Journal of Biochemistry and Molecular Biology, Vol. 39, No. 4, July 2006, pp. 394-399

JOURNAL OF Biochemistry and Molecular Biology

Putrescine Transport in a Cyanobacterium *Synechocystis* sp. PCC 6803

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Received February 8 2006, Accepted April 3 2006

The transport of putrescine into a moderately salt tolerant cyanobacterium *Synechocystis* sp. PCC 6803 was characterized by measuring the uptake of radioactively-labeled putrescine. Putrescine transport showed saturation kinetics with an apparent K_m of 92 ± 10 μM and V_{max} of 0.33 ± 0.05 nmol/min/mg protein. The transport of putrescine was pH-dependent with highest activity at pH 7.0. Strong inhibition of putrescine transport was caused by spermine and spermidine whereas only slight inhibition was observed by the addition of various amino acids. These results suggest that the transport system in *Synechocystis* sp. PCC 6803 is highly specific for polyamines. Putrescine transport is energy-dependent as evidenced by the inhibition by various metabolic inhibitors and ionophores. Slow growth was observed in cells grown under salt stress. Addition of low concentration of putrescine could restore growth almost to the level observed in the absence of salt stress. Upshift of the external osmolality generated by either NaCl or sorbitol caused an increased putrescine transport with an optimum 2-fold increase at 20 mosmol/kg. The stimulation of putrescine transport mediated by osmotic upshift was abolished in chloramphenicol-treated cells, suggesting possible involvement of an inducible transport system.

Keywords: Cyanobacterium, Osmotic activation, Putrescine transport, *Synechocystis* sp. PCC 6803

Introduction

Living organisms have evolved strategies for the maintenance of metabolism subject to fluctuations in osmotic strength. The response to hyperosmotic stress can be separated into two main processes. First, the reduction of the water potential after hyperosmotic shock causes the cell to lose water and shrink. Consequently, this process is counteracted by an increase in organic solutes entering the cells along chemical gradients (Wood, 1999; Heide *et al*., 2001). Many organisms that thrive in high salinity use two strategies to acquire high intracellular amounts of compatible solutes: *de novo* synthesis and/or transport from the surroundings. Salt-loaded cells accumulate compatible solutes, which are low molecular weight compounds that do not interfere with cell metabolism, such as carnitine (Fraser and O'Byrne, 2002), choline (Incharoensakdi and Karnchanatat, 2003), ectoine (Jebbar *et al*., 1997), glycine betaine (Incharoensakdi and Wutipraditkul, 1999), proline (Dunlap and Csonka, 1985) and polyamines (Flores and Galston, 1984).

Polyamines, represented by putrescine, spermidine and spermine, play important roles in all aspects of cellular processes such as growth, development and biosynthesis (Tabor and Tabor, 1985; Bouchereau *et al*., 1999; Thomas and Thomas, 2001). Uptake systems specific for polyamines are reported in both prokaryotic and eukaryotic cells (Rinehart and Chen, 1984; Igarashi and Kashiwagi, 1999; Tassoni *et al*., 2002). The properties of three polyamine uptake systems were characterized by cloning the genes for these systems in *Escherichia coli* (Igarashi *et al*., 2001). The first system which is spermidine-preferential consists of PotA, PotB, PotC and PotD proteins whereas the second system which is putrescine-specific consists of PotF, PotG, PotH and PotI proteins. Both systems are classified as ABC [ATP binding cassette]-type transporters. The third system is involved in the excretion of putrescine by a putrescine-ornithine antiporter activity consisting of only the PotE protein. The importance of polyamine uptake by living cells is far from clear, since all organisms have enzymes able to synthesize them and maintain optimum levels for their metabolism. Indeed, most of studies for the uptake of polyamine were carried out in *Escherichia coli*. There are a few scattered reports on putrescine transport in cyanobacteria. Perhaps the earliest one was the study in *Anacystis nidulans* where the mechanism of putrescine transport was passive diffusion and ion trapping within the cells (Guarino and Cohen, 1979).

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Synechocystis sp. PCC 6803, which is a moderately salt-tolerant cyanobacterium, can accumulate glucosylglycerol as an osmoprotective compound by *de novo* synthesis (Mikkat *et al*., 1997; Ferjani *et al*., 2003). Previously, we reported that the effect of long-term salt and osmotic stresses resulted in the increase of the cellular putrescine contents of this cyanobacterium (Jantaro *et al*., 2003). In the present study, we have investigated putrescine transport in *Synechocystis* sp. PCC 6803 and found that putrescine transport was energy dependent, specific for polyamines and could be stimulated by moderate osmotic upshift.

Materials and Methods

Organism and culture conditions. Axenic cells of *Synechocystis* sp. PCC 6803 were grown photoautotrophically in BG-11 medium at 30°C under continuous illumination (warm white fluorescent tubes) of 50 μE/m^2/s. The cultivation was performed in cotton-plugged 250-ml conical flasks on a rotatory shaker at 160 rpm. The growth rate was monitored by measuring the optical density of the culture at 730 nm with a Spectronic® Genesys™2 spectrophotometer.

Transport assays. Cells at late log phase were harvested by centrifugation (8,000 × *g*, 10 min, 4°C), washed twice with 50 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid(Hepes)-KOH buffer pH 7.6 containing 0.4% glucose and suspended in the same buffer to yield a protein concentration of 0.1 mg cell protein/ml. The cell suspension (95 μl) was preincubated at 37°C for 5 min. The uptake experiment was initiated by the addition of 5 μl [methylene-^{14}C] putrescine (Radiochemical Centre) with a specific activity of 2 mCi/mmol at a final concentration of 50 μM. After incubation, the cells were rapidly collected on membrane filters (cellulose acetate, 0.45 μm pore size; Millipore Corp.). The filters were washed twice with 1 ml of cold buffer containing 1 mM putrescine to remove the adsorbed [methylene-^{14}C] putrescine. The amount of amine adsorbed to the cell surface and the filter was less than 0.1% of the added amine under these experimental conditions. The radioactivity on the filter was determined with a liquid scintillation counter. Initial putrescine uptake rates were determined from the linear increase of uptake and are expressed as nanomoles of putrescine taken up per minute per milligram protein. Cellular protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Cells were lysed by the addition of the Bradford reagent. After centrifugation, the supernatant was measured for optical density at 595 nm.The osmolality of the uptake assay medium was measured with a Wescor vapor pressure osmometer model 5520. For the assay of inhibition by substrate analogs, cells were added to a mixture of labeled substrate and 20-fold excess of unlabeled analogs. In inhibitory assays, cells were preincubated with the inhibitor for 30 min at 37°C before the addition of [methylene-^{14}C] putrescine.

Results

Protection against growth inhibition by putrescine. Growth of *Synechocystis* cells in the medium containing 550 mM NaCl was not as rapid as that in the absence of NaCl (Fig. 1). Addition of 0.5 mM putrescine could alleviate the effect of salt stress with apparently normal growth up to 6 days. The addition of 1 mM putrescine caused similar growth pattern to that of 0.5 mM putrescine during the first 3 days. Slower growth occurred after 3 days with the apparent cessation of growth observed after 6 days.

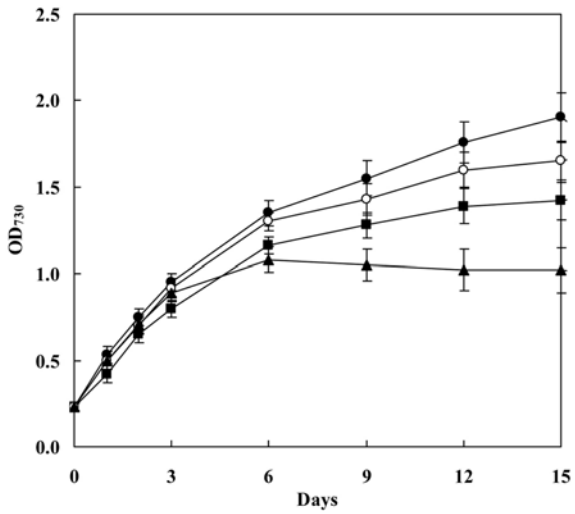

Fig. 1. Growth promoting effect of putrescine on salt-stressed *Synechocystis* sp. PCC 6803. Cells were grown under normal condition (●), salt stress condition by the addition of 550 mM NaCl (■), 550 mM NaCl plus 0.5 mM putrescine (○), 550 mM NaCl plus 1.0 mM putrescine (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

Kinetics of putrescine uptake. Incubation of *Synechocystis* cells with increasing concentration of putrescine up to 500 μM resulted in a saturable initial uptake rate (Fig. 2). A Lineweaver-Burk transformation of the data yielded a straight line typical of Michaelis-Menten kinetics. The apparent affinity constant (K_m) value of 92 ± 10 μM and the maximal velocity (V_{max}) value of 0.33 ± 0.05 nmol/min/mg protein were obtained.

Effect of external pH on putrescine uptake. Since putrescine carries a net positive charge, we therefore further tested whether the changes in external pH could affect its uptake. Among the three pH values tested, highest uptake occurred at neutral pH of 7.0 (Fig. 3). Alkaline condition yielded a higher rate of putrescine uptake than acidic condition. The initial rates of putrescine uptake in 100 mM Tris-maleate or Tris-HCl buffer at pH 5.5, 7.0, and 8.2 were estimated to be 0.024 ± 0.002, 0.167 ± 0.004, and 0.080 ± 0.004 nmol/min/mg protein, respectively.

Specificity of putrescine uptake. The specificity of putrescine uptake in intact cells was studied by addition of various amine

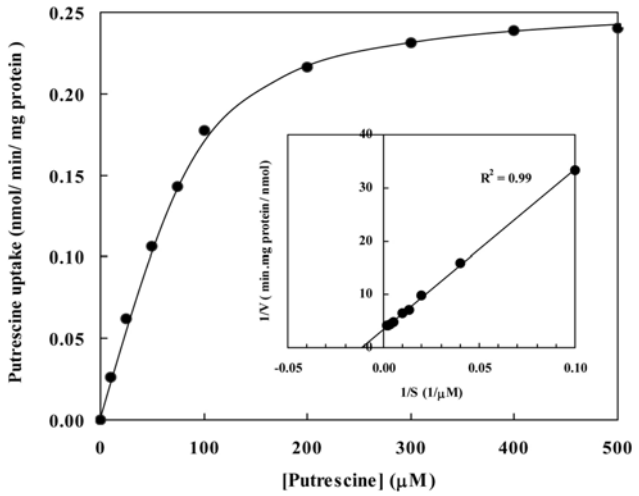

Fig. 2. Kinetics of putrescine uptak by *Synechocystis* sp. PCC 6803 incubated with 0-500 μM putrescine. Initial rates were determined with 1 min incubation time. Inset represents a Lineweaver-Burk transformation of the data. The line drawn is that derived from regression analysis of the data and the points shown are typical of this result from triplicate experiments.

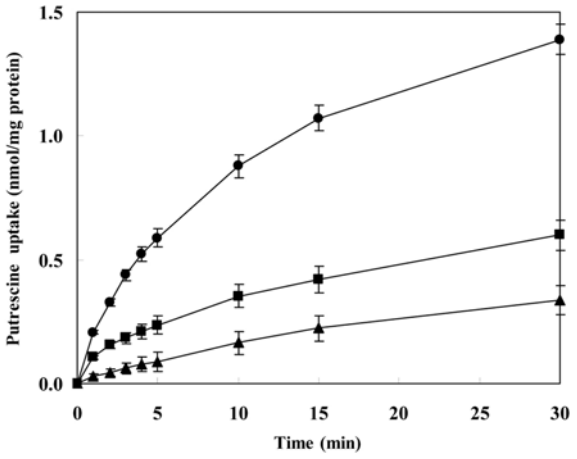

Fig. 3. Dependence of putrescine uptake on external pH. Putrescine uptake assay was done with the modification using 100 mM Tris-maleate for pH 5.5 (▲) and 100 mM Tris-HCl for pH 7.0 (●) and pH 8.2 (■). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

Table 1. Effect of putrescine analogs on the putrescine uptake of *Synechocystis* sp. PCC 6803[a]

Analog compound	Concentration	Putrescine uptake (%)
None		100 ± 2
Agmatine	1 mM	98 ± 2
Alanine	1 mM	91 ± 4
Glycine	1 mM	87 ± 3
Glutamic acid	1 mM	85 ± 5
Serine	1 mM	84 ± 3
Spermidine	1 mM	60 ± 5
Spermine	1 mM	40 ± 6

[a]Cells were incubated in the mixture containing 1 mM unlabeled analog and 50 μM [Methylene-^{14}C] putrescine. Initial rates were determined with 1 min incubation time. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 nmol/min/mg protein.

Table 2. Effect of metabolic inhibitors on the putrescine uptake of *Synechocystis* sp. PCC 6803[a]

Inhibitor	Concentration	Putrescine uptake (%)
None		100 ± 2
N-Ethylmaleimide	1 mM	38 ± 5
p-Chloromercurisulfonic acid	1 mM	20 ± 5
Sodium arsenate	1 mM	13 ± 2
Sodium fluoride	1 mM	26 ± 2
Potassium cyanide	1 mM	39 ± 7
Gramicidin D	10 μM	42 ± 5
2,4-Dinitrophenol	1 mM	36 ± 6
N,N'-Dicyclohexylcarbodiimide	80 μM	26 ± 5
Valinomycin	10 μM	9 ± 3
Amiloride	50 μM	11 ± 4

[a]Cells were preincubated with inhibitors for 30 min before the addition of 50 μM [methylene-^{14}C] putrescine to initiate the uptake with 1 min incubation as described in Materials and Methods. The data shown are the means of three independent experiments representing the percent uptake rate relative to the control rate which was 0.12 nmol/min/mg protein.

analogs into the assay medium and following the rate of uptake. As shown in Table 1, agmatine, a putrescine precursor, had no effect on putrescine uptake. Slight inhibition of putrescine uptake occurred in the presence of alanine, glycine, glutamic acid, and serine. In contrast, spermidine and spermine, which are structurally similar to putrescine but with additional amino groups, showed 40 and 60% inhibition, respectively.

Inhibition of putrescine uptake by metabolic inhibitors. To determine whether putrescine uptake was energy-dependent, the effects of some inhibitors on the uptake activity were studied. Results summarized in Table 2 show that *N*-ethylmaleimide and *p*-chloromercurisulfonic acid, which modify the protein structures, were effective inhibitors of putrescine uptake. The inhibitors for ATP formation, sodium arsenate and sodium fluoride also reduced the uptake activity suggesting the requirement of ATP for putrescine uptake. Interference of electron transport by potassium cyanide resulted in effective inhibition of putrescine uptake. Transport uncouplers such as gramicidin D and dinitrophenol, which dissipate proton motive force, could significantly inhibit putrescine uptake to a similar extent by about 60-65%. *N, N'*

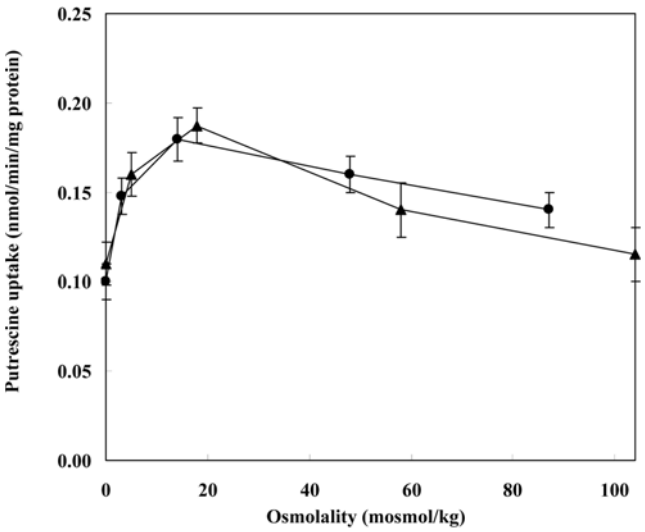

Fig. 4. Effect of external osmolality on putrescine uptake by *Synechocystis* sp. PCC 6803. Initial uptake rates (1 min incubation) were determined in the presence of increasing osmolality generated by NaCl (●) or sorbitol (▲). The data are means from three independent experiments with vertical bars representing standard errors of the means, n = 3.

dicyclohexylcarbodiimide, an ATPase inhibitor causing a reduction in pH gradient, also effectively inhibited putrescine uptake. Valinomycin, an ionophore collapsing the electrical potential with a reduction in ψ, strongly inhibited putrescine uptake. Similarly strong inhibition of putrescine uptake was also observed with amiloride, an inhibitor of Na^+/H^+ exchanger. All these results collectively indicate that the proton motive force with the contribution of both pH and ψ (electrical or membrane potential), plays an important role in putrescine transport in *Synechocystis* cells.

Stimulation of putrescine uptake by osmotic upshift. When testing the effect of osmotic upshift on putrescine uptake, increasing osmolality generated by either NaCl or sorbitol up to 20 mosmol/kg caused about 2-fold increase of putrescine uptake compared to the control (Fig. 4). When the osmolality was higher than 20 mosmol/kg the extent of the stimulation of the uptake was reduced. Moreover, the osmotic upshift higher than 100 mosmol/kg caused a progressive decline in putrescine uptake (data not shown). It is noteworthy that the uptake of putrescine responded to changes in osmolality with similar pattern regardless of the source generating the osmotic upshift.

Induction of putrescine uptake. Since NaCl-imposed osmotic upshift led to an increase of initial putrescine uptake rate, we investigated whether protein synthesis was involved in the stimulation of putrescine uptake. Pretreatment of cells in the presence of 50 mM NaCl with chloramphenicol dramatically lowered putrescine uptake rate, i.e. it was decreased to 50% of that without chloramphenicol pretreatment (Table 3). Cells

Table 3. Effect of chloramphenicol on putrescine uptake of *Synechocystis* sp. PCC 6803 [a]

Condition	Concentration	Putrescine uptake (%)
Control		100 ± 2
NaCl	50 mM	142 ± 3
Chloramphenicol	100 μg/ml	63 ± 5
NaCl + Chloramphenicol	50 mM, 100 μg/ml	74 ± 4

[a]Cells with or without NaCl were preincubated with chloramphenicol for 30 min before the addition of 50 μM [methylene-^{14}C] putrescine to initiate the uptake with 1 min incubation as described in Materials and Methods. The data shown are the means of three independent experiments. One hundred percent represents the uptake rate of 0.12 nmol/min/mg protein.

pretreated with chloramphenicol in the absence of NaCl also showed considerable reduction in the rate of putrescine uptake suggesting the involvement of protein synthesis in the uptake process.

Discussion

Here we have described the existence of a transport system for putrescine in *Synechocystis* sp. PCC 6803. In a previous study, we demonstrated that *Synechocystis cells* were able to accumulate polyamines after exposure to long-term osmotic stresses (Jantaro *et al*., 2003). However, the synthesis of polyamines requires an investment of organic carbon and metabolic energy. It is likely that the portion of cell carbon and energy diverted for polyamine synthesis is at least partially responsible for the observed reduction in the growth rate of cells under osmotic stress. Therefore, the ability to accumulate polyamines via the uptake from the growth medium may be advantageous in terms of the adaptive response to high salinity environment. The data in Fig. 1 indicated that exogenous putrescine could be transported into *Synechocystis* cells and could act as growth promoter in the presence of high salt concentration. These results seem to suggest the role of putrescine as a compatible solute in this cyanobacterium. Nevertheless, the protective role by putrescine against inhibition of growth under hyperosmotic stress was not as efficient as that by glucosylglycerol, suggesting that putrescine per se is not an osmoprotectant (Ferjani *et al*., 2003; Mikkat *et al*., 1997). Indeed, we previously showed that the levels of putrescine accumulated in *Synechocystis* cells after salt and osmotic stress treatments were not sufficient to account for any osmotic importance (Jantaro *et al*., 2003). Furthermore, high concentration of intracellular putrescine is detrimental to the growth of cells since the oxidation of polyamines can generate toxic compounds such as acrolein (Sakata *et al*., 2003). It is worth mentioning here that the addition of exogenous putrescine higher than 1.0 mM was

inhibitory to the growth of *Synechocystis* cells. Similar results were reported for *Chlamydomonas reinhardtii* where cytotoxic effects were observed when exogenous putrescine was higher than 1.5 mM (Theiss *et al*., 2004). The results in Fig. 1 clearly showed that the growth of *Synechocystis* cells was inhibited after 6 days in the presence of 1 mM putrescine. This could be ascribed to the toxic effects caused by the accumulation of intracellular putrescine. Previously, it has been shown in *Anacystis nidulans* that exposure of cells to 150 μM putrescine resulted in cell death within 3 h concomitant with high intracellular putrescine concentration of 100 mM (Guarino and Cohen, 1979).

The putrescine transport system in *Synechocystis* sp. PCC 6803 was saturable displaying typical Michaelis-Menten type kinetics (Fig. 2). *Synechocystis* cells exhibited a low affinity (K_m 92 μM) for putrescine transport, which is in contrast to a rather high affinity for *E. coli* with the K_m value of 1.4 μM (Kashiwaki *et al*., 1986). Low affinity for putrescine transport indicated the slow uptake of putrescine by *Synechocystis* cells. This was corroborated by the observations that rather long period of exposure to putrescine was required for growth inhibition and growth stimulation to manifest in the presence of 1.0 and 0.5 mM putrescine, respectively (Fig. 1). The transport system for putrescine in *Synechocystis* sp. PCC 6803 is an active transport. Putrescine transport is highly inhibited by various energy generation inhibitors (Table 2) suggesting the requirement of energy for the transport system. The coupling mechanism between Na^+ electrochemical gradient and transport systems has been extensively studied. Previously, we have shown that a halotolerant cyanobacterium *Aphanothece halophytica* contains Na^+-dependent choline and nitrate transport systems (Incharoensakdi and Karnchanatat, 2003; Incharoensakdi and Wangsupa, 2003; Incharoensakdi and Laloknam, 2005). Na^+-gradient has been shown to be a major source of energy, coupling with the active transport for polyamine in mouse cells (Rinehart and Chen, 1984). Moreover, the dependency on Na^+ has been reported for the transport of potassium and bicarbonate into *Synechocystis* PCC 6803 (Matsuda *et al*., 2004; Shibata *et al*., 2002). Although we observed a reduction of putrescine uptake in the presence of amiloride, a Na^+-gradient dissipator, it is premature to suggest the involvement of Na^+-gradient in the transport of putrescine into *Synechocystis* cells. The ionophores, valinomycin and 2,4-dinitrophenol and DCCD, an ATPase inhibitor, strongly inhibited the uptake of putrescine, suggesting that the energy required is provided by electron transport in the cytoplasmic membrane through a proton motive force, with the hydrolysis of ATP. Previously, proton motive force-dependent transport of polyamine has been shown in *Saccharomyces cerevisiae* (Kakinuma *et al*., 1992) and *E. coli* (Kashiwaki *et al*., 1986).

The present study revealed that spermidine and spermine could significantly inhibit the uptake of putrescine in *Synechocystis* cells (Table 1) suggesting the same transport system for these three polyamines. Similar observations were reported for the uptake of putrescine by a green alga *Chlamydomonas reinhardtii* (Theiss *et al*., 2002). However, in contrast to *C. reinhardtii* the transport of putrescine by *Synechocystis* cells was influenced by inhibition of protein synthesis (Table 3). On the other hand, no significant inhibition of putrescine transport by various amino acids was observed suggesting a distinctly different transport system for putrescine and amino acids.

Synechocystis cells appeared to possess putrescine transport system with optimal activity around neutral pH (Fig. 3). This was in agreement with a previous study in a parasitic protozoan, *Leishmania mexicana* Promastigote (Basselin *et al*, 2000).

We have shown here that the accumulation of putrescine in *Synechocystis* sp. PCC 6803 in the presence of low concentration of NaCl and sorbitol was a result of an osmotic effect rather than an ionic effect. This was based on the results showing that sorbitol with no ionic effect also produced similar pattern of stimulation seen for NaCl (Fig. 4). It is noted that a marginal level of putrescine uptake could be detected in the absence of osmotic upshift. Without stress this low level of putrescine taken up might serve as nutrient for metabolic function. Increasing the osmolality resulted in the enhancement of the uptake rate since cells require putrescine to better thrive against osmotic upshift. However, at much higher osmolality a decline in putrescine uptake was detected which might be due to less energy available for transport as a consequence of impaired metabolic function. All these results suggested that putrescine transport in *Synechocystis* was efficient at moderate osmolalities. This was similarly reported in *E. coli* (Munro *et al*., 1974). Worth mentioning here is that the slight stimulation of putrescine uptake by both NaCl and sorbitol could not be ascribed to the osmotic or salt stress effect. The increased uptake might reflect a better physiological state of *Synechocystis* cells rather than the direct effect on the uptake system. The increase in putrescine uptake would not be related to salt or osmotic acclimation since no parallel increase in the uptake was observed with the increase in the external osmolality.

Results in Table 3 also indicated that protein synthesis is required for the functional putrescine transport since it was significantly inhibited by chloramphenicol. Future work on the characterization of genes as well as the transporters involved in putrescine uptake in *Synechocystis* sp. PCC 6803 is needed for a better understanding of the putrescine transport system.

Acknowledgments This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (PHD/0115/2546) to W. Raksajit and A. Incharoensakdi and partly by the Academy of Finland to P.Mäenpää.

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