Nutrient utilization and transport in the thermoacidophilic archaeon Sulfolobus shibatae

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Growth of the thermoacidophilic archaeon Sulfolobus shibatae was tested on a range of carbon and nitrogen sources. Optimal defined and complex growth media were developed and growth conditions in both shake flask and fermenter cultures were optimized. Better growth was observed on maltose in particular and disaccharides in general than on monosaccharides. Moreover, maltose utilization was not repressed in the presence of glucose which suggests that glucose is not the preferred substrate of S. shibatae. Uptake studies putatively identified two saturable, constitutive maltose transport systems, a high-affinity, possible membrane-binding system with a $K_m$ of 20 $\mu$M and a $V_{max}$ of 218 nmol min$^{-1}$ (mg protein)$^{-1}$, and a low-affinity, proton-dependent system with a $K_m$ of 158 $\mu$M and a $V_{max}$ of 680 nmol min$^{-1}$ (mg protein)$^{-1}$. Both systems showed differential responses to treatment with 2,4-dinitrophenol and arsenate, and differed from other maltose transport mechanisms described in being constitutive under all conditions tested and not repressed by glucose.

**Keywords:** Sulfolobus shibatae, Archaea, thermoacidophile, maltose transport, disaccharides

INTRODUCTION

The factors affecting the growth of members of the Archaea are relatively poorly understood. Knowledge in this area will provide insights into archaeal metabolism and may be important for their future exploitation (Romano, 1986).

The strain chosen for this study, Sulfolobus shibatae, grows optimally at pH 3.5 and 78 °C and is an thermoacidophilic crenarchaeote member of the *Sulfolobales*, which are a diverse group of organisms including the genera Sulfolobus, Acidilimobius, Metallosphaera and Desulfurolobus (Stetter et al., 1990). Members of the genus Sulfolobus are the only strict aerobes of the order, different species growing either as strict autotrophs, strict heterotrophs or facultative heterotrophs (Grogan, 1989; Stetter et al., 1990). *S. shibatae* differs significantly from the other members of the genus, particularly in its growth physiology, showing poor autotrophic growth but good heterotrophic growth on a wide range of carbon and nitrogen substrates (Grogan, 1989). We have studied the growth physiology of *S. shibatae* by measuring growth on a range of different carbon and nitrogen substrates and studying the transport of nutrients into the cell. To date, only one other nutrient transport system has been described for the Archaea, a pH-dependent glucose transport system in Sulfolobus solfataricus (Cusdin et al., 1996).

METHODS

Growth of *S. shibatae*. *S. shibatae* DSM 5389, formerly isolate B12 (Grogan et al., 1990) isolated near Beppu, Japan (Yeats et al., 1982) was used in all experiments. Cultures were maintained for up to 6 months at −20 °C in a 10% (v/v) glycerol solution supplemented with basal salts and 0.01% (w/v) peptone at pH 3.5–4.0. Shake flask experiments were carried out in 250 ml Erlenmeyer flasks at 250 r.p.m. and 78 °C using 10% (v/v) inocula of a 48-h-old culture in 70 ml of the indicated medium at pH 3.5. All media contained a basal salts mixture consisting of a modified Allen's medium (Brock et al., 1972). All substrates were autoclaved with the basal medium with the exception of sugars and amino acids which were filter-sterilized separately and added to the medium after autoclaving. Microbial biomass (growth) was monitored by measuring the optical density of cultures at 440 nm. Batch and continuous culture experiments were carried out using a Bioflo III fermenter unit (New Brunswick Scientific).

Measurement of disaccharide stability. Disaccharide stability was measured by monitoring the release of glucose with time using the Glucose Oxidase kit (procedure 510; Sigma).

**Abbreviations:** PEP, phosphoenolpyruvate; PMF, proton motive force.
Measurement of maltose and sucrose utilization. Sucrose utilization was measured using the sucrose phosphorylase assay (Birnberg & Brenner, 1984). Sucrose samples (40 μl) were mixed with 750 μl 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM MgSO₄, 0.5 mM NAD (Boehringer Mannheim), 3 U phosphoglucomutase, 1 U glucose 6-phosphate and 0.08 U sucrose phosphorylase. After incubation at room temperature (approx. 22 °C) for 30 min, A₄₅₀ was determined against a blank to which no sucrose had been added. Maltose utilization was measured by hydrolysis with maltase (Sigma) for 1 h at 37 °C and then assaying for glucose using the Glucose Oxidase kit (procedure 510; Sigma).

Sugar uptake studies. Cells were harvested after 24 h growth in a medium containing basal salts supplemented with the appropriate sugar (0.25%, w/v), peptone (0.05%) and ammonium sulphate (0.15%, w/v). The OD₄₅₀ was measured, cells were pelleted at 2870 g and washed twice in basal salts solution. Cells were then resuspended to an OD₄₅₀ of 20. Aliquots (200 μl) of cell suspension were pre-incubated at 78 °C for 1 h at pH 3.5 (unless otherwise stated) in a Dri-Block (Techni). Pre-warmed aliquots (200 μl) of the appropriate labelled sugar (1 μCi ml⁻¹) were then added [specific activities: sucrose 677 mCi mmol⁻¹ (25.1 GBq mmol⁻¹), maltose 664 mCi mmol⁻¹ (24.6 GBq mmol⁻¹)]. Uptakes were measured by mixing the cell suspensions and the sugar solutions and stopping the reaction after the appropriate interval by adding 10 ml chilled basal salts medium (pH 3.5) and filtering through 0.45 pm Millipore filters. Samples were washed three times, each with 3 ml chilled basal salts medium, and dried in scintillation vials before covering with 20 ml scintillation fluid (Optiphase Hi-Safe 2). Samples were read in a scintillation counter. Cell protein was measured by the Lowry method.

Effects of arsenate and 2,4-dinitrophenol (DNP). The effects of arsenate and DNP on transport were tested by their addition, at concentrations ranging from 0.05 to 1.5% for ammonium sulphate, and 0.05 to 0.5% for glutamate and glutamine. Sucrose (0.2%) was present as the carbon source. Ammonium sulphate supported the best growth, 0.5% giving optimum growth (Fig. 2b). Growth on 0.5% ammonium sulphate over a range of sucrose concentrations showed that sucrose concentration had little effect on biomass with 0.5% giving only marginally better growth. A decrease in culture pH from 3.5 to 2.5–2.75 occurred during growth at all concentrations of sucrose and ammonium sulphate tested. The utilization of the ammonium sulphate therefore appeared not to affect culture pH.

Growth on complex nitrogen substrates

Growth was tested on different nitrogen substrates at concentrations ranging from 0.1 to 15%, with 0.5% sucrose added as the carbon source. Maximum biomass was obtained on 0.2% peptone (Fig. 2a). Peptone concentrations above 0.2% sharply inhibited growth with complete inhibition above 0.75%. A similar pattern was repeated for all complex nitrogen sources tested. Peptone was therefore selected as the optimum complex nitrogen source and growth tested at different peptone concentrations combined with different sucrose concentrations. Maximum growth (OD₄₅₀ of 3.0) occurred on 1.0% sucrose and 0.2% peptone (data not shown). Changes in culture pH were observed and appeared to be due to the relative concentrations of sucrose and peptone, for example culture pH decreased from pH 3.5 to 2.9 during growth on 1.0% sucrose/0.1% peptone but increased from pH 3.5 to pH 6.0 during growth on 0.2% sucrose/0.2% peptone (data not shown). Decreases in culture pH probably resulted from the metabolism of sucrose while increases in pH may have been due to the deamination of amino acids from peptone. When culture pH remained unchanged or decreased, growth did not appear to be affected. However, when culture pH increased above pH 5.0, growth was inhibited (data not shown).
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**Fig. 1.** Growth of *S. shibatae* on various carbon substrates. *S. shibatae* was grown in shake flasks as described in Methods, on basal salts supplemented with a range of carbon substrates and 0.2% peptone. Substrate concentrations are given in % (w/v). The data represent means of at least three repeats. Variation was less than 10%.

**Fig. 2.** Growth of *S. shibatae* on various defined nitrogen substrates. *S. shibatae* was grown in shake flasks as described in Methods, on basal salts supplemented with (a) complex and (b) defined nitrogen substrates, with 0.2% sucrose. Substrate concentrations are given in % (w/v). The data represent means of at least three repeats. Variation was less than 10%.

**Growth in continuous culture**

Batch experiments carried out in shake flasks can provide information on final growth yields but growth rates can only accurately be determined in a fermenter. A further advantage is that it is possible to accurately control culture conditions. Optimum conditions for growth of *S. shibatae* in continuous cultures in the fermenter were determined to be 78 °C, pH 3.5, aeration of 2.5 l min⁻¹ and an impeller speed of 500 r.p.m. for a working volume
Fig. 3. Growth of *S. shibatae* in continuous culture. *S. shibatae* was grown in continuous culture by pumping in fresh medium at a set rate and removing waste medium through an overflow pipe when a set volume had been reached. Cells were cultured on basal salts supplemented with (a) a range of peptone concentrations plus 0.5% sucrose and (b) a range of sucrose concentrations plus 0.1% (○) or 0.2% (■) peptone, at 78°C, pH 3.5, an aeration of 2.5 l min⁻¹ and an impeller speed of 500 r.p.m. Growth rates were calculated from the maximum dilution rates at which steady state was observed and above which wash-out occurred. The data are typical of at least three repeats. Variation was less than 10%.

**Fig. 4. Utilization of sucrose and maltose by *S. shibatae* in continuous culture.** Maltose-grown cultures of *S. shibatae* were inoculated into basal salts supplemented with 0.25% sucrose, 0.25% maltose and 0.5% ammonium sulphate and grown in continuous culture as described in Methods. Growth was measured by OD₆₀₀ (■). Utilization of glucose (○) and maltose (□) was measured as described in Methods. The data are typical of at least two repeats and variation was less than 10%.

Utilization of disaccharides

Preferential utilization of glucose over other sugars is common for many organisms. The utilization of glucose and a range of disaccharides by *S. shibatae* was tested in a fermenter using a medium containing basal salts supplemented with ammonium sulphate (0.5%) and both glucose and the appropriate disaccharide (each 0.25%). No difference was observed between the utilization of glucose and the disaccharide tested whether cultures were grown previously on either glucose or the appropriate disaccharide. Fig. 4 shows the utilization of glucose and maltose using a culture grown on maltose. Glucose does not therefore appear to be the preferred substrate of *S. shibatae*.

Sugar transport experiments

The better growth of *S. shibatae* on disaccharides compared to monosaccharides (Fig. 1) led us to examine their uptake into the cell. Experiments using radiolabelled sucrose identified a saturable sucrose transport mechanism with a *Kₐ* of 2.5 mM and a *Vₐₐₛ* of 18 μmol min⁻¹ (mg protein)⁻¹. However, preliminary competition studies found a 50% inhibition of sucrose transport by a tenfold
lower concentration of maltose suggesting maltose may be a better substrate for the transport system responsible for sucrose uptake. Experiments using radiolabelled maltose confirmed this and identified the possible presence of two saturable maltose uptake systems (Fig. 5), a high-affinity uptake system with a $K_m$ of 20 μM and a $V_{max}$ of 218 nmol min$^{-1}$ (mg protein)$^{-1}$ and a low-affinity uptake system with a $K_m$ of 158 μM and a $V_{max}$ of 680 nmol min$^{-1}$ (mg protein)$^{-1}$. Sugar stability studies revealed no chemical or enzymic hydrolysis of the sugars at 78 °C and pH 3.5. It was therefore concluded that the disaccharides undergo hydrolysis after entry into the cell as has been previously shown in S. solfataricus and S. shibatae (Grogan, 1991; Rolfsmeier & Blum, 1995) in addition to our own studies (data not shown).

The effects of pH on maltose and sucrose uptake was measured by pre-incubating cells at pH 3.5 for 1 h, then resuspending in basal salts of the appropriate pH for the assay. Sucrose and low-affinity maltose uptake appeared to be pH-dependent with 100% activity at pH 3.5, approximately 50% activity at pH 5.0 and no activity at pH 6.5 (Fig. 6). High-affinity maltose uptake appeared independent of pH, showing unchanged activity at pH 3.5, 5.0 and 6.5. The effects of pH were further studied by pre-incubating cells at pH 6.5 for up to 3 h. Sucrose and low-affinity maltose uptake did not occur when cells were assayed at pH 6.5, but activity was recovered when assayed at pH 3.5, confirming their pH dependence. However, high-affinity maltose uptake, whilst active for 1 h at pH 6.5, gradually lost activity over the next 2 h. Activity was recovered when assayed at pH 3.5 (data not shown). High-affinity uptake did not therefore appear, as was first thought, to be independent of pH. The ability to show uptake, albeit only transiently, following prolonged pre-incubation at pH 6.5 may indicate that transport may not be directly proton-dependent, but may be dependent upon indirect effects. The same results were observed when experiments were carried out with cells starved of endogenous energy (pre-incubation in basal salts at 78 °C for 96 h). Cells starved of endogenous energy are commonly used to distinguish proton-dependent uptake. In neutrophilic bacteria such as Escherichia coli, at pH 7.5 the proton motive force (PMF) is composed chiefly of the membrane potential ($ΔΨ$), which requires the active pumping of $H^+$ ions across the membrane, with the pH gradient ($ΔpH$) playing a minor role. At pH 5.5 the PMF is composed chiefly of the $ΔpH$, which requires no endogenous energy, with $ΔΨ$ playing a minor role (Joshi et al., 1989). Proton-dependent uptake can therefore be distinguished in starved cells by detecting uptake following a decrease in pH. It would appear from the above results that all three transport systems tested were proton-dependent; however complications may exist. As S. shibatae exists naturally at low pH, neutral pH may indirectly affect uptake, such as affecting cell metabolism (cells remain viable but not active at pH 6.5) or affecting transport proteins which may require a low external pH.

Maltose uptake was also tested in the presence of the protonophore DNP which increases the permeability of the membrane to protons. DNP dissipates the PMF and hence inhibits proton symport. Concentrations below 500 μM DNP did not affect any of the three transport systems, and only exceptionally high concentrations (1 mM) were found to have an effect, causing 100% inhibition of sucrose and low-affinity maltose transport, and 50–60% inhibition of high-affinity uptake. It is widely accepted that acidophiles possess a neutral or near-neutral cytoplasmic pH and that under normal conditions of growth, pH 3.5 for S. shibatae, there is an influx of $H^+$ ions into the cell down a concentration gradient (Cobley
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As described in Methods in Tris/HCl buffer with additions of P, (■), PEP (▲), ATP (□) and with no addition (○). The data represent means of two repeats and variation was less than 10%.

**Fig. 7.** Maltose phosphorylation by cell extracts of *S. shibatae*. Extracts of *S. shibatae* cells grown on maltose as the sole carbon source were prepared and assayed for maltose phosphorylation as described in Methods in Tris/HCl buffer with additions of P, (■), PEP (▲), ATP (□) and with no addition (○). The data represent means of two repeats and variation was less than 10%.

& Cox, 1983; Booth, 1985). To counteract this and to maintain cytoplasmic pH the cell actively pumps out H⁺ ions, a process which is linked to respiration and occurs via the electron transport system (Anemuller et al., 1985; Moll & Schafer, 1988; Lubben & Schafer, 1989; Schafer et al., 1990). The PMF which exists and which drives the conversion of ADP to ATP is made up almost entirely of the ApH with Δφ playing a minor role (Lubben & Schafer, 1989). However, as energy is depleted and the rate of H⁺ efflux is reduced the cell cytoplasm would rapidly acidify, resulting in cell death, if it were not for a number of mechanisms. *S. shibatae* cells possess a membrane which shows a decreased permeability to H⁺ ions and the cytoplasm shows considerable buffering capacity. However, these are only transitory defences. The mechanism which is thought to allow long term survival is the ability of the Δφ to turn increasingly inside-positive. This is thought to occur by the concentration of H⁺ ions by a Donnan potential and by a H⁺ diffusion potential (Goulbourne et al., 1986; Matin, 1990a) and by the concentration of other positive ions such as Na⁺ and K⁺ (Bakker, 1990; Glaser et al., 1990). This positive Δφ balances the influx of H⁺ ions and maintains cytoplasmic pH as well as the pH gradient, even in non-viable cells. A similar effect is thought to occur in the presence of protonophores (Matin, 1990b; Bakker, 1990). As H⁺ conductance of the cell membrane is increased with addition of the protonophore so the Δφ becomes inside-positive and limits the influx of H⁺ ions. The activity of sugar uptake in the presence of DNP below 500 μM either indicates that the cell is resistant to the effects of DNP, or that a pH gradient is maintained despite permeabilization of the membrane with transport continuing to operate with an inside-positive Δφ. The complete loss of activity at 1 mM DNP could be due to non-specific effects on the cell and/or cell membrane as described in previous studies (Matin, 1990b). However, if non-specific effects are responsible they would affect all systems equally and this did not happen as only partial inhibition of high-affinity maltose transport (50–60%) occurred. This result suggests that sucrose and low-affinity maltose transport are proton-dependent while the partial inhibition caused by DNP is indicative of a transport system that is not reliant on the PMF, for example the phosphotransferase system (PTS) or periplasmic binding system (Erni, 1992; Ames, 1992; Postma et al., 1993).

The operation of a PTS system can be revealed by PEP-dependent phosphorylation of the sugar substrate in cell extracts. Extracts of *S. shibatae* grown on maltose were assayed for maltose phosphorylation in the presence of phosphate buffer and phosphorylation was observed not only in the presence of PEP and ATP but also in the presence of the phosphate buffer alone (data not shown). It was therefore possible that maltose PTS activity was being masked by the formation of high levels of maltose phosphate from the phosphate buffer. To counter this, cell extracts were prepared in Tris/HCl. The addition of inorganic phosphate again resulted in maltose phosphorylation, but the addition of PEP had no effect (Fig. 7). This result suggests that whilst *S. shibatae* is capable of phosphorylating maltose, it does not possess a maltose PTS system. A periplasmic binding system analogous to that of Gram-negative bacteria may be the mechanism of maltose uptake. This is theoretically possible in members of the Archaea as they possess an S-layer surrounding the cell membrane with an interspace between, which may be similar to the periplasmic space in Gram-negative bacteria.

Uptake was tested in the presence of the phosphate analogue arsenate which inhibits the conversion of ADP to ATP. Arsenate can therefore be used to distinguish between transport systems which depend directly upon ATP and those which are energized by a transmembrane PMF generated by respiration. Unstarved cells and cells incubated in basal salts for 96 h were tested for maltose transport in the presence of 5 mM arsenate. Both maltose transport systems were inhibited in cells starved for 96 h and in the presence of arsenate. However, in unstarved cells only high-affinity maltose uptake was inhibited by arsenate with low-affinity uptake operating normally. This result suggests that high-affinity maltose uptake is dependent directly upon ATP and is similar to periplasmic binding protein systems, and that low-affinity maltose uptake is dependent directly upon the PMF and is similar to a proton-dependent system. The inhibition of both maltose transport systems in starved cells (96 h) in the presence of arsenate was due to cell death. However, cell death could not be attributed to the effects of either starvation or arsenate alone as viability was maintained in cultures exposed to starvation and arsenate individually. It therefore appeared that starvation combined with exposure to arsenate was responsible for cell death. One possible explanation is the tight coupling of cellular energy and the maintenance of cytoplasmic pH and existing pH gradient. Acidophiles do not need to generate a transmembrane PMF as they exist naturally with an often considerable pH gradient. They do however need to
mechanisms for ATP production may have adverse effects on the cell not seen in neutrophilic organisms.

actively pump out H⁺ ions to prevent acidification of cellular cytoplasm, and thus any interference with mechanisms for ATP production may have adverse effects on the cell not seen in neutrophilic organisms.

Competition studies with both maltose uptake systems confirmed their identification as primarily maltose uptake systems and showed that sucrose uptake was taking place via low-affinity maltose uptake (Fig. 8a, b). Lactose was observed to compete most effectively with both maltose uptake systems, (17- and 67-fold lower affinities for the low- and high-affinity systems, respectively). Sucrose, glucose and galactose had similar effects on low-affinity maltose uptake, (23-fold lower affinities). Approximately a 50-fold lower affinity was shown towards fructose, while cellobiose and trehalose had a relatively small effect (Fig. 8a). Galactose, glucose and fructose had similar effects on high-affinity maltose uptake (80-85-fold lower affinities), see Fig. 8(b). Cellobiose, trehalose and sucrose had no effect on high-affinity maltose uptake (Fig. 8b). The competition of sucrose, cellobiose and trehalose with low-affinity but not high-affinity uptake, and the poor transport of fructose relative to other monosaccharides by high-affinity but not low-affinity uptake, further differentiates the two transport processes. The ability of a range of sugars to be transported by the two maltose transport systems may play a role in the effective survival and competition of the organism in the extreme conditions which it inhabits. In other micro-organisms maltose is known to be transported by two inducible primary mechanisms, which are repressed when grown on glucose, the high-affinity maltose-binding protein system, for example E. coli, Aerophila hydrophila and Yersinia enterocolitica (Hengge & Boos, 1983; Höner zu Bentrup et al., 1994; Brzostek et al., 1993), or lower affinity H⁺ symport, for example Bacillus liebeniformis NCIB 6346 (Tangney et al., 1992a), Bacillus subtilis (Tangney et al., 1992b) and Saccharomyces cerevisiae (Loureiro-Dias & Peinado, 1982; Busturia & Lagunas, 1985; Benito & Lagunas, 1992; Van Leeuwen et al., 1992). However, a few reports exist of an organism possessing both transport systems, for example Thermoaerobacterium thermosulfurigenes (Sahm et al., 1996). Moreover, the two maltose transport mechanisms of S. shibatae appeared to be constitutive, showing equal transport in sucrose-, lactose-, glucose- and peptone- (no sugar present) grown cultures, and were not repressed when grown on glucose. Sucrose transport by the low-affinity maltose uptake system was also found to be constitutive and not repressed by growth on glucose. The failure of maltose transport to be repressed by growth on glucose, together with the non-preferential utilization of glucose, indicates that there is no preferred sugar for S. shibatae. The $K_m$ of high-affinity uptake (20 μM) is approximately 10-20-fold that of reported periplasmic binding systems of other bacteria (for example 0.3-2 μM for E. coli and A. hydrophila (Höner zu Bentrup et al., 1994; Nikaido, 1994)). The $K_m$ of low-affinity uptake (158 μM) compares favourably to the proton symport mechanisms of other organisms such as the yeast S. cerevisiae with $K_m$ values of 2-5.9 mM (Loureiro-Dias & Peinado, 1982; Busturia & Lagunas, 1985; Benito & Lagunas, 1992; Van Leeuwen et al., 1992), and also to proton symport mechanisms of other sugars, for example arabinose uptake transport (145-160 μM) in E. coli (Daruwalla et al., 1981).

In conclusion, the initial biphasic uptake data, which gave two $K_m$ values, combined with the differential effects of pH, treatment with DNP and arsenate, and sugar competition studies, suggests that two distinct maltose uptake systems are present, both of which appear to be constitutive and not repressed when grown on glucose. This preliminary study provides the basis for further physiological and genetic studies on sugar uptake in S. shibatae.
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REFERENCES


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