Factors Affecting the Accumulation of Glycinebetaine in a Halophilic Cyanobacterium, *Aphanothece halophytica*

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The effects of various factors on the accumulation of glycinebetaine have been investigated in a halophilic cyanobacterium *Aphanothece halophytica*. The increase of NaCl in the growth medium from 0.5 to 1.0 and 2.0 M caused an elevation of intracellular glycinebetaine by about 2- and 8-fold, respectively. Salt-stressed cells could synthesize glycinebetaine at a higher rate than the control cells and the longer the duration of stress, the higher the synthetic rate. Cell growth was retarded under hypersalinity conditions. Determination of glycinebetaine by either ^{1}H-NMR or tri-iodide method gave closely agreeing results; although the latter method required preliminary purification of the sample. The presence of 1 M sorbitol in the growth medium could also induce the increase of glycinebetaine by about 5-fold. Supplementation of 20 mM $NaNO_3$ to the growth medium caused about a 5-fold increase of glycinebetaine in salt-stressed cells but no change was observed in non-stressed cells. Similarly the increased glycinebetaine by about 3-fold occurred only in salt-stressed cells under the influence of light.

Key words: Glycinebetaine accumulation, halophilic cyanobacterium, *Aphanothece halophytica*.

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ปัจจัยที่มีผลต่อการสะสมไกลซีนบีเทนในไซยาโนแบคทีเรียมชนิดชอบเค็ม, *อะฟาโนทีคี ฮาโลฟิทิคา*

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ทำการศึกษาผลของปัจจัยต่างๆ ต่อการสะสมไกลซีนบีเทนในไซยาโนแบคทีเรียมชนิดชอบเค็ม *อะฟาโนทีคี ฮาโลฟิทิคา* พบว่าความเข้มข้นของโซเดียมคลอไรด์ในอาหารเลี้ยงที่เพิ่มขึ้นจากปกติ 0.5 โมลาร์เป็น 1.0 และ 2.0 โมลาร์ ทำให้เกิดการเพิ่มขึ้นของไกลซีนบีเทนภายในเซลล์ ประมาณ 2 และ 8 เท่าตามลำดับ เซลล์ที่เลี้ยงภายใต้สภาวะความเครียดอันเนื่องจากเกลือสามารถสังเคราะห์ไกลซีนบีเทนในอัตราที่สูงกว่าเซลล์ที่เลี้ยงภายใต้สภาวะปกติ นอกจากนี้การเลี้ยงในสภาวะความเครียดที่นานขึ้นก็จะทำให้อัตราการสังเคราะห์ยิ่งสูงขึ้น การเจริญของเซลล์ลดลงภายใต้สภาวะความเค็มของเกลือที่เพิ่มขึ้น การหาปริมาณไกลซีนบีเทนในเซลล์ทำได้โดยวิธีโปรตอนเอนเอมอาร์ หรือวิธีไทรไอโอไดด์ซึ่งค่าที่หาได้จาก 2 วิธีนี้ใกล้เคียงกันมาก แต่ถ้าใช้วิธีที่ 2 ในการหาค่าจำเป็นจะต้องผ่านขั้นตอนทำให้บริสุทธิ์บางส่วนก่อน การเติม 1 โมลาร์ ซอร์บิทอลในอาหารเลี้ยงก็สามารถชักนำให้ปริมาณไกลซีนบีเทนภายในเซลล์เพิ่มขึ้นประมาณ 5 เท่า การเสริม 20 มิลลิโมลาร์โซเดียมไนเตรทลงไปเพิ่มในอาหารเลี้ยง หรือการให้แสงสว่างขณะเลี้ยงเซลล์ทำให้ไกลซีนบีเทนภายในเซลล์เพิ่มขึ้น 5 เท่า และ 3 เท่าตามลำดับ เฉพาะในกรณีที่เลี้ยงเซลล์ในสภาวะความเครียดอันเนื่องจากเกลือเท่านั้น

คำสำคัญ การสะสมไกลซีนบีเทน, ไซยาโนแบคทีเรียมชนิดชอบเค็ม, *อะฟาโนทีคี, ฮาโลฟิทิคา*

INTRODUCTION

Organisms living in hypersaline environments have specific mechanisms that enable them to adjust their internal osmotic status. One such mechanism usually involves

the accumulation of inorganic ions and some organic solutes. Since high intracellular concentrations of inorganic ions may be detrimental to cellular functions, many organisms appear to overcome this problem by accumulating one or more organic solutes of low molecular weight such as amino acids, polyols, and quaternary ammonium compounds, that can act as compatible solutes.[1] Among the so-called compatible solutes, the quaternary ammonium compound glycinebetaine (N, N, N-trimethylglycine: betaine) has recently become the subject of intensive studies.[2] The osmoprotective role of glycinebetaine is evident in a number of diverse microbial systems, including enteric bacteria,[3] soil bacteria,[4] halophilic bacteria, methanogenic archaebacteria[5] and cyanobacteria.[6] In higher plants the accumulation of glycinebetaine has been demonstrated to be an adaptive response to hyperosmotic stress.[7] The function of glycinebetaine as an osmolyte has also been reported in mammalian renal cells[8] as well as in invertebrate cardiac cells.[9] Besides its physiological role as an osmoprotectant, glycinebetaine also functions as a methyl group donor where this methyl group is incorporated into alkaloids,[10] methionine[11] and cobalamin.[12] Furthermore, glycinebetaine can be utilized as a carbon or nitrogen source by some microorganisms.[13]

In cyanobacteria, the highly halotolerant strains accumulate glycinebetaine as a major osmolyte whereas the less tolerant strains accumulate either sucrose or glucosylglycerol.[6, 14, 15] Glycinebetaine was shown to prevent the dissociation of cyanobacterial ribulose-1, 5-bisphosphate carboxylase and restore the activity inhibited by 0.25 M KCl[16] and to reverse the inhibition of glutamine synthase by up to 2 M NaCl in two cyanobacteria.[17]

Despite the importance of glycinebetaine in many physiological functions, the mechanism governing its accumulation inside the cells has been well studied only in higher plants.[18, 19] In the present study we looked at the factors that could affect the accumulation of glycinebetaine in a halophilic cyanobacterium, *Aphanothece halophytica*.

MATERIALS AND METHODS

Culture conditions

A.halophytica was grown photoautotrophically in a BG 11 medium plus 18 mM $NaNO_3$ and Turks Island Salt Solution.[20] Cells were grown in 250-ml flasks containing 100 ml of medium on a rotary shaker with an incident white light illumination of 60 μmol $m^{-2}s^{-1}$ at 30°C without CO_2 supplementation. The concentration of NaCl in the culture-medium was adjusted by adding NaCl as required (0.5 M, 1.0 M, 1.5 M, 2.0 M). Growth of the cells was followed by measuring the turbidity of the culture at 750 nm. The cell number was determined by a haemocytometer.

Determination of glycinebetaine in *A. halophytica*

Culture of *A. halophytica* was centrifuged at 8,000 g for 20 min to collect the cells. The cell pellet was extracted by incubation in boiling 80% (v/v) ethanol for 5 min as described by Reed.[21] To ensure complete extraction the pellet was re-extracted with 80% (v/v) ethanol and incubated for 18 h at 25°C. The combined suspension after removal of the cell pellet was pooled and evaporated to dryness before subjecting it to analysis by ^{1}H-NMR and UV-spectrophotometry.

The dried residue was dissolved in 1.5 ml of D_2O and 0.6 ml of solution was transferred to a 5 mm NMR tube to which was added 5 μl of 1% sodium 2, 2-dimethyl-2-silapentate-5-sulfonic acid (DSS). The ^{1}H-NMR spectrum was run on a JEOL JMN-A 500 Fourier transform NMR spectrometer operating at a frequency of 500 MHz and a probe temperature of 29°C.[22] The quantitation of glycinebetaine was obtained by comparing the integrated peak intensity against a standard curve.

The analysis for glycinebetaine content by UV-spectrophotometry was done by dissolving the dried residue in 1.5 ml of distilled water and loading onto a Dowex-50W column (1 x 3 cm, H^+ form) to remove other quaternary ammonium compounds. The column was washed with 10 ml of distilled water followed by 20 ml of 2 M NH_3 to elute glycinebetaine. The eluate obtained was dried by lyophilization. The dried pellet was analyzed for glycinebetaine by tri-iodide assay according

to Storey and Wyn Jones.[23] The dried pellet dissolved in 0.25-0.6 ml of distilled water was added to 0.2 ml of acid potassium tri-iodide solution. The mixture was shaken for at least 90 min in an ice bath; 2 ml of ice-cold water was then added rapidly to the mixture to reduce the absorbance of the blank. This was quickly followed by 5 ml of 1, 2-dichloroethane and the two layers were mixed by stirring. The absorbance of the lower organic layer was measured spectrophotometrically at 365 nm. The quantitation of glycinebetaine was obtained by comparing the absorbance against a standard curve. The values shown in the figures and tables represent the mean of 2 independent experiments.

Biosynthesis of glycinebetaine in *A. halophytica*

The biosynthesis of glycinebetaine was determined by measuring the rate of conversion of radioactive choline into radioactive glycinebetaine. The culture of *A. halophytica* grown at various times in medium containing either 0.5 M or 2 M NaCl was centrifuged at 2,000 g for 10 min. The cell pellet was suspended in 0.5 ml of 50 mM HEPES-NaOH buffer pH 7.5 containing either 0.5 M or 2 M NaCl. The suspension was incubated with 40 μM [methyl-^{14}C] choline (55 mCi/mmol) at 30°C for 3 h. The reaction was stopped by adding 1 ml of methanol followed by centrifugation. The pellet was washed twice with either 0.5 M or 2 M NaCl to remove excess label before subjecting to extraction with boiling 80% (v/v) ethanol for 5 min. The re-extraction with the same solvent was carried out at room temperature for 18 h. The combined extract was centrifuged at 2,000 g for 5 min and the supernatant was dried at 65°C. The dried residue was suspended in 0.5 ml of distilled water containing 1 mM glycinebetaine. This suspension was loaded onto a Dowex 50 W (50x4-200, H^+ form, Sigma) column (0.9x1.3 cm) and the elution of [^{14}C] glycinebetaine was done by 15 ml of 2 M NH_3. The radioactivity of [^{14}C] glycinebetaine in the eluate was determined by liquid scintillation counting. The mean value of 2 independent experiments is shown in the results.

RESULTS AND DISCUSSION

Effect of salinity on the growth of *A. halophytica.*

The growth of *A. halophytica* previously maintained in 0.5 M NaCl in response to increased salinity is shown in Figure 1. Although *A. halophytica* requires NaCl for its normal growth, the increased concentration of NaCl to 1 M and 2 M retarded the growth rate during the first 6 days of cultivation. However, after 6 days the growth rate did not appear to be affected by NaCl. High salinity can cause the cessation of growth and eventually lead to cell death if the organism does not have the mechanism to withstand salinity stress. *A. halophytica* responded to high salinity in the manner that its growth was delayed during the initial period of salinity stress. An analogous phenomenon was also observed in a fresh water cyanobacterium *Synechococcus* 6311.[24] After transferring *Synechococcus* 6311 to salinity in the range 0.2 to 0.4 M NaCl, the photosynthetic activity initially decreased. However, the cells later increased in photosynthetic activity by 2-fold. The capacity of *A. halophytica* to sustain growth at high salinity has been attributed to the increased photosynthetic activity and the increase in the ribulose-1, 5-bisphophate carboxylase activity and content of the cells.[25] Since the growth rate of the salinity-stressed *A. halophytica* did not increase, it is likely that the products resulting from increased photosynthetic activity were diverted to the process of osmoregulation. To test this possibility we attempted to find out how the increased salinity could alter the content of glycinebetaine inside *A. halophytica.*

Fig. 1 Effect of external salinity on the growth of *A. halophytica.*

Fig. 2 ^{1}H-NMR spectrum of extract from *A. halophytica* grown in a normal medium. Peaks marked GB (glycinebetaine) and Ch (choline) have their ^{1}H-NMR resonance positions for N-methyl protons at 3.253 and 3.191 ppm, respectively. Dimethyl silapentate sulfonic acid (DSS) serves as a reference position.

Effect of salinity on the glycinebetaine content of *A. halophytica*

The quantitation of glycinebetaine was done by 2 methods, namely ^{1}H-NMR spectrometry and tri-iodide assay by UV-spectrophotometry. Figure 2 represents the ^{1}H-NMR spectrum of quaternary ammonium compounds extracted from *A. halophytica* grown in 0.5 M NaCl-containing medium. Glycinebetaine was clearly separated from choline with peaks at 3.25 ppm and 3.19 ppm, respectively. The tri-iodide assay for glycinebetaine was based on the precipitation of quaternary ammonium compounds by iodine to form periodides.[26] The periodides were subsequently extracted by 1, 2-dichloroethane and subjected to ultraviolet absorption at 365 nm. Figure 3 shows the linear standard curve of glycinebetaine. The linear regression analysis gave the equation $y = 5.752x$. This method was able to detect a nanomol level of glycinebetaine. Nevertheless, before using

this method it is essential that other co-existing quaternary ammonium compounds must be removed by ion-exchange column. We used a Dowex-50 W column to purify glycinebetaine and the purity (checked by thin layer chromatography) as well as the yield were very satisfactory; only one spot of glycinebetaine was detected and the column gave 100% recovery of glycinebetaine.

The accumulation of glycinebetaine induced by salinity in *A. halophytica* was determined by transferring the cells normally grown in 0.5 M NaCl-containing medium to the medium containing 1.0 and 2.0 M NaCl. Table 1 shows that higher salinity could induce the increased content of glycinebetaine inside the cells. The increase of glycinebetaine appeared to be proportional to the increase of NaCl. It is therefore likely that glycinebetaine serves as a major osmolyte in *A. halophytica.* The accumulation of glycinebetaine not only plays a role in osmoregulation but also contributes to the beneficial effect on the metabolic activities of the cells. In *Synechocystis* DUN 52 glycinebetaine exhibited protective effects on glutamine synthase activity.[17] Also in *Spirulina subsalsa*, glycinebetaine was able to protect glucose-6-phosphate dehydrogenase against inhibition by NaCl.[27] Moreover, previous studies in *A. halophytica* have shown that the inhibition of ribulose-1, 5-bisphosphate carboxylase by Cl^- can be relieved in the presence of glycinebetaine.[16, 28] It is worth mentioning here that the analysis of glycinebetaine as determined by ^{1}H-NMR and tri-iodide methods gave comparable results (Table 1). In later determinations of glycinebetaine only the tri-iodide method was used.

Glycinebetaine (nmol)

Fig. 3 Standard curve of glycinebetaine assayed by the tri-iodide method.

Table 1. Effect of external salinity on intracellular glycinebetaine and growth of *A. halophytica.* Log phase cells grown in medium containing 0.5 M NaCl were transferred and grown in fresh medium containing various NaCl concentrations. The cells were harvested after 10 days and were analyzed for cell density and glycinebetaine level as described in Materials and Methods.

NaCl (M)	Cell density (x10^6 /ml)	Glycinebetaine (nmol/10^6 cells)	
		^{1}H-NMR	Tri-iodide
0.5	23.4	9.7	9.1
1.0	16.3	26.9	26.4
2.0	4.3	76.0	78.7

Effect of salt stress on the glycinebetaine synthesis of *A. halophytica.*

To investigate the effect of salt stress on the synthesis of glycinebetaine, *A. halophytica* was grown in 0.5 M (control) and 2 M (salt stress) NaCl for various times. The cells were then withdrawn and analyzed for [^{14}C] glycinebetaine synthesis using [^{14}C] choline as a precursor. Glycinebetaine synthesis in the control was relatively unchanged up to day 4 and increased about 1.5-fold at day 6 (Table 2). When cells were subjected to salt stress glycinebetaine synthesis increased about 2-fold at day 2 and a drastic rise in the synthesis occurred at day 4. The overall results indicated an increase of about 10-fold for glycinebetaine synthesis during 6 days of salt stress. The data supported the result in Figure 2 showing the presence of endogenous choline inside *A. halophytica*. If the conversion of choline to glycinebetaine is the only pathway responsible for the synthesis of glycinebetaine we will be able to follow the relative increase and decrease for glycinebetaine and choline respectively due to salt stress. However, it is essential that two assumptions must be taken into account. First, glycinebetaine must not be catabolized further, which is the case in many organisms including higher plants.[29] Second, the intracellular content of choline must be relatively constant, i.e., the pathways generating choline and converting choline to other unrelated products should be negligible. The conversion of choline to glycinebetaine in radiotracer experiment in this study was substantiated by the findings that the two enzymes responsible for the pathway, namely choline and betaine aldehyde dehydrogenases, were functioning in *A. halophytica*. Details of such findings will appear in a subsequent communication. Future experiments are required to determine whether the increase in glycinebetaine synthesis due to salt stress can approach or keep up with the rate of glycinebetaine accumulation. Nevertheless it is clear that the accumulation of glycinebetaine in *A. halophytica* occurred as a result of increased synthesis.

Effect of sorbitol on the glycinebetaine content of *A. halophytica*

The increase of salt in the growth medium can increase the level of glycinebetaine in *A. halophytica*. This increase can be attributed to either the direct influence of increased salinity or to the indirect influence of the decrease in the external water potential. To test this hypothesis, an organic solute, namely sorbitol, was used instead of NaCl as an external osmoticum. Glycinebetaine content in *A. halophytica* grown in the presence of 1 M sorbitol was about 5-fold higher than that in the absence of sorbitol (Table 3). The result suggested that the increased content of glycinebetaine was induced by an osmotic effect rather than by a salinity-specific effect.

Table 2. Effect of salt stress on [^{14}C] glycinebetaine synthesis in *A. halophytica.* Log phase cells grown in medium containing 0.5 M NaCl were transferred and grown in fresh medium containing either 0.5 M (control) or 2.0 M (salt stress) NaCl for various times as indicated. The synthesis of [^{14}C] glycinebetaine was determined as described in Materials and Methods.

Day	Synthesis of [^{14}C] glycinebetaine ($nC_i/10^9$ cells)	
	control	salt stress
0	0.32	0.32
2	0.28	0.64
4	0.34	2.65
6	0.52	3.11

The osmotic effect due to sorbitol also resulted in a retardation of cell growth. Cell density was increased by about 1.5-fold in sorbitol-grown cells as compared to about 10-fold in control cells. Although *A. halophytica* was able to grow in the presence of 1 M sorbitol its growth rate was much lower than that in NaCl at the same osmolarity (data not shown). Sorbitol is a non-ionizable carbohydrate compound and cannot penetrate into the cells whereas Na^+ and Cl^- can be taken up by the cells. This suggests that the uptake of ions by the cell is an important first step to trigger the cell mechanism for readjustment of the cell volume at the initial stage of osmotic stress. The osmotic stress signal elicited by sorbitol was probably received by the cells later rather than sooner as compared to that by NaCl.

Table 3. Effect of sorbitol on intracellular glycinebetaine and growth of *A. halophytica.* Log phase cells grown in medium containing 0.5 M NaCl were transferred and grown in fresh medium containing either 0.5 M NaCl without sorbitol or 0.5 M NaCl with 1 M sorbitol. Cell density and glycinebetaine level were determined at the onset and after 7 days growth as described in Materials and Methods.

Day	Without Sorbitol		With 1 M Sorbitol	
	Glycinebetaine (nmol/10^6 cells)	Cell density (10^6/ml)	Glycinebetaine (nmol/10^6 cells)	Cell density (10^6/ml)
0	2.9	1.4	2.9	1.4
7	3.1	14.2	15.1	2.2

Effect of nitrate supplementation on the glycinebetaine content of *A. halophytica*

As glycinebetaine is a nitrogen-containing compound, it is therefore relevant to study whether $NaNO_3$ which is one major component in the growth medium *of A. halophytica* can affect the content of glycinebetaine. The level of glycinebetaine in cells grown in a non-stressed condition was hardly affected by the supplementation of 20 mM $NaNO_3$ whereas in salt-stressed cells such supplementation induced the increase of glycinebetaine by about 6-fold after 7 days growth (Table 4). When cells were grown in salt stress conditions without the supplementation of $NaNO_3$, the level of glycinebetaine increased about 3-fold, whereas no increase was observed in control condition. This 3-fold increase of glycinebetaine occurred as a result of the combined effect of salt stress and $NaNO_3$ originally present in the growth medium. It should be noted here that the condition of salt stress by itself without $NaNO_3$ already contributed to about a 2-fold increase of glycinebetaine (data not shown). Since nitrogen for this increased glycinebetaine was not provided in the growth medium, the sources of available nitrogen were likely to derive from the metabolic degradation of some nitrogen-containing biomolecules like proteins or nucleic acids. The reduced level of many proteins has been reported in tobacco cells adapted to salt or water stress.[30] This could be

a result of either reduced gene expression of the affected proteins or the increased degradation of the proteins. It remains to be clarified whether salt stress in *A. halophytica* can cause an alteration in gene expression of some proteins, especially those responsible for the synthesis of glycinebetaine. However, overall results in Table 4 suggested that the supply of $NaNO_3$ could alleviate the salt stress in *A. halophytica* through the availability of nitrogen for the synthesis of glycinebetaine.

Table 4. Effect of supplementation of nitrate to medium containing different NaCl concentrations on intracellular glycinebetaine of *A. halophytica.* Log phase cells grown in medium containing 0.5 M NaCl were transferred and grown in fresh medium containing either 0.5 M or 1.5 M NaCl with or without additional 20 mM $NaNO_3$.

Day	Glycinebetaine (nmol/10^6 cells)			
	Without $NaNO_3$ Supplementation		With $NaNO_3$ Supplementation	
	0.5 M NaCl	1.5 M NaCl	0.5 M NaCl	1.5 M NaCl
0	2.5	2.4	2.5	2.4
7	2.4	7.5	2.8	15.3

Effect of light on the glycinebetaine content of *A. halophytica*

Photosynthetic organisms require light for their initial energy transduction process. *A. halophytica* is a unicellular cyanobacterium capable of O_2-evolving photosynthesis characteristic of plant cells. The relation of light to salt stress can be investigated with respect to the accumulation of glycinebetaine. As shown in Table 5 light did not change the level of glycinebetaine in non-stressed cells. When stressed with 1.5 M NaCl, a 3-fold increase in glycinebetaine was observed. In the dark the cells did not change their glycinebetaine regardless of the salt stress condition. The natural occurrence of *A. halophytica* is in the hypersaline lakes with abundant sunshine. The organism is able to use the light to create the proton gradient which finally results in the generation of ATP. This ATP would spare the use of organic nutrients for energy generation, and thereby release a higher proportion of those nutrients for biosynthesis of required compounds, including glycinebetaine. In this respect it would be worth investigating how an inhibitor of ATP synthesis could affect the process of glycinebetaine accumulation and synthesis.

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Table 5. Effect of light on intracellular glycinebetaine of *A. halophytica.* Log phase cells grown in medium containing 0.5 M NaCl were transferred and grown in fresh medium containing either 0.5 M or 1.5 M NaCl. Growth condition was either in the dark or under white light illumination at 60 μ mol m^{-2} s^{-1}.

Day	Glycinebetaine (nmol/10^6 cells)

	0.5 M NaCl		1.5 M NaCl	
	Dark	Light	Dark	Light
0	2.2	2.2	2.4	2.4
7	2.0	2.4	2.2	7.5

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