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Sucrose transport by the alkaliphilic, thermophilic *Bacillus* sp. strain TA2.A1 is dependent on a sodium gradient

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Abstract An alkaliphilic *Bacillus* designated strain TA2.A1, isolated from a thermal spring in Te Aroha, New Zealand, grew optimally at pH 9.2 and 70°C. Sodium chloride (>5 mM) was an obligate requirement for the growth of strain TA2.A1 on sucrose, and growth on sucrose was inhibited by monensin, an ionophore that collapses the sodium gradient (ΔpNa^+) across the cell membrane. Sucrose transport by strain TA2.A1 was sodium dependent and was inhibited by monensin. The K_t for sucrose transport was 33 μM and the Eadie–Hofstee plot was linear, suggesting one high-affinity uptake system for sucrose. The affinity for sodium was low (0.5 mM), and the Hill plot had a slope of 1.6, suggesting that sodium binding was noncooperative and that the sucrose transporter had more than one binding site for sodium. Based on these results, *Bacillus* strain TA2.A1 uses a sodium gradient for sucrose uptake, in addition to the sodium-dependent glutamate uptake system reported previously.

Key words Sucrose transport · Alkaliphile · Thermophile · Sodium gradient

Introduction

In natural environments, energy sources are often limiting for bacterial growth, and the acquisition of a high-affinity nutrient transport system is a key factor in an organism's ability to survive. Bacteria have evolved a variety of mechanisms by which solutes are transported into and out of the cell, and these systems can be classified according to the source of energy coupled to the transport process. Some bacterial transport systems utilize an electrochemical gradient of sodium for solute transport (Maloy 1990; Tolner et al. 1992). Sodium-driven transport systems are common in microorganisms (e.g., alkaliphiles) that inhabit environments rich in Na^+ and require Na^+ for growth (Krulwich and Guffanti 1989; Krulwich 1986; Prowe et al. 1996). Furthermore, Konings and others (Tolner et al. 1997; van de Vossenberg et al. 1995) have demonstrated high proton permeability of membranes at high temperature, suggesting that bacteria that grow under these conditions may have membranes that are leaky to protons. To overcome this bioenergetic limitation, some thermophilic bacteria use sodium as a coupling ion for solute transport (Speelmans et al. 1989, 1993; De Vrij et al. 1989, 1990; Heyne et al. 1991; Holtom et al. 1993).

In comparison to the number of bacterial species described that are capable of growth at either high pH or high temperature, few species have been described which are capable of growth under both alkaliphilic and thermophilic conditions and those that have are anaerobes (Li et al. 1993, 1994; Wiegel 1998). The majority of the alkaliphilic/alkalitolerant and thermophilic/thermotolerant aerobic species described belong to the genus *Bacillus* (Heinen et al. 1982; Demharter and Hensel 1989; Sarkar 1991; Fritze 1996).

We have isolated an alkaliphilic thermophilic bacillus, designated *Bacillus* strain TA2.A1, from an alkaline thermal bore in New Zealand. When characteristics of both temperature and pH are taken into account, *Bacillus* strain TA2.A1, with a temperature optimum of 65°C and a pH optimum of 9.2 at 70°C, is the most alkaliphilic thermophilic aerobic bacteria yet described. This strain uses sodium as a coupling ion for the uptake of glutamate (Peddie et al. 1999). Because this strain also utilizes sucrose as a carbon source, investigations were conducted into whether sodium was also required for sucrose transport.

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Materials and methods

Chemicals, growth, and strain maintenance

[U-^{14}C]Sucrose was obtained from Du Pont NEN. Amiloride-HCl, monensin, and 2-deoxyglucose were obtained from Sigma (St. Louis, MO, USA). Strain TA2.A1 was isolated from a continuously enriched pool sample. The growth medium contained (per liter) Na_2SO_4, 0.5 g; $(NH_4)_2SO_4$, 0.1 g; $MgSO_4{\cdot}7H_2O$, 0.1 g; K_2HPO_4, 0.2 g; $NaHCO_3$, 9.0 g; dictyglomous trace elements, 5.0 ml (Saiki et al. 1985); trypticase (Oxoid), 0.1 g; and sucrose, 0.5 g (BDH). The pH of the media was adjusted to pH 10 using 2 N NaOH. Then, 100-ml aliquots of the media were dispensed into 500-ml flasks, which were stoppered with non-absorbent cotton wool bungs and autoclaved at 15 lb/in.^{2} for 15 min. Cells were cultured in a 65°C orbital shaking incubator and aerated by shaking at 100 rpm. Exponential-phase cultures were used for each experiment: flasks were inoculated (1% inoculum) from 18-h stock cultures and grown to midexponential phase (0.2–0.25 units of optical density at 450 nm).

Sucrose transport assays

Cells were harvested by centrifugation (12000 × *g*, 5 min, 4°C) during exponential growth (0.22 mg protein ml^{-1}) and washed twice in Tris-HCl buffer (50 mM, pH 9.2). The cell pellets were resuspended in the same buffer to achieve a concentration of 14–20 mg protein ml^{-1}. Aliquots of 200 µl of cell suspension were placed into tubes in a shaking water bath (Julabo, Labortechnik, GMBH, Germany) at 60°C with a shaking speed of 70 rpm, and transport was initiated by the addition of [U-^{14}C]sucrose (442 mCi mmol^{-1}) diluted 1:1 in 1 mM cold sucrose, giving a final sucrose concentration per assay of 12 µM. Four microliters of diluted [^{14}C]sucrose was used for each assay of 200 µl of washed cells. After 0–60 s, transport was terminated by the addition of ice-cold LiCl (2 ml, 100 mM) and rapid filtration (0.45-µm-pore-size cellulose-nitrate filter). Preliminary experiments indicated that the transport rate was first order with respect to protein from 0 to 20 s, and initial rates were always measured over this time interval. The filter was washed once with 2.0 ml of LiCl, dried for 30 min at 105°C, and counted by liquid scintillation. Preliminary experiments indicated that virtually all the [^{14}C]sucrose was recovered by being trapped in the cells or remaining in the extracellular supernatant.

Competition and metabolic inhibitor experiments

Competitive substrates and metabolic inhibitors were added to the transport assay medium 5 min before initiating uptake. Unlabeled carbohydrates were added at a final concentration of 50 µM. Inhibitors were added to give final concentrations of amiloride at 5 µM and of monensin at 10 µM. Water-insoluble inhibitors were dissolved in 95% ethanol and compared with ethanol-treated controls. Results of competition experiments were expressed as the mean of three determinations ± the standard error of the mean, and the level of inhibition was expressed as the percent inhibition on the initial rate of uptake compared to controls (nominally 100%) in the absence of competitive substrate (Table 1).

Table 1. Effect of competing carbohydrates on [^{14}C]sucrose uptake by *Bacillus* strain TA2.A1 grown on sucrose

Competing carbohydrate	Inhibition of control (%)
Melibiose	21
Raffinose	60
Trehalose	56
Turanose (500 µM)	78

No inhibition of [^{14}C]sucrose uptake by 2-deoxyglucose, fructose, glucose, or maltose was observed
[^{14}C]Sucrose was used at a final concentration of 12 µM, and competing carbohydrates were added at a concentration of 50 µM except where stated

Results

Growth of *Bacillus* strain TA2.A1 on sucrose

Strain TA2.A1 utilized a number of di- and trisaccharides as the sole carbon and energy source for growth including sucrose, trehalose, and raffinose (data not shown). Strain TA2.A1 grew rapidly on sucrose, and the specific growth rate was 2.5 h^{-1}. Strain TA2.A1 also showed good growth on oligosaccharides, but was unable to grow on the monosaccharide components of di- or trisaccharides, namely fructose and glucose. During growth on sucrose, no extracellular fructose or glucose was detectable in the growth medium, suggesting that sucrose was transported into the cell before hydrolysis. Further evidence for this hypothesis were the observations that sucrose-grown cells did not transport [^{14}C]glucose or [^{14}C]fructose at a range of external concentrations tested and that no sucrase activity could be detected extracellularly (data not shown). An inducible intracellular sucrase was detected in cell-free extracts (data not shown).

Previous work had demonstrated that sodium (>5 mM) was essential for the growth of strain TA.2A1 on amino acids (Peddie et al. 1999). No growth on sucrose was noted below a concentration of 5 mM NaCl, and the yield and growth rate increased as the sodium ion concentration in the growth medium was increased from 5 to 100 mM, with sodium concentrations greater than 100 mM being inhibitory to growth (data not shown).

To investigate the sodium requirement in more detail, monensin, an ionophore that disrupts sodium (ΔpNa^+) or potassium gradients or both across bacterial membranes (Pressman 1976), was added to cells growing on sucrose. On monensin addition, there was an immediate cessation of growth, even when as little as 0.1 µM was added (Fig. 1). Growth was completely inhibited, cells did not become

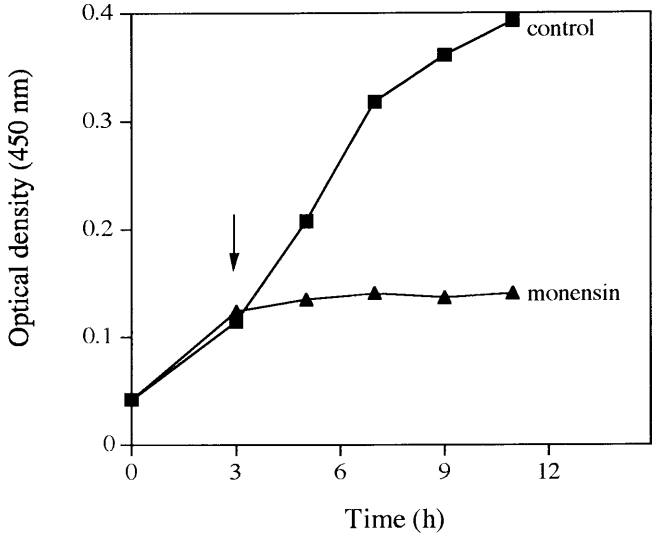

Fig. 1. The effect of monensin (0.1 μM) addition on the growth of *Bacillus* strain TA2.A1 in sucrose-supplemented minimal medium. Amiloride (500 μM) had no effect on growth. One hundred microliters of a 100% ethanol stock was added to the control culture. *Arrow* indicates addition of inhibitor or ethanol

resistant to monesin even after prolonged incubation, and cell lysis was not evident (Fig. 1). Amiloride (500 μM), an inhibitor of Na^+/H^+ antiporters (Pressman 1976), had no effect on the growth of strain TA2A1 (data not shown).

[^{14}C]Sucrose transport by *Bacillus* strain TA2.A1

Cells grown on sucrose that were washed twice in Tris-HCl (100 mM, pH 10.0) containing 100 mM NaCl transported [^{14}C]sucrose at an initial rate of 0.13 nmol min^{-1} mg protein^{-1} (Fig. 2a). In cells washed twice in Tris-HCl (100 mM, pH 10.0) containing 100 mM KCl, [^{14}C]sucrose uptake was significantly reduced (Fig. 2a). Cells grown on glutamate were unable to transport [^{14}C]sucrose, suggesting that the sucrose transporter was inducible (data not shown). When sodium-washed cells were preincubated with monensin (10 μM), the uptake of [^{14}C]sucrose was completely abolished (Fig. 2b), suggesting a sodium gradient was required for the uptake of [^{14}C]sucrose.

To better understand this requirement for extracellular sodium, we performed experiments in which the extracellular concentration of sodium was varied. Cells resuspended in Tris-HCl (pH 10.0) containing 100 mM KCl did not transport [^{14}C]sucrose at a rate that could be differentiated from that of nonspecific [^{14}C]sucrose binding to cells (see Fig. 2a; K^+-washed cells). When the extracellular concentration of NaCl was increased to more than 1 mM, the rate of [^{14}C]sucrose uptake increased and reached a maximum rate at 30 mM extracellular NaCl (Fig. 3). Further increases in the sodium concentration did not increase the rate of [^{14}C]sucrose transport significantly (Fig. 3). The K_m for sodium was 0.5 mM, and the slope of the Hill plot was approximately 1.6, suggesting more than one binding site

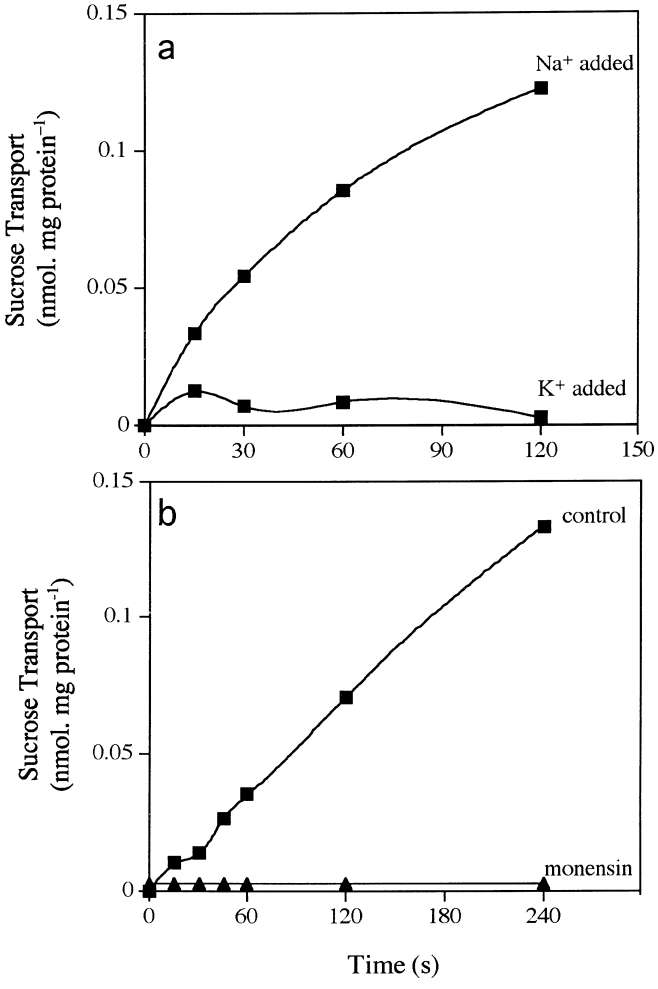

Fig. 2. a [^{14}C]Sucrose transport by washed cells of *Bacillus* strain TA2.A1 with 100 mM NaCl or with 100 mM KCl. **b** The effect of monensin (10 μM) on [^{14}C]sucrose transport by washed cells of *Bacillus* strain TA2.A1

for sodium (see Fig. 3, inset). The specific requirement for Na^+ could not be met by other cations (e.g., K^+ and Li^+) (data not shown).

The rate of sucrose uptake was studied over a range of sucrose concentrations (Fig. 4). When the extracellular sucrose concentration was increased from 1 to 48 μM, the rate of sucrose transport increased and typical Michaelis–Menton saturation kinetics were observed (Fig. 4). The Eadie–Hofstee plot was linear; the K_t for sucrose was 33 μM and the V_{max} was 0.96 nmol min^{-1} mg protein^{-1} (Fig. 4, inset).

The substrate specificity of the Na^+-dependent sucrose transport system was determined by measuring the uptake of [^{14}C]sucrose in the presence of a 50-fold excess of other competing sugars. Sucrose uptake was inhibited (56%) by an excess of cold trehalose, which suggests that both carbohyrates may be competing for the same uptake system. Cold maltose, glucose, fructose, and 2-deoxyglucose did not have a significant effect on sucrose uptake (Table 1).

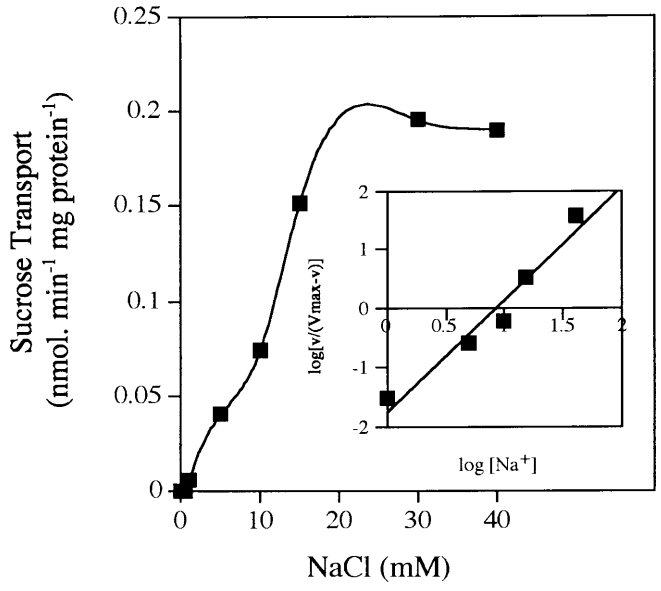

Fig. 3. The effect of extracellular sodium chloride concentration on [^{14}C]sucrose (1 μM) uptake by washed cells of *Bacillus* strain TA2.A1. Hill plot of data is shown in *inset*

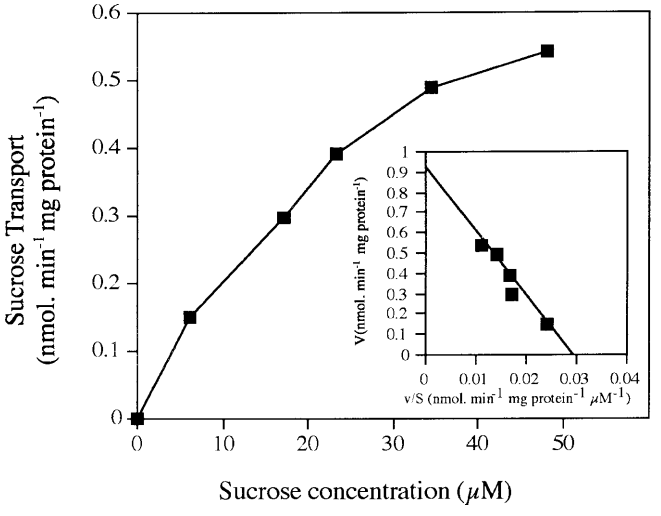

Fig. 4. The effect of sucrose concentration on the rate of [^{14}C]sucrose transport by washed cells of *Bacillus* strain TA2.A1. The Eadie–Hofstee plot of sucrose transport is shown in *inset*

Discussion

A wide range of bacteria have the ability to grow on disaccharides and these include those living in extreme environments. For example, *Pyrococcus furiosus* can grow on maltose and cellobiose (Fiala and Stetter 1986; Schäfer and Schönheit 1992; Kengen and Stams 1994a,b). Like many hyperthermophiles, *P. furiosus* can transport and metabolize glucose, but cannot grow on glucose or any other monomeric sugar (Usenko et al. 1993; Kengen et al. 1994; Schäfer et al. 1994). Kengen et al. (1996) stated that there was at present no explanation for this phenomenon, which is observed for almost all hyperthermophiles. *Bacillus* strain TA2.A1 is able to utilize sucrose, but not the component monosaccharides of sucrose as a growth substrate. The addition of fructose or glucose to a growing sucrose culture caused inhibition of growth. The inability of *Bacillus* strain TA2.A1 to utilize fructose, while being able to utilize fructose-containing compounds such as fructo-oligosaccharides, might be explained by either the different structures of fructose (for example, in aqueous solution a six-membered pyranose ring structure) or the instability of fructose at high pH and temperature. As stated by Driskill et al. (1999), it is not completely clear what specific effects the intermediates generated by the thermochemical modifications of carbohydrates (e.g., Maillard reactions, caramelization) have on the nutritional value of sugars and how these effects are influenced by pH, temperature, and salt concentrations.

An increasing body of evidence has accumulated indicating that bacteria use Na^+ ions in addition to or instead of protons to couple exergonic reactions with endergonic reactions in the membrane. Sodium symporters for solute transport have been reported in alkaliphilic bacteria (Krulwich et al. 1982), marine bacteria (Droniuk et al. 1987), *E. coli* (Tsuchiya et al. 1977), *Salmonella typhimurium* (Cairney et al. 1984), rumen bacteria (Russell et al. 1988; Chen and Russell 1989, 1990), thermophilic bacteria (De Vrij et al. 1989, 1990; Speelmans et al. 1989; Heyne et al. 1991), and halophilic bacteria (Severina et al. 1991). These systems are common to bacteria that inhabit environments rich in Na^+ and require Na^+ ions for growth. Symport of both Na^+ and H^+ ions with the same solute has been observed for the L-glutamate transport system of *E. coli* (Fujimura et al. 1983) and *Bacillus stearothermophilus* (De Vrij et al. 1989; Heyne et al. 1991). Prowe et al. (1996) reported that an anaerobic thermoalkaliphilic bacterium used sodium cycling as the primary method of energy transduction. Despite the high specificity for Na^+, the L-leucine transport system showed poor affinity for Na^+ ions, with more than 100 mM required to saturate the system (Prowe et al. 1996). In contrast, a higher affinity for Na^+ (>5 mM) has been shown for the sodium/glutamate symporter of the aerobic thermoalkaliphilic *Bacillus* strain TA2.A1 (Peddie et al. 1999).

A number of different mechanisms of sucrose transport have been described in bacteria. Studies with mesophilic bacteria, *Bacillus subtilis* (Lepesant and Dedonder 1968; Kunst et al. 1974; Fouet et al. 1987), *Streptococcus lactis* (Thompson and Chassy 1981), and *Streptococcus mutans* (Slee and Tanzer 1979; St. Martin and Wittenberger 1979) have reported that sucrose is accumulated in the cell by the phosphoenolpyruvate-dependent phosphotransferase system (PTS). In contrast, the marine bacterium *Vibrio alginolyticus* actively transports sucrose using a Na^+ electrochemical potential, and the sucrose is then hydrolyzed to glucose and fructose by a sucrase (Kakinuma and Unemoto 1985).

Growth of strain TA2.A1 in minimal medium supplemented with sucrose required sodium (>5 mM) and was inhibited by the ionophore monensin. Growth on trehalose

was also inhibited by monensin, suggesting that a ΔpNa^+ was required for sucrose and trehalose uptake by strain TA2.A1. In fact, both disaccharides may utilize the same transport system because sucrose uptake was inhibited by an excess of cold trehalose, suggesting competition for a common permease. Sucrose uptake by *Bacillus* strain TA2.A1 required a ΔpNa^+, demonstrating that transport was an active process and most probably by a sucrose/Na^+ symport. On entering the cell, sucrose was hydrolyzed by the enzyme sucrase, which was detected in cell-free crude extracts but not in cell-free culture supernatants of cells grown on sucrose. Because sucrose appeared to enter the cell unmodified, this further supported the hypothesis that sucrose uptake was via an active mechanism. This mechanism of sucrose uptake and metabolism resembles that of *Vibrio alginolyticus* (Kakinuma and Unemoto 1985).

The results reported here support and extend the previous findings with respect to the role of Na^+ in the growth and solute transport of alkaliphilic bacilli (Krulwich et al. 1982; Krulwich 1986: Krulwich and Guffanti 1989; Peddie et al. 1999). Uptake of all growth substrates (e.g., amino acids and sugars) tested thus far by thermophilic alkaliphilic *Bacillus* strain TA2.A1 are sodium dependent, suggesting that this bacterium may depend on sodium for all bioenergetic processes such as transport, motility, pH homeostasis, and ATP synthesis.

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