compared with mutants or acid-Al sensitive strains. This result indicates that there was a disruption of the gene for metabolising phosphate. Aluminium was able to enter the cells of tolerant, sensitive, and mutants strains, but the amount accumulated intracellularly was less in the tolerant strains. Johnson and Wood (1990) reported that the absorption of aluminium by cells of both acid-Al- tolerant and -sensitive strains took place however amounts of Al absorbed by the sensitive strains was 2X higher than for the tolerant strains. Thus, Al tolerant strains appeared to have a mechanism to protect the cells by limiting the uptake of Al from the culture solution. The differences among the strains in their ability to store phosphate internally and use it under adverse conditions could be one of the determining factors to acid-Al stress tolerance or sensitivity (Mukherjee and Asanuma 1998). It seems that available phosphate plays a major role in detoxification of Al lost intra- and extra-cellularly.

There are reports on the role of phosphate metabolism and metal detoxification in microbial systems. Alvarez and Jerez (2004) reported on the role of polyphosphate on copper tolerance in *Acidithiobacillus ferrooxidans*. They propose that one of mechanisms for heavy metal tolerance involved the hydrolysis polyphosphate and the formation of metal-phosphate complexes which are then transported out of the cell. Keasling and Hupf (1996) demonstrated that not only formation, but also the hydrolysis of polyphosphate play a significant role in detoxification of cadmium in *E. coli*. They also found that two enzymes i.e. polyphosphate kinase and polyphosphatase were involved in the detoxification of cadmium (Keasling and Hupf 1996). The same hypothesis was proposed by Remonsellez *et al.* (2006) who showed that the ability to accumulate and hydrolyze polyphosphate may play an important role for a copper tolerance mechanism in members of genus *Sulfolobus*. Acid conditions also stimulated an increase in intracellular polyphosphate and cellular polyphosphate kinase activity of newly isolated environmental strain *Candida humicola* (Remonsellez *et al*. 2006). When the cells were grown at pH 5.5, phosphate removal was found to be 4.5 fold higher than when grown at pH 7.5. This increase in phosphate removal was associated with an increase in free intracellular polyphosphate (McGrath and Quinn 2000). Mukherjee and Asanuma (1998) reported that in Al tolerant strains, cellular absorption of Al was related positively to the release of phosphate from the cells and the accumulation of intracellular phosphate (Pi). From our results it is clear that the mutants, AAS11 and AAS15 showed a weaker ability to store phosphate internally and absorbed aluminium more strongly than for their wild type. We therefore conclude that the phosphate metabolic status of bradyrhizobial cells seems to play a major role in countering Al toxicity in acid and in extracellular P-limited conditions.

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