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Identification of two clusters of genes involved in salt tolerance in *Sinorhizobium* sp. strain BL3

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Abstract
Sinorhizobium sp. strain BL3 was isolated from the nodules of *Phaseolus lathyroides* plants that were found growing under saline conditions in the northeastern region of Thailand. It can tolerate up to 600 mM NaCl and is effective in N_2 fixation. A genomic DNA clone library of BL3 was constructed and transferred to the salt-sensitive *Rhizobium* sp. strain TAL1145 by conjugation and the transconjugants were selected on medium containing 100 mM NaCl. Two hundred colonies that grew under this salt condition were isolated. From twenty-four of those colonies, two clusters of genes involved in salt tolerance were isolated. The deduced proteins encoded by the genes of first region include an ATPase, and a transcriptional regulator of AraC family, while the deduced proteins of the second region showed high similarities with enzymes involved in glycine betaine biosynthesis in *Sinorhizobium meliloti*. When these gene clusters were transferred to salt-sensitive *Rhizobium* and *Bradyrhizobium*, the transconjugants showed increased tolerance to salt stress.

Keywords: Genes, salt tolerance, *Sinorhizobium*

1. Introduction

Root nodule bacteria, known as rhizobia, of genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium* form nitrogen-fixing symbiosis with various leguminous plants. Environmental factors such as low or high pH, high temperature, low soil moisture, nutrient deficiency, mineral toxicity, and soil salinity can reduce survival and growth of rhizobia in the soil and inhibit rhizobia-legume symbiosis (Dowling and Broughton, 1986; Triplett and Sadowsky, 1992; Zahran, 1999). Soil salinity adversely affects the nodulation and nitrogen fixation capacities of rhizobia, resulting in lower productivity of legumes (Miller and Wood, 1996). Growth of most rhizobial strains is inhibited by 100 mM NaCl, while some strains can tolerate more than 300 mM concentrations of NaCl (Embalomatis et al., 1994; Mohammad, 1991; Muller and Pereira, 1995).

Nearly 40% of the world land surface can be categorized as having potential salinity problem (Cordovilla et al., 1994). Moreover, salinity of soil in cultivated area is expected to rise as a result from local salt accumulation due to irrigations and applications of chemical fertilizers (Miller and Wood, 1996). In Thailand, there is a widespread salinity problem, especially in the northeastern region. Many areas in this region are not suitable for agriculture because of salinity. One particular wild leguminous species, *Phaseolus lathyroides*, grows successfully forming effective root nodules in these saline soils. The objectives of this investigation are to isolate and characterize a high salt-tolerant rhizobial strain from the nodules of *P. lathyroides*, and clone DNA fragments containing genes involved in salt tolerance from the selected rhizobial strain.

2. Materials and Methods

Microbiological methods

Bacterial strains and plasmids used in this study are listed in Table 1. Rhizobia were isolated from *P. lathyroides*

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Table 1. Bacterial strains and plasmids.

	Relevant characteristics	Marker	Source
Rhizobial strains			
BL3	*Sinorhizobium* sp., wild-type, highly salt tolerant	Rf^r, Sm^r	This study
TAL1145	*Rhizobium* sp. Nod^+ on *Leucaena* and bean	Rf^r	Moawad and Bohlool, 1984
THA6	*B. japonicum*	Tc^r, Sm^r	Department of Agriculture, Thailand
Escherichia coli			
VCS257	*E. coli*, host strain for library construction	–	Gigapack II, Stratagene
DH5αMCR	Used for transformation	–	Bethesda Research Laboratories
Plasmids			
pLAFR3	Wide-host-range P1 group cloning vector, used for BL3 genomic DNA library construction	Tc^r	Staskawicz et al., 1987
pRK2013	Helper plasmid used for mobilizing plasmids of triparental mating	Km^r	Figurski and Helinski, 1979
pUC18	Cloning vector	Ap^r	Maniatis et al., 1982
pUHR307	pLAFR3-based cosmid clone isolated from the genomic library of strain BL3 containing 18.3-kb of salt tolerant genes	Tc^r	This study
pUHR310	pLAFR3-based cosmid clone isolated from the genomic library of strain BL3 containing betaine production operon (*bet*) with 23.5-kb	Tc^r	This study

Table 2. Number of nodules, dry weight of nodules and shoots of *P. lathyroides* plants inoculated with *Sinorhizobium* sp. strain BL3 grown in the presence of 0, 50, 100 and 150 mM NaCl.

Salinity condition	Nodules (no. per 5 plants)*	Nodule dry mass (mg per 5 plants)*	Plant dry mass (g per 5 plants)*
0 mM NaCl	49.6 a	19.2 a	0.21 a
50 mM NaCl	45.2 a	15.8 ab	0.15 b
100 mM NaCl	44.4 a	15.0 b	0.09 c
150 mM NaCl	30.6 b	8.8 c	0.07 d
Uninoculated control	0	0	0.16 b

*Means with different letters are significantly different at $p<0.05$.

Table 3. Betaine aldehyde dehydrogenase activity in THA6 and THA6(pUHR310).

Medium	Specific activity unit (mg^{-1} protein)*	
	THA6	THA6(pUHR310)
Minimal medium (MM)	3.68 ± 0.18	3.80 ± 0.27
MM + 100 mM choline	3.75 ± 0.32	4.99 ± 0.18
MM + 100 mM choline + 50 mM NaCl	3.55 ± 0.57	5.30 ± 0.10

*1 unit (U) is defined as the activity that catalyzes the formation of 1 μmol of product (NADH) per minute (μmol × mg^{-1} protein × $minute^{-1}$). Data represent means ± SD of 3 replications.

nodules and grown in yeast extract mannitol (YEM) medium as described by Somasegaran and Hoben (1994).

Amplification of 16S rRNA

Chromosomal DNA of selected strain was amplified for the 16S rRNA gene by PCR with forward primer 5'AGAGTTTGATCCTGGCTC3' and reverse primer 5'CACCCCAGTCGCTGACCCTAC3'. The PCR reaction was performed in a 100 µl mixture containing 200 µM dNTP, 100 pM of each primer, 300 ng of chromosomal DNA, 2.0 mM $MgCl_2$ and 1U of Taq DNA polymerase (Promega, USA), using the following temperature profile: initial denaturation at 95°C for 10 min, 30 cycles at 94°C for 1.5 min, 55°C for 1.5 min and 72°C for 2 min, final extension at 72°C for 3 min. The PCR product was purified for sequencing by a plasmid mini prep kit (Qiagen Inc., USA). The purified PCR-amplified DNA fragment was sequenced using an automated DNA sequence (Model 310A; Applied Biosystems, Foster City, CA, USA) at the school of Biotechnology, Suranaree University of Technology).

Cosmid clone library construction

Genomic DNA was prepared according to standard protocol (Sambrook and Russell, 2000). For cosmid library construction, genomic DNA was partially digested with the restriction enzyme *Sau3*AI. DNA fragments were size-fractionated using a sucrose gradient centrifugation and fragments of 20–30 kb were selected. The fragments were ligated to the cosmid vector pLAFR3, which was previously digested with the restriction enzyme *Bam*HI and dephosphorylated. The ligation mixture was packaged *in vitro* with Gigapack® II XL packaging extracts and used to transfect *E. coli* strain CVS257 following the protocol specified by manufacturer (STRATAGENE, USA). The transfected *E. coli* cells were plated onto LB containing 5 $\mu g.ml^{-1}$ tetracycline, and incubated at 37°C for 24 h. 7231 colonies from 20 plates were pooled to constitute the cosmid clone library.

Betaine aldehyde dehydrogenase activity

From freshly grown cultures, cell pellets were collected by centrifugation at 7000 x g, washed twice with 50 mM phosphate buffer, pH 7, and resuspended in 1 ml of the same buffer containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 1 mM DTT, 4 $mg.l^{-1}$ DNase and 1 $mg.l^{-1}$ lysozyme. Cells were disrupted by sonication, and the lysate was centrifuged twice at 12,000 x g for 10 min to remove unbroken cells. Protein concentration was measured by Lowry's method (Lowry et al., 1951). Betaine aldehyde dehydrogenase activity was determined spectrophotometrically at room temperature by monitoring the reduction of NAD^+ at 340 nm. The reaction mixture consisted of 50 mM HEPES-KOH, pH 8.0, 5 mM DTT, 1 mM EDTA, 10 mM betaine aldehyde, 1 mM NAD^+ and 100 µl of sample solution. The reaction mixture without NAD^+ was used as the blank. The reaction was started by adding NAD^+ to the mixture. The enzyme activity was determined by incubation of cell extract with 10 mM betaine aldehyde at 30°C according to the method of von Tigerstrom and Razzell (1968). One unit is defined as the activity that catalyzes the formation of 1 µmol of product (NADH) per minute.

Plant experiments

Seeds of *P. lathyroides* (wild) were surface-sterilized and germinated and grown in Leonard jars using standard methods (Somasegaran and Hoben, 1994). Plant inoculation and nodulation competition experiments were conducted as described previously (Payakapong et al., 2004).

3. Results

Isolation of the salt-tolerant Sinorhizobium *strain BL3*

Three hundred and seventy three rhizobial isolates were collected from the nodules of the wild bean *P. lathyroides* that grew in the saline uncultivated areas of the Nakhon Ratchasima province, northeastern region of Thailand. These isolates were then tested on YEM broth containing 300 mM NaCl and eight isolates that grew well were selected. One of these isolates, strain BL3, could tolerate up to 600 mM NaCl in YEM broth, although at salt concentrations above 300 mM, its growth was much inhibited (Fig. 1). Therefore, this strain was selected for further studies on salt tolerance.

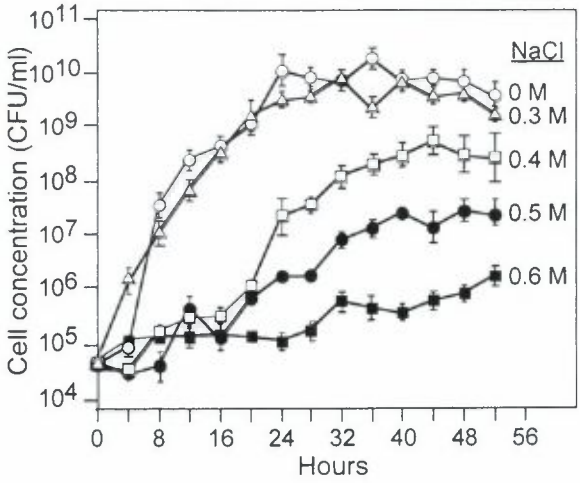

Figure 1. Growth of *Sinorhizobium* sp. BL3 in YEM medium containing different salt concentrations; 0 mM NaCl (open circle), 0.3 M NaCl (open triangle), 0.4 M NaCl (open square), 0.5 M NaCl (close circle), 0.6 M NaCl (close square). The error bars represent the standard deviation from three replications.

The strain BL3 is fast-growing and forms opaque creamy wet colonies on YEM agar within 3 days. The colonies show acidic reactions on YEM agar containing bromothymol blue. BL3 can utilize esculin, glucose and mannitol, but not arginine, gelatin, citrate, and arabinose. It forms effective nodules on *P. lathyroides*, *Centrocema pascuorum*, *Macroptilium atropurpureus*, and ineffective nodules on *Vigna radiata*, *V. umbellate* and *V. sinensis*. When BL3 was used to inoculate with *P. lathyroides* grown in the presence of 100 mM NaCl, the plants were able to form effective nodules. In the presence of 150 mM NaCl, although the inoculated plants produced effective nodules, their number was significantly reduced (Table 2).

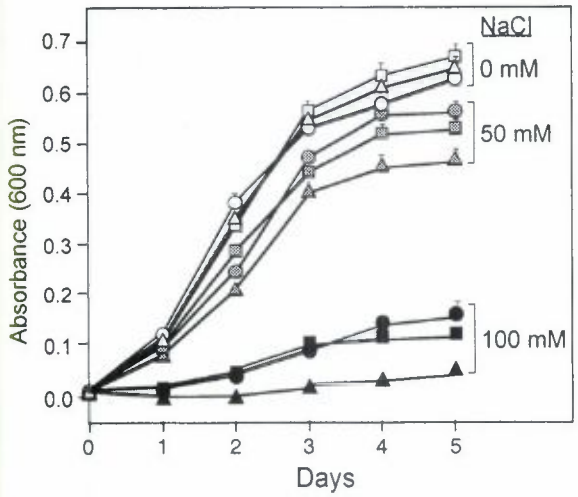

Figure 2. Growth of *B. japonicum* THA6 (triangle) and its transconjugant derivatives THA6(pUHR307) (circle) and THA6(pUHR310) (square) in minimal medium containing 0 mM (open symbols), 50 mM (shaded symbols) and 100 mM NaCl (close symbols). The error bars represent the standard deviation from three replications.

To analyze the taxonomic relationship of the strain BL3, the 16S rRNA gene was sequenced. The GenBank accession number for the 16S rRNA gene sequence of this strain is AY943949. It shows 99% similarities with the 16S rRNA gene sequences of *Sinorhizobium meliloti* strain LMTR32 and *Sinorhizobium* sp. SEMIA 6161. Therefore, BL3 was named as *Sinorhizobium* sp. strain BL3.

Identification of two clusters of BL3 genes for salt tolerance

To isolate the genes for salt tolerance, the cosmid clone library of genomic DNA of BL3 was transferred to the salt-sensitive *Rhizobium* sp. strain TAL1145. The transconjugants were plated on YEM agar containing 100 mM NaCl to select salt-tolerant colonies. Two hundred such salt-tolerant colonies were selected. From 24 of these transconjugants, the cosmid DNA was isolated. The restriction analyses of these clones using *Hind*III showed two different digestion patterns, indicating that the cloned DNA represented two regions of BL3 genome. The first region showed five similar restriction patterns. Restriction analysis of these cosmid clones using *Hind*III revealed three overlapping fragments of sizes 8.8, 4.9 and 3.2-kb. One of these cosmid clones, pUHR307, was used for further analyses. The second group of cosmid clones, represented by pUHR310, showed only one restriction pattern with five *Hind*III fragments of sizes 6.5, 6.2, 4.8, 3.8 and 2.2-kb. When pUHR307 and pUHR310 were each reintroduced into the salt-sensitive strain TAL1145, the resulting transconjugants could grow in the presence of 100 mM NaCl, but not in 200–600 mM NaCl as BL3, indicating that each of these two cosmids contains only some of the genes required for salt tolerance.

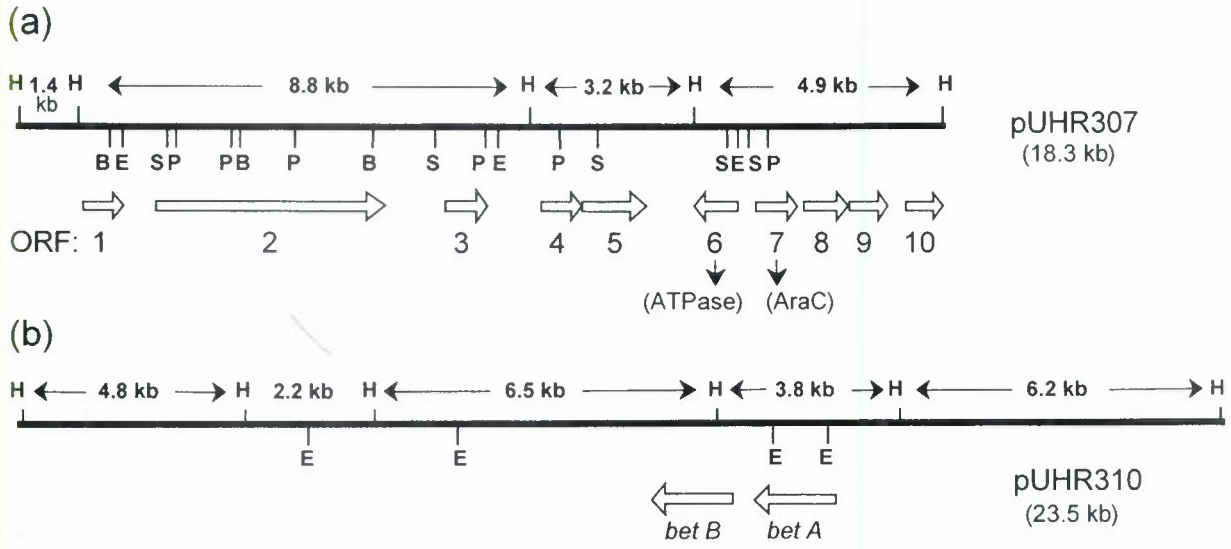

Figure 3. Restriction maps of the two regions of BL3 DNA containing genes involved in salt tolerance. The first region contains 18.3-kb DNA, cloned in pUHR307 (a) and the second region contains 23.5-kb DNA, cloned in pUHR310 (b). The positions and directions of ORFs are indicated with open arrows. The sizes of the *Hind*III fragments are indicated.

The transconjugants of salt-sensitive Bradyrhizobium japonicum *THA6 containing pUHR307 and pUHR310 have higher salt tolerance than THA6*

To determine if the cloned genes in pUHR307 and pUHR310 confer salt tolerance in slow-growing rhizobia, these two cosmids were introduced to a salt-sensitive *B. japonicum* strain THA6 by conjugation. The transconjugants were tested for ability to grow and survive under salt stress conditions using minimal medium containing different concentrations of NaCl. The salt-tolerance ability of the transconjugants was elevated compared to THA6 in the presence of 50 mM or 100 mM NaCl (Fig. 2). This suggests that genes present in pUHR307 or pUHR310 provide some degree of salt tolerance in *Bradyrhizobium*, although the level of salt tolerance in these transconjugants is not as high as in BL3.

DNA of cluster-I includes genes for ATPase and transcriptional regulation, while cluster-II contains genes for glycine betaine biosynthesis

To identify the cluster-I genes for salt tolerance, the three *Hind*III fragments of pUHR307 were subcloned and sequenced. Ten open reading frames (ORF) were identified by sequence analyses (Fig. 3). Among the ten ORFs, ORF6 and ORF7 may be involved in salt tolerance, based on their homologies to genes with known functions. The ORF6-encoded protein shows 76% of identities with ATPase of *Rhodospirillum rubrum*, and therefore, it may be involved in Na^+ export channel for enhancing salt tolerance of BL3. The ORF7-encoded protein may be a transcriptional regulator of AraC family that is known to regulate diverse bacterial functions including responses to stress. To identify genes involved salt tolerance from the second region of BL3 genome, the *Hind*III and *Eco*RI fragments of pRUH310 were subcloned in pUC18 and sequenced from both ends. Sequence analysis of a 7.7-kb *Eco*RI fragment and a 3.8-kb *Hind*III fragment showed that the insert DNA in pUHR310 contains the *bet* operon for biosynthesis of glycine betaine, which is known to be an osmoprotectant in many bacteria. The deduced amino acid sequence exhibited 92% identities with the choline dehydrogenase (BetA) and 95% identities with betaine aldehyde dehydrogenase (NADH) oxidoreductase NAD protein (BetB) of *S. meliloti*.

The expression of the *bet* genes in the transconjugants of THA6 containing pUHR310 was verified by measuring the betaine aldehyde dehydrogenase activity. The transconjugants exhibited 1.35-fold and 1.43-fold higher enzyme activities than THA6 when grown in the presence of 100 mM choline, and 100 mM choline plus 50 mM NaCl, respectively (Table 3). These results indicate that the *bet* genes in pUHR310 are expressed in THA6 transconjugants, which probably results in an increase in salt tolerant ability. When the transconjugants of THA6 containing pUHR307and pUHR310 were tested on soybean for nodulation competition under salt stress conditions in paired inoculation tests, the transconjugants did not show any detectable level of superiority over THA6 in nodule occupancy at 50 mM or 100 mM salt conditions (data not shown). Thus, the improvement of salt tolerance ability in THA6 transconjugants did not lead to increased nodule occupancy under salt stress conditions.

4. Discussion

Sinorhizobium sp. strain BL3 shows high-level of salt tolerance and forms effective nodules under salt-stress conditions. Numerous osmotic adaptation mechanisms for survival under salt stress condition have been reported among microorganisms. Among rhizobia, many strains of *R. fredii* show increased levels of intracellular free glutamate and/or K^+ at high salt concentrations (Fujihara and Yoneyama, 1994). Trehalose accumulates to higher levels in cells of peanut bradyrhizobia under conditions of hyper-salinity (Streeter and Bhagwat, 1999). *S. meliloti* responded to salt stress by accumulation of several compatible solutes, including glycine betaine (Pocard et al., 1997; Talibart et al., 1994; Gouffi et al., 1999). In the present study, the isolation of the gene cluster for biosynthesis of glycine betaine suggests that one of the mechanisms of salt tolerance in the strain BL3 is through production of glycine betaine. The *bet* gene cluster in pUHR310 provided only partial tolerance to salt-stress in the transconjugants of *Rhizobium* TAL1145 and *B. japonicum* THA6. These results indicate that besides glycine betaine production to maintain osmotic balance, BL3 must have additional mechanisms for salt stress tolerance.

Bacterial cells maintain turgor by first increasing their potassium (K^+) content and then replacing part of the accumulated K^+ with compatible solutes in the second phase of osmoadaptation (Wood, 1999). *E. coli* possesses two Na^+/H^+-antiporters, both of which are implicated in the maintenance of pH homeostasis and salinity tolerance (Padan and Schuldiner, 1994). The retranslocation of Na^+ is coupled to inverse H^+-gradients created by H^+-ATPases. ATPase was shown to be involved in salt stress response of several other organisms. *Saccharomyces cerevisiae* plasma membrane H^+-ATPase activity was observed by the addition of NaCl into the culture medium (Watanabe et al., 1993). Co-expression of the cell membrane associated H^+-ATPase and Na^+/H^+-antiporter can induce salt tolerance in *S. cerevisiae* cells (Watanabe et al., 2005). In the present study, the ATPase identified in pUHR307 may be involved in maintaining H^+-gradient in the cell during salt stress.

The AraC family of transcriptional activators regulates diverse bacterial functions including sugar catabolism, virulence, and responses to stress. A large number of these proteins were classified as stress response activators. For example, Ada in *E. coli* is produced in response to

alkylating agents (Demple et al., 1985); SoxS in *E. coli* and *S. typhimurium* are synthesized in response to oxidative stress (Ama'bile-Cuevas et al., 1991; Wu and Weiss, 1991). Similarly, transcriptional activators MarA and Rob in *E. coli*, and RamA in *Klebsiella pneumoniae* are involved in tolerance to antibiotics, organic solvents, and heavy metals (George et al., 1995; Skarstad et al., 1993). It is possible that the transcriptional activator protein encoded by ORF7 in the present study is also involved in inducing tolerance to salt stress in BL3.

The two gene clusters for salt tolerance identified in this study express in both fast and slow growing rhizobia. Each of these two clusters confers only a low level of tolerance to the transconjugants of salt-tolerant strains. For high level of salt tolerance, both clusters of genes have to be transferred to salt-sensitive strains. Characterization of these genes for salt tolerance will help to develop more effective strains of rhizobia for salinity conditions.

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