CHAPTER IV

DISCUSSION

The unicellular cyanobacterium *A. halophytica* is a highly halotolerant organisms. It can grow at high external NaCl concentration up to 3.0 M (Takabe et al., 1988). One of the mechanism enabling the halotolerance is the accumulation of glycine betaine. Glycine betaine is a major osmoprotectant, accumulated up to 2.0 M in response to changes of extracellular salinity (Reed et al., 1984). Biosynthesis of glycine betaine results from the conversion of choline to betaine aldehyde by choline dehydrogenase, and betaine aldehyde dehydrogenase converts betaine aldehyde to glycine betaine (Kumarb, 1997; Wutipraditkul, 1998). In *E. coli* and *R. meliloti*, genes coding for these two enzymes are called *betA* and *betB*, respectively (Styrvold et al., 1986; Pocard, et al., 1997). When *A. halophytica* was cultured under salt stress, the activity of betaine aldehyde dehydrogenase in the cells increased (Kumarb, 1997). The level of glycine betaine inside the cells was also increased. Therefore, choline-glycine betaine pathway was an important mechanism of salt tolerance in *A. halophytica*. To study the system in detail, an attempt to clone *betB* gene was considered.

Three selective strategies were used in this study. The so-called "bet phenotypic test" was the first strategy used to select the *bet* containing recombinant clones in the *A. halophytica* chromosomal DNA library. The *bet* deletion mutant host, *E. coli* strain JM109, was employed as the host for transformation. In the presence of 0.7 M NaCl, the growth rate of *E. coli* JM109 was retarded by about 50% from that in the absence of NaCl (Fig. 3.3). The concentration of NaCl used was similar to that used by Andresen et al.(1988) who cloned the *betABIT* genes from *E. coli* wild type strain CSH7, into the *bet* deletion mutant strain, *E. coli* MC4100, and

was selected using 0.65 M NaCl. The *gbsAB* genes from *B. subtilis* were cloned into the *bet* deletion mutant strain, *E. coli* MKH13 and was selected using 0.7 M NaCl (Boch et al., 1996). The recombinant plasmid containing *bet* genes would render the host cell halotolerant, i.e. the growth rate was not retarded in the presence of salt. Under the selection pressure, choline and/or betaine aldehyde were provided as substrate for the choline-glycine betaine pathway. The choline and betaine were expected to be converted to glycine betaine in the *bet* containing transformant making it tolerant to salt. This expectation was seen in Fig. 3.4, when 1 mM glycine betaine was added into the salt medium, the growth rate of cell was close to normal. Fig. 3.4 also confirmed the *bet* deletion genotype of *E. coli* JM109 since choline and betaine aldehyde could not alleviate the suppressive effect of salt. The *Sau*3AI partially digested DNA fragments were cloned into *Bam*HI site of pUC18 and transformed into *E. coli* JM109. The transformants were selected for the BET phenotype on M63 agar containing 0.7 M NaCl and 1 mM choline or betaine aldehyde as the precursor of glycine betaine. In *E. coli* and *B. subtilis*, genes coding for choline dehydrogenase and betaine aldehyde dehydrogenase are adjacent in the chromosomal DNA (Lamark et al., 1991; Boch et al., 1996). The corresponding genes in *A. halophytica* are expected to be also tightly genetically linked. It could be expected that the positive recombinant clone should contain *betAB* when choline was used as substrate.

Unfortunately, after the screening of several thousands of transformants, no clones exhibited salt tolerance. The failure of screening for the *A. halophytica* genes encoding the glycine betaine synthesis enzymes by the phenotypic selection might be due to the inability of *E. coli* host cells to express the cyanobacterium *bet* genes. Nomura et al. (1995) had shown that the *bet* genes of *E. coli* could be expressed in the cyanobacterium *Synechococcus* PCC7942 but the reverse had not been confirmed. The drawback of this strategy was the lack of positive control to show that the selection system worked.

The second strategy was the use of oligonucleotides specific to the genes of interest in the selection system using colony hybridization. The oligonucleotide probes were derived from the nucleotide sequence comparison of *betB* related genes from bacteria; *E. coli*, *R. meliloti* and *B. subtilis*, and plants; *S. bicolor*, *A. hortensis*, *H. hypochondrocus*, *B. vulgaris*, *H. vulgare* and *S. oleracea*. Oligonucleotide number were from the N-terminal coding sequence and the C-terminal coding sequence, respectively (Fig. 3.5). The homology among organisms within these 2 sequences were about 60%. The sequences of probe no.4402 and 4403 were 5′ TGGAACTTG GCGGTAAAA 3′ and 5′ GCCCCTGCGCTGGCCGCTGG 3′, respectively. Some colonies were selected from colony hybridization. The recombinant plasmids were prepared and analyzed by agarose gel electrophoresis. It was, however, suspicious that the plasmids were not the desired clones. The *A. halophytica* chromosomal DNA was run on an agarose gel, transferred onto nylon membrane and hybridized with both probes. It turned out that the probes were not specific to *A. halophytica* chromosomal DNA. Therefore, the recombinant plasmids were not the desired clones. Since the probes were designed from homologous sequences of *betB* related genes from several organisms but not that of *A. halophytica*, the probes might not be suitable for use in this selection.

Since the *betB* related genes from other organisms had some degrees of homology to the *betB* gene of *A. halophytica*, it was possible to use *betB* gene from other organism as probe. The *gbsA* gene, a *betB* related gene from *B. subtilis*, was kindly provided by Dr. E. Bremer. Nucleotide sequence comparison of the *gbsA* gene with the *betB gene* of *E. coli* and *R. meliloti* revealed 52% and 45% of identical sequence, respectively (Appendix 4 and 5), while the homology between *E. coli* and *R. meloloti* was 62% (Appendix 6). Thus, the *gbsA* probe was used to probe the *betB* in *A. halophytica*. In order to find a single fragment containing the *betB* gene, the chromosomal DNA was digested separately with several restriction enzymes and

subjected to agarose gel electrophoresis. Southern blot hybridization revealed a band of about 9.4 kb for *Eco*RI digested DNA. The *Bam*HI, *Hind*III and *Pst*I, gave the hybridization signals of more larger 23.1 kb. Other restriction enzymes should be tested in order to obtaine smaller fragments suitable for cloning. Once suitable fragment(s) was obtained, the fragment could be eluted and cloned. The *gbsA* could be used further to probe the cloned gene.