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Minireview

Energy Transduction and Transport Processes in Thermophilic Bacteria

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Bacterial growth at the extremes of temperature has remained a fascinating aspect in the study of membrane function and structure. The stability of the integral membrane proteins of thermophiles make them particularly amenable to study. Respiratory enzymes of thermophiles appear to be functionally similar to the mesophilic enzymes but differ in their thermostability and unusual high turnover rates. Energy coupling at extreme temperatures seems inefficient as suggested by the high maintenance coefficients and the high permeability of the cell membrane to protons. Nevertheless, membranes maintain their structure at these extremes through changes in fatty acid acyl chain composition. Archaebacteria synthesize novel membrane-spanning lipids with unique physical characteristics. Thermophiles have adapted to life at extreme temperatures by using sodium ions rather than protons as coupling ions in solute transport. Genetic and biochemical studies of these systems now reveal fundamental principles of such adaptations. The recent development of reconstitution techniques using membrane-spanning lipids allows a rigorous biochemical characterization of membrane proteins of extreme thermophiles in their natural environment.

KEY WORDS: Thermophiles; archaebacteriae; extremophiles; solute transport; energy transduction; reconstitution; membrane fusion; tetraether lipids.

INTRODUCTION

Because biological properties of proteins arise primarily from their native conformation, considerable research effort has been directed at a molecular elucidation of the mechanisms which determine thermostability and -activity. This aspect is not only of fundamental but also of biotechnological interest (Brock, 1985, 1986; Deming, 1986). Physiological and genetic studies on thermophilic bacteria now reveal new biotechnological applications. Thermophiles are equiped with enzymes that are stable up to a temperature of 100°C (Brock, 1985). At moderate temperatures, the activities of these enzymes can be low or even absent while their mesophilic counterparts are optimally active. Temperature optima of thermophilic enzymes are usually found at or even above the optimum temperature for growth of the bacterium from which they have been isolated. Evolutionary adaptations which have produced thermophiles must have involved mutations which increased the thermostability of their proteins. Conversely, mesophiles may have evolved from thermophiles which developed at an early stage in evolution. Molecular biological approaches are now widely used to reveal differences in primary structure among mesophilic and thermophilic enzymes that determine thermostability and thermal activity. The stability of thermophilic enzymes at room temperature can prolong the shelf life of commercial products that use such enzymes. Temperature also affects physicochemical parameters such as diffusion rates, substrate solubility, viscosity, and water surface tension. The influence of high temperature on

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these factors can be beneficial in certain microbial fermentations. Heat production associated with bacterial metabolic activities is less of a problem when thermophiles are used. On the other hand, these bacteria are often endowed with low growth rates, cell or product yield, incomplete substrate utilization, and often suffer from a genetic instability. The lack of fundamental knowledge on genetic manipulation of these bacteria can severely limit their application in industrial processes (Deming, 1986). In our attempts to gain more insight into different physiological aspects of thermophiles, we have focussed on energy conservation and mechanisms of solute transport in this diverse group of bacteria, including extreme thermophilic archaebacteria.

EXPERIMENTAL APPROACHES IN THE STUDY OF ENERGY TRANSDUCING PROCESSES

Cytoplasmic Membrane Vesicles

The difficulty in elucidating the molecular mechanisms of energy transduction in intact cells has led to the development of procedures for the isolation of closed cytoplasmic membrane vesicles that retain the functional and structural properties of the cytoplasmic membrane (Kaback, 1986). Such procedures are based on two experimental steps: (i) the conversion of the bacterial cell into an osmotically sensitive form (protoplast or spheroplast) by removal of the cell wall with the use of peptidoglycan degrading enzymes in a hypertonic medium, and (ii) osmotic lysis by dilution of the proto- or spheroplasts into a hypotonic medium in the presence of (deoxy-)ribonuclease to remove DNA and RNA. The resulting vesicles retain the *in vivo* orientation of the cytoplasmic membrane, and preserve their structural and functional properties. The activities of interfering cytoplasmic enzymes are usually minor, whereas residual energy sources are completely absent. In many cases, only minor adaptations are required to prepare functional membrane vesicles from moderate thermophiles. For instance, membrane vesicles derived from thermophilic bacilli have been extensively used for the study of energy-transducing processes such as solute transport. Membrane vesicles retain their membrane-associated enzymes and the ability to generate a Δp through electron flow. On the other hand, it has not been possible to prepare functional membrane vesicles of extreme thermophilic archaebacteria. Their cell envelope is covered with a stable proteinaceous layer, the S-layer, which is firmly associated with the cytoplasmic membrane (see paper by W. Baumeister in this issue). This layer confers stability to the cell envelope and may prevent proper membrane vesicle formation. Procedures which rely on mechanical lysis, i.e., french press treatment or sonication, cause a major loss of peripheral membrane-bound enzymatic activities whereas the polarity of the cytoplasmic membrane is inside-out or scrambled.

Membrane Fusion

Fermentative thermophilic bacteria lack electron transfer chain components, and rely on the hydrolysis of ATP for the generation of a Δp. The same constraints apply to isolated membrane vesicles derived from these bacteria with the experimental handicap that ATP cannot be used as energy source for the generation of a Δp since these membrane vesicles have the *in vivo* polarity of the cytoplasmic membrane. Alternative methods which have been developed to create a Δp in such membrane systems rely on the imposition of diffusion gradients of weak acids and/or ions in the presence of ionophores. Such gradients are useful for a qualitative characterization, but their transient character, which is particularly evident at elevated temperatures, preludes further quantitative uses. An efficient solution for this problem has been found in the incorporation of foreign primary proton pumps in the cytoplasmic membrane (Driessen *et al.*, 1987). For this purpose membrane vesicles are fused with proteoliposomes containing a Δp-generating enzyme, such as cytochrome *c*-oxidase (Driessen *et al.*, 1985a), the light-driven bacteriorhodopsin (Driessen *et al.*, 1985b), or photochemical reaction centers (Crielaard *et al.*, 1988). The fusion procedure yields closed hybrid membranes which retain the energy-conserving properties of the original membrane vesicles while the presence of Δp-generating systems allows the long-term generation of a Δp when supplied with a suitable energy source. Fused membranes have proven to be very useful for the study of solute transport in membranes of fermentative mesophilic organisms like lactococci and *Clostridium acetobutylicum* (Driessen *et al.*, 1987). With fermentative thermophiles, thermostable Δp-generating systems have been used to extent the workable temperature range. Thermostable proteins used are cytochrome *c*-oxidase from *Bacillus stearothermophilus* (De Vrij *et al.*, 1989a) and reaction

centers from *Chloroflexus aurantiacus* (G. Speelmans, unpublished results).

Proteoliposomes Composed of Membrane-Spanning Tetraether Lipids

Membrane lipids of archaebacteria differ considerably from conventional lipids in their chemical structure (see paper by D. Sprott in this issue). They are characterized by ether linkages instead of ester linkages and contain biphytanyl chains instead of fatty acyl chains. Lipids of extreme thermophiles are essentially composed of tetraethers with two polar heads linked by two C_{40} phytanyl chains (De Rosa *et al.*, 1991). Based on the bipolar structure, tetraether lipids can span the entire cytoplasmic membrane in a monomolecular arrangement. The extreme thermophile *Sulfolobus acidocaldarius* contains 95–98% of these tetraether lipids when grown at 85°C and an external pH of 2.5. Closed and stable unilamellar liposomes can be formed from a specific lipid fraction obtained from a total lipid extract (Elferink *et al.*, 1992). The size and structure of these liposomes resembles that of liposomes composed of the bilayer-forming lipids of *E. coli*. However, a remarkable difference was observed after freeze-fracturing of both liposome preparations. With conventional bilayer lipids, the preferential fracture plane in freeze-fracturing is in the middle of the phospholipid bilayer, yielding convex and concave halves (Fig. 1A). In contrast, with tetraether lipids the fracture plane is absent and only cross-fracturing of the total membrane is observed (Fig. 1B). Thus, liposomes composed of tetraether lipids from a true monolayer. Beef heart cytochrome *c*-oxidase, bacteriorhodopsin, as well as the leucine transport carrier of *L. lactis* have been functionally reconstituted in these monolayers (Elferink *et al.*, 1992; G. In't Veld, unpublished data). Reconstitution of cytochrome *c*-oxidase can be achieved by dialysis followed by freeze–thaw and sonication. The leucine carrier has been introduced into these liposomes by fusion with membrane vesicles of *L. lactis* (see previous section). In this system, the Δp generated by cytochrome *c*-oxidase drives the uphill transport of leucine. The monolayer lipids from *S. acidocaldarius* activate the leucine transport system (G. In't Veld, unpublished data). Like a conventional phospholipid bilayer, archaebacterial monolayers form a competent matrix for the function of exogenous membrane proteins irrespective of their origin.

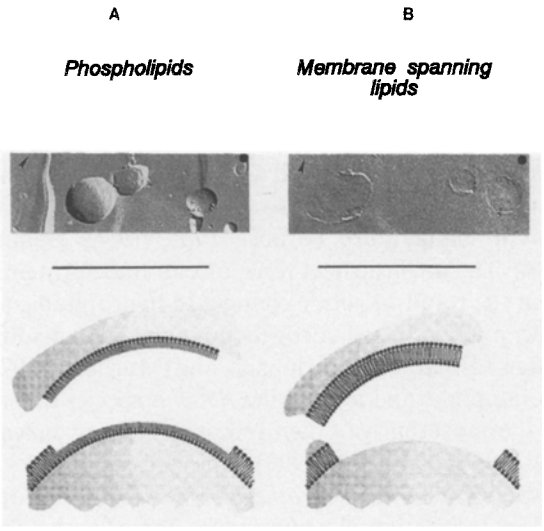

Fig. 1. Freeze-facture replicas of cytochrome *c*-oxidase liposomes composed of *E. coli* (A) and *S. acidocaldarius* (B) and lipid. The arrow indicates the direction of shadowing. The cartoon shows a schematic representation of the fracture plane in freeze-fracturing of the phospholipid bilayer and the cross-fracturing of the membrane-spanning archaebacterial lipid.

EFFICIENCY OF ENERGY TRANSDUCTION

Thermophiles grow up to 80°C, whereas extreme thermophiles are capable of growing up to the boiling point of water. This ability is not only determined by the stability and activity of enzymes at these extremes but also by the structure of the membrane. The lipid composition of membranes of thermophiles is remarkably different from that of mesophiles with respect to fatty acid and polar headgroup content (Mendoza and Cronan, 1983; Russell 1984). As discussed in the previous section, membranes of extreme thermophilic archaebacteria contain lipids with distinct chemical structure which confer a high stability to the cytoplasmic membrane. In the aerobic moderate thermophile *B. stearothermophilus* changes in growth temperature affect alterations in phospholipid composition (Reizer *et al.*, 1985). The ratio of phosphatidylglycerol (PG) and cardiolipin (CL), which constitute approximately 90% of cellular phospholipid, increases with increasing growth temperature, whereas the content of unsaturated fatty acids gradually depletes. Iso and anteiso fatty acids are replaced by saturated linear fatty acids with long chain length. Such changes in fatty acid composition are considered to maintain membrane fluidity at a constant level over the entire growth temperature range, i.e., homoviscous adaptation

(Sinensky, 1974). Consequently, physiological features like protein–lipid interactions have to be adjusted to accommodate the growth temperature. With archaebacterial lipids, the degree of cyclization of the biphytanyl chains increases with increasing growth temperature (De Rosa and Gambacorta, 1988). Thermophilic enzymes are also adapted to the elevated growth temperature (Brock, 1985, 1986; Deming, 1986). The intrinsic heat resistance of these proteins is often the result of subtle changes in hydrophobic and ionic interactions, hydrogen bonding, and disulfide bridge formation (Amelunxen and Murdoch, 1978). Thermophilic and mesophilic *Bacillus* species differ in the thermostability of their redox-converting enzymes (De Vrij *et al.*, 1988, 1990). The activity profiles of these enzymes in *B. stearothermophilus* are nearly optimal at the optimum temperature for growth (T_g = 65°C), with a much higher turnover rate than the functionally comparable enzymes of the mesophile *B. subtilis*. The efficiency of energy conversion is low in thermophiles compared to the mesophiles. This has been attributed to the increased H^+ permeability of membrane of thermophiles at elevated temperatures (De Vrij *et al.*, 1988). High rates of redox-energy conversion are required to overcome the high H^+ permeability. In order to maintain a significant Δp at higher growth temperatures, cells have to invest more of their metabolic energy in maintenance. Many thermophiles exhibit a low growth yield and high maintenance requirement at their growth temperatures compared to the mesophilic counterparts (Kuhn *et al.*, 1980; McKay *et al.*, 1982; Farrand *et al.*, 1983). Obviously, these organisms are not capable of adjusting the membrane fatty acid composition in such a way that at high temperatures efficient energy transduction is possible and inefficient energy coupling may be a general characteristic of thermophiles.

PRIMARY TRANSPORT IN THERMOPHILES

Bacterial cytoplasmic membranes are equipped with energy-transducing enzymes, capable of converting redox, chemical, or light-energy into electrochemical energy (Mitchell, 1966). We will briefly discuss two redox enzymes, i.e., cytochrome *c*-oxidase from *B. stearothermophilus* and reaction centers of *Chl. aurantiacus*, both of which have been used to study solute transport mechanisms in membrane vesicles of thermophilic fermentative bacteria (Speelmans *et al.*, 1989).

The purified cytochrome *c*-oxidase from *B. stearothermophilus* shows the typical spectral characteristics of a caa_3-type oxidase (De Vrij *et al.*, 1989a). Both heme *a* and heme a_3 bind CO. Cytochrome *c*-oxidases typically contain three Cu atoms, i.e., Cu_a, Cu_b, and an additional Cu atom and 2 Fe atoms per enzyme molecule (Steffens *et al.*, 1987). The Fe atoms correspond to the two heme *a* groups. In contrast, the *B. stearothermophilus* enzyme contains three Fe atoms per monomer, suggesting the presence of three heme moieties per enzyme monomer. The structural complexity of this enzyme is relatively low in comparison to that of eukaryotic aa_3-type oxidases (Azzi, 1980). The protein consists of three subunits with apparent molecular masses (M_w) of 55, 37 and 22 kDa, respectively. The 37-kDa subunit contains a covalently attached heme *c* group, a characteristic shared by other thermophilic *c*-type oxidases (Ludwig, 1987). The *B. stearothermophilus* cytochrome *c*-oxidase oxidizes not only cytochrome *c* (E_m = +210 mV) but also nonphysiological electron donors like PMS (E_m = +80 mV) and TMPD (E_m = +260 mV). Electron transfer from reduced TMPD may proceed via the enzyme-bound heme *c* to the other components (heme *a*, a_3, Cu_a and Cu_b atoms). The enzyme confers a high thermostability both in detergent solution and reconstituted form, whereas it is relative insensitive to changes in phospholipid microviscosity (De Vrij *et al.*, 1989a). The oxidase is capable of generating a high Δp (up to −160 mV) when reconstituted into proteoliposomes, an activity which is prevented by the typical oxidase inhibitors like cyanide and azide. The thermostability and high turnover rates (V_{max} = 250–300 electrons per second) at elevated temperatures of the *B. stearothermophilus* cytochrome *c*-oxidase is of advantage for its use as a Δp-generating system in fused membranes. Electron-transport components like the terminal aa_3-oxidase and succinate dehydrogenase have been isolated from the archaebacterium *S. acidocaldarius* (Anemüller and Schäfer, 1990; Moller and Schäfer, 1991). The reconstituted thermal aa_3-oxidase generates a Δp upon energization with synthetic electron donors (Gleißner *et al.*, 1992). The construction of liposomes composed of archaebacterial lipids now allows the study of these enzymes in their natural environment.

The photochemical reaction center of the thermophilic green filamentous gliding bacterium *Chl. aurantiacus* has been purified and characterized (Pierson and Thornber, 1983; Shiozawa *et al.*, 1987). This protein is thermostable and consists of two subunits

(L and M) with similar M_w, i.e., 24 and 24.5 kDa (Shiozawa *et al.*, 1987). The L-subunit of the *Chloroflexus* reaction center shares a high degree of homology with the L-subunit of purple sulfur bacteria (Ovchinnikov *et al.*, 1988). Purple bacteria contain four photoactive bacteriochlorophyll (P-865) and two bacteriopheophytines per reaction center, whereas *Chloroflexus* contains three of each (Pierson and Thornber, 1983). Studies on the primary photochemistry indicate the presence of a two-menaquinone acceptor system (Venturoli and Zannoni, 1988), whereas a membrane-bound cytochrome c_{554} operates as electron donor to the photo-oxidized P-865 (Ovchinnikov *et al.*, 1988). The turnover of the *Chloroflexus* reaction center is temperature dependent and maximal at 50–60°C ($V_{max} = 40\,s^{-1}$). When reconstituted, activity decreases at higher temperatures due to thermal inactivation of horse heart cytochrome *c*. Cyclic electron transfer can be reconstituted in liposomes bearing the purified reaction center by the use of the membrane-permeable electron-mediator UQ_0 and cytochrome *c* as described for reaction centers of *Rhodobacter sphaeroides* (Molenaar *et al.*, 1988). At temperatures up to 45°C and low ionic strength, the reconstituted *Chloroflexus* reaction center generates a $\Delta\psi$ up to -160 mV illumination (D. Hillenga, unpublished results). These liposomes have been fused with membrane vesicles of *Cl. fervidus* to generate a Δp which functions as a driving force for amino acids uptake (G. Speelmans, unpublished results). Reaction centers do not suffer from the intrinsic limitation of oxidases in which oxygen supply can become a limiting factor at higher temperatures, while strict anaerobic conditions can be used with obligate anaerobic thermophiles.

SECONDARY TRANSPORT IN THERMOPHILES

Protons are believed to be the most widely used energy-coupling cation in secondary solute transport systems. Na^+ ions are essential for growth of marine, halophilic, certain alkalophilic, and rumen bacteria that live in a Na^+-rich environment (Skulachev, 1985; Dimroth, 1987; Unemoto *et al.*, 1990). Na^+ often participates as coupling ion in secondary solute transport and energy conservation (Dimroth, 1987; Maloy, 1990), and fulfills an essential role in pH homeostasis in most bacteria (Padan *et al.*, 1981; Booth, 1985). Bacteria may use two strategies to generate an electrochemical gradient of Na^+ (Δp_{Na+}) (Table 1) (Dimroth, 1987): (i) primary transport systems, such as Na^+-translocating decarboxylases, ATPases, or redox-converting enzymes, or (ii) through secondary Na^+/H^+ antiporters which convert Δp into an Δp_{Na+} (Fig. 2). Na^+-dependent solute transport is common in alkalophilic and marine bacteria but in bacteria which grow optimally at neutral pH it is an unusual feature. The advantage of the use of Na^+ instead of H^+ is not always clear (De Vrij *et al.*, 1990); however, at high growth temperatures, Na^+ often represents the preferred coupling ion. As Na^+ exhibits a lower membrane permeability than protons (De Vrij *et al.*, 1990), its use as coupling ion may circumvent major energy losses. Up to now, no evidence is available for primary transport mechanisms for Na^+ in thermophiles, and it is generally assumed that the Δp_{Na+} results from a secondary Na^+/H^+ antiport mechanism.

Biochemical Studies

Membrane vesicles of *B. stearothermophilus* accumulate most amino acids in response to Δp (De Vrij *et al.*, 1989b). A thermostable high-affinity transport system for L-glutamate and L-aspartate has been studied in detail (Table II). Membrane vesicles accumulate glutamate to a high level when supplied with a suitable electron donor, such as succinate. Maximal transport activity is observed at 60–65°C (De Vrij *et al.*, 1989b). Studies with ionophores and imposed ion-diffusion gradients established that both ΔpH and $\Delta\psi$ as well as Δp_{Na+} function as driving forces. Quantitative studies on the relation between the steady-state accumulation level of glutamate and Δp at different pH values indicate that the anionic glutamate is translocated in symport with a H^+ and a Na^+ ion. The system exhibits a high affinity for Na^+ below 5–6 μM, a concentration present as contaminant in most buffer solutions. The rate of glutamate transport is affected by the internal pH. In *B. stearothermophilus*, high-affinity transport systems have been established for the following groups of L-amino acids: leucine, isoleucine, valine; alanine and serine; and lysine and arginine. Δp-dependent transport of leucine and alanine is inhibited by the Na^+ ionophore nonactin and stimulated by monensin, which converts ΔpH into $\Delta\bar{\mu}_{Na+}$. Uptake of leucine and alanine required high concentrations of Na^+ (up to 50 mM) and exhibits an apparent K_m for Na^+ in the millimolar range. Except for Li^+, other monovalent cations (NH_4^+, Cs^+, Rb^+,

Table I. Occurrence of Sodium-Coupled Transport Systems

Environment	High salt	High pH	Extreme	Nonextreme
Bacteria	Halophiles Marine bacteria Rumen bacteria	Alkaliphiles Alkalitolerants	Thermophiles Methanogens	Enterobacteria
Mode of Δp_{Na+} generation	Na^+/H^+ antiport Respiratory chain Decarboxylase ATP hydrolysis	Na^+/H^+ antiport ATP hydrolysis	Na^+/H^+ antiport Decarboxylase	Na^+/H^+ antiport

and K^+) are ineffective (Heyne *et al.*, 1991). A catalytic role of H^+ could be excluded for the alanine transport system, and the system is inhibited by protons with an apparent p*K* of 7.6. The alanine transport systems of *B. stearothermophilus* resembles the Na^+-linked system found in *Bacillus PS3*, which has been purified and functionally reconstituted into proteoliposomes (Hirata *et al.*, 1976, 1984). Lysine transport depends on $\Delta\psi$ only, and does not require Na^+. Lysine is most likely translocated by an electrogenic uniport mechanism (Heyne *et al.*, 1991). The mechanism by which Δp_{Na+} is generated in *B. stearothermophilus* is unknown, although the involvement of a Na^+/H^+ antiport has been implicated.

Cl. fervidus is a thermophilic fermentative bacterium which has been isolated from a hot pool in New Zealand (Patel *et al.*, 1987). Membrane vesicles of *Cl. fervidus* accumulate neutral (serine, leucine, glycine, alanine), basic (lysine, arginine), acidic (glutamate, aspartate), and aromatic (phenylalanine) amino acids in the presence of Δp_{Na+}. Serine uptake is observed only in the presence of a Δp_{Na+}, $\Delta\bar{\mu}_{Na+}$, or a $\Delta\psi$ in the presence of Na^+. Membrane vesicles of *Cl. fervidus* have been fused with proteoliposomes harboring cytochrome *c*-oxidase of *B. stearothermophilus* to maintain a Δp for a considerable length of time. In this system, monensin promotes the generation of a Δp_{Na+} through the conversion of a ΔpH into a $\Delta\bar{\mu}_{Na+}$. Monensin remarkably stimulates serine transport, suggesting a Na^+-linked transport mechanism. The Na^+-serine cotransport stoichiometry estimated from the steady-state level of serine uptake and $\Delta\Psi$ in the

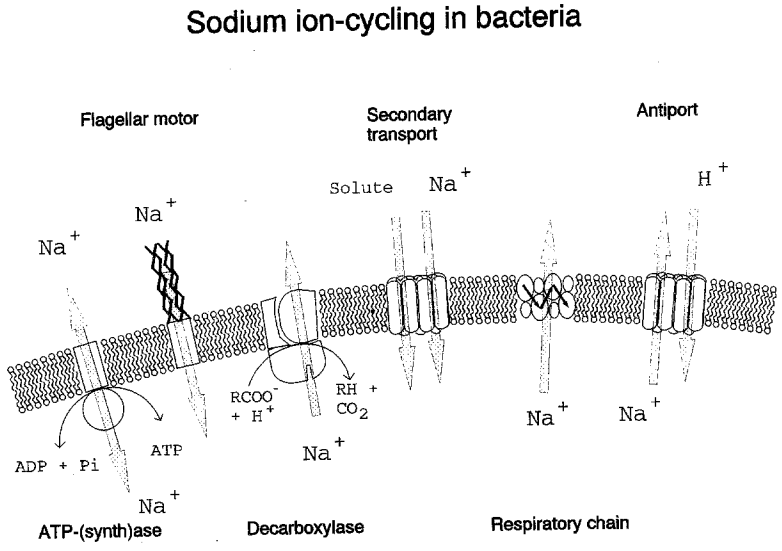

Fig. 2. Putative elements of a sodium cycle in bacteria. Indicated are the primary transport systems such as the Na^+-translocating decarboxylases, ATPases, and electron transfer chains, the secondary Na^+/H^+ antiporter. Other membrane proteins utilize the sodium-motive force for the synthesis of ATP, solute transport, or motility. From Tolner *et al.*, 1992c.

Table II. Amino acid transport systems in Thermophilic Bacteria[a]

Bacterium	Substrate	K_m (μM)	Mechanism	K_m for Na^+ (mM)
B. Caldotenax	Glutamate	2.9	Na^+/H^+ symport	—
B. stearothermophilus	Glutamate	4.7	Na^+/H^+ symport	< 0.005
	Aspartate	3.8		
	Alanine	12.5	Na^+ symport	1.0
	Serine	49		
	Leucine	0.9	Na^+ symport	0.4
	Isoleucine	1.0		
	Valine	2.5		
	Lysine	12.9	Uniport	—
	Arginine	3.4		
Cl. fervidus	Serine	10	Na^+ symport	2.5
	Leucine	?		
	Glutamate	?	Na^+ symport	0.8
	Lysine	?	Na^+ symport	?
	Arginine			

[a]L-Amino acids.

absence of $\Delta\tilde{\mu}_{Na+}$ equals 0.9. Direct estimates of the stoichiometry from the coupled efflux of ^3H-labelled serine and $^{24}Na^+$ yield a value of 1 (G. Speelmans, unpublished results). Uptake requires either Na^+ or Li^+, while the affinity for Na^+ is rather low (Table II). Arginine uptake is driven by Δp_{Na+}, while Na^+ can be replaced for Li^+. In the fused membranes, a steady-state level of accumulation could not be achieved within a reasonable time-span precluding an estimation of the Na^+-arginine stoichiometry. Direct estimates indicate a one-to-one stoichiometry. Uptake of glutamate appears to be similarly dependent on the presence of Na^+. Glutamate is hardly accumulated by the fused-membrane system containing cytochrome-*c* oxidase unless the ionophore monensin is present. In the presence of Na^+, monensin promotes the interconversion of ΔpH into $\Delta\tilde{\mu}_{Na+}$. This observation argues in favor of electroneutral symport of Na^+ and glutamate. This conclusion is confirmed by experiments demonstrating artificial imposed $\Delta\tilde{\mu}_{Na+}$-driven glutamate uptake, whereas the simultaneous imposition of $\Delta\psi$ has only a slight stimulatory effect. However, at low concentrations of Na^+, $\Delta\psi$ drives glutamate uptake. ΔpH cannot drive glutamate uptake. A direct assessment of the stoichiometry suggest a value of 1 (G. Speelmans, unpublished results). The transport system for glutamate in *Cl. fervidus* is specific for glutamate and Na^+. The system is not inhibited by aspartate, glutamine, or asparagine, whereas Li^+ cannot replace Na^+.

Genetic Studies

To elucidate the molecular properties of glutamate transport systems of thermophilic bacteria in more detail, a strategy was devised to clone the gene encoding the glutamate transport protein. This strategy is based on complementation of an *E. coli* K12 strain which grows poorly on glutamate as sole source of energy, nitrogen, and carbon, as a result of an insufficient capacity to accumulate glutamate (Halpern *et al.*, 1965). So far the genes encoding the glutamate transport systems have been cloned from *B. caldotenax* ($gltT_{Bc}$) and *B. stearothermophilus* ($gltT_{Bs}$) (Tolner *et al.*, 1992a). Functional expression of the gene products was shown by increased uptake of glutamate. The K_m values for uptake of glutamate mediated by $GltT_{Bs}$ and $GltT_{Bc}$ expressed in *E. coli* are lower than the values estimated in native *Bacillus* membrane vesicles (Tolner *et al.*, 1992a). Both genes have been sequenced (Tolner *et al.*, 1992a), and their translated amino acid sequences are highly homologous (96.7% identity). The $gltT_{Bs}$ and $gltT_{Bc}$ genes encodes a protein of 421 residues, which corresponds with a M_w of 45 kDa while the apparent M_w estimated from SDS-PAGE is 33 kDa. Such aberrant electrophoretic behavior seems to be general property of hydrophobic integral membrane proteins. Hydropathy analysis suggests that both proteins traverse the membrane 12 times. $GltT_{Bs}$ and $GltT_{Bc}$ bear extensive similarity (57.2% identity) with $GltP_{Ec}$, a H^+/glutamate transport protein of *E. coli* K12 (Tolner *et al.*,

1992b), and not with $GltS_{Ec}$, a Na^+/glutamate transport protein of *E. coli* B (Deguchi, 1990) and K12 (Kalman, 1991). A conserved amino acid sequence has been proposed to be involved in Na^+ recognition or binding (_-G-_-A---L---Gr-_) (Deguchi, 1990). This sequence is also present in $GltT_{Bs}$ and $GltT_{Bc}$, except that Arg is replaced for Lys (_-G_{38}-_-A_{62}---L_{67}---$G_{71}K_{72}$——). A similar motif is found in the $GltP_{Ec}$ protein (_-G_{45}-_-A_{69}---A_{74}---G_{78} R_{79}——), and though speculative, the mismatch could explain the inability of $GltP_{Ec}$ to use Na^+ as coupling ion despite its extensive similarity with $GltT_{Bs}$. On the other hand, the SOB-motif of $GltT_{Bs}$ and $GltT_{Bc}$ is located in a short hydrophilic region and might be involved in stabilization of protein structure (Henderson, 1990).

CONCLUDING REMARKS

Na^+ plays a pivotial role as energy-transducing coupling ion in thermophiles, although little is known about the mechanisms of solute transport in extreme thermophilic archaebacteria. Current strategies are directed at the cloning of genes coding for transport proteins of thermophiles. These proteins possess high thermostability, and their high level of expression in a mesophilic host-strain such as *E. coli* may allow rapid purification merely on the basis of the thermostability. The stability of these proteins may facilitate their crystallization, and allow the elucidation of the structure of a secondary transport system at the molecular level.

REFERENCES

Amelunxen, R. E., and Murdoch, A. L. (1978). *CRC Crit. Rev. Microbiol.* **6**, 343–393.

Anemüller, S., and Schäfer, G. (1990). *Eur. J. Biochem.* **191**, 297–305.

Azzi, A. (1980). *Biochim. Biophys. Acta* **594**, 231–252.

Booth, I. A. (1985). *Microbiol. Rev.* **49**, 359–378.

Brock, T. D. (1985). *Science* **230**, 132–138.

Brock, T. D. (1986). *Thermophiles; General, Molecular and Applied Microbiology*, Wiley, New York.

Crielaard, W., Driessen, A. J. M., Molenaar, D., Hellingwerf, K. J., and Konings, W. N. (1988). *J. Bacteriol.* **170**, 1820–1824.

Deguchi, Y., Yamato, I., and Anraku, Y. (1990). *J. Biol. Chem.* **265**, 21704–21708.

Deming, J. W. (1986). *Microbiol. Ecol.* **12**, 111–119.

De Vrij, W., Bulthuis, R. A., and Konings, W. N. (1988). *J. Bacteriol.* **170**, 2359–2366.

De Vrij, W., Heyne, R. I. R., and Konings W. N. (1989a). *Eur. J. Biochem.* **178**, 763–770.

De Vrij, W., Bulthuis, R. A., Van Iwaarden, P. R., and Konings, W. N. (1989b). *J. Bacteriol.* **171**, 1118–1125.

De Vrij, W., Speelman, G., Heyne, R. I. R., and Konings, W. N. (1990). *FEMS Microb. Rev.* **75**, 183–200.

De Rosa, M., and Gambacorta, A. (1988). *Prog. Lipid. Res.* **27**, 153–175.

De Rosa, M., Trincone, A., Nicolaus, B., and Gambacorta, A. (1991). In *Life under Extreme Conditions* (di Prisco, G., ed.), Springer Verlag, Berlin, pp. 61–87.

Dimroth, P. (1987). *Microbiol. Rev.* **51**, 320–340.

Driessen, A. J. M., de Vrij, W., and Konings, W. N. (1985a). *Proc. Natl. Acad. Sci. USA* **82**, 7555–7559.

Driessen, A. J. M., Hellingwerf, K. J., and Konings, W. N. (1985b). *Biochim. Biophys. Acta* **808**, 1–12.

Driessen, A. J. M., Hellingwerf, K. J., and Konings, W. N. (1987). *Microb. Sci.* **4**, 173–180.

Elferink, M. G. L., de Wit, J., Demel, R., Driessen, A. J. M., and Konings, W. N. (1992). *J. Biol. Chem.* **267**, 1375–1381.

Farrand, S. G., Jones, C. W., Linton, J. D., and Stephenson, R. J. (1983). *Arch. Microbiol.* **135**, 276–283.

Gleißner, M., Elferink, M. G. L., Driessen, A. J. M., Konings, W. N., and Schäfer, G. (1992). 7th EBEC reports, Helsinki, Finland, pp. 52.

Halpern, Y. S., and Lupo, M. (1965). *J. Bacteriol.* **90**, 1288–1295.

Henderson, P. J. F. (1990). *Res. Microbiol.* **141**, 316–328.

Heyne, R. I. R., de Vrij, W., Crielaard, W., and Konings, W. N. (1991). *J. Bacteriol.* **173**, 791–800.

Hirata, H., Sone, N., Yoshida, H., and Kagawa, Y. (1976). *J. Biol. Chem.* **79**, 1157–1166.

Hirata, H., Kambe, T., and Kagawa, Y. (1984). *J. Biol. Chem.* **259**, 10653–10656.

Kaback, H. R. (1986). *Annu. Rev. Biophys. Chem.* **15**, 279–319.

Kalman, M., Gentry, D. R., and Cashel, M. (1991). *Mol. Gen. Genet.* **225**, 379–386.

Kuhn, H. J., Cometta, S., and Fiechter, A. (1980). *Eur. J. Appl. Microbiol. Biotechnol.* **10**, 303–315.

Ludwig, B. (1987). *FEMS Microbiol. Rev.* **46**, 41–56.

Maloy, S. (1990). In *The Bacteria, Vol. XII: Bacterial Energetics* (Krulwich, T. A., ed), Academic Press, San Diego, pp. 203–224.

McKay, A., Quilter, J., and Jons, C. W. (1982). *Arch. Microbiol.* **131**, 43–50.

Mendoza, D., and Cronan, J. E. (1983). *Trends Biochem. Sci.* **8**, 49–52.

Mitchell, P. (1966). *Physiol. Rev.* **41**, 445–502.

Molenaar, D., Crielaard, W., and Hellingerf, K. J. (1988). *Biochem.* **27**, 2014–2023.

Möller, R., and Schäfer, G. (1991). *Eur. J. Biochem.* **201**, 593–600.

Ovchinnikov, Y. A., Abdulaev, N. G., Zolotarev, A. S., Shmukler, B. E., Zargarov, A. A., Kutuzov, M. A., Telezhinnskaya, I. N., and Levina, N. B. (1988). *FEBS Lett.* **231**, 237–242.

Padan, E., Züberstein, D., and Schuldiner, S. (1981). *Biochim. Biophys. Acta* **650**, 151–166.

Patel, B. K. C., Monk, C., Littleworth, H., Morgan, H. W., and Daniel, R. M. (1987). *Int. J. Syst. Bacteriol.* **37**, 123–126.

Pierson, B., and Thornber, J. P. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 80–84.

Reizer, J., Grossowicz, N., and Barenhoiz, Y. (1985). *Biochim. Biophys. Acta* **815**, 268–280.

Russell, N. J. (1984). *Trends Biochem. Sci.* **9**, 108–112.

Shiozawa, J. A., Lottspeich, F., and Feick, R. (1987). *Eur. J. Biochem.* **167**, 595–600.

Sinensky, M. (1974). *Proc. Natl. Acad. Sci. USA* **71**, 522–525.

Skulachev, V. P. (1985). *Eur. J. Biochem.* **151**, 199–208.

Speelmans, G., De Vrij, W., and Konings, W. N. (1989). *J. Bacteriol.* **171**, 3788–3795.

Steffens, G. C. M., Biewald, R., and Buse, G. (1987). *Eur. J. Biochem.* **164**, 295–300.

Tolner, B., Poolman, B., and Konings, W. N. (1992a), *Mol. Microbiol.*, in press.

Tolner, B., Poolman, B., Wallace, B., and Konings, W. N. (1992b). *J. Bacteriol.*, **174**, 2391–2393.

Tolner, B., van der Rest, M. E., Speelmans, G., and Konings, W. N. (1992c). In *Molecular mechanisms of transport* (Quagliariello, E., and Palmieri, F., eds.), Elsevier, Amsterdam, pp. 43–50.

Unemoto, T., Tokuda, H., and Hayashi, M. (1990). In *The Bacteria, Vol. XII: Bacterial Energetics* (Krulwich, T. A., ed), Academic Press, San Diego, pp. 33–54.

Venturoli, G., and Zannoni, D. (1988). *Eur. J. Biochem.* **178**, 503–509.