NA+ AS COUPLING ION IN ENERGY TRANSDUCTION IN EXTREMOPHILIC BACTERIA AND ARCHAEA

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Na⁺ as coupling ion in energy transduction in extremophilic Bacteria and Archaea

G. Speelmans, B. Poolman and W.N. Konings

For microorganisms to live under extreme physical conditions requires important adaptations of the cells. In many organisms the use of Na⁺ instead of protons as coupling ion in energy transduction is associated with such adaptation. This review focuses on the enzymes that are responsible for the generation and utilization of Na⁺ gradients in extremophilic microorganisms. Aspects that are dealt with include: bioenergetics and ion homeostasis in extremophilic Bacteria and Archaea; the molecular mechanism of Na⁺ translocation; and (dis)advantages of Na⁺ as coupling ion in energy transduction.

Key words: Bioenergetics, energy transduction, extremophiles, Na⁺, transport.

Our knowledge about the functioning of the prokaryotic cell is, for the greater part based on observations made on cells functioning under 'moderate' or 'normal' physical conditions, i.e. at a pressure of 1 atm, near neutral pH, moderate osmolarities and a temperature of 30-37°C. Many microorganisms belonging to the Archaea and Bacteria have been discovered that live in the most diverse and bizarre environments, and these organisms have developed special properties to survive and prosper in these habitats. Adaptations are found in the cellular lipids, proteins and other macromolecular compounds, and in the mechanisms for energy transduction and the regulation of the intracellular milieu. The following groups of extremophiles have been discriminated: (1) alkali- and acido-philes, growing optimally at high and low pH, respectively; (2) halophiles, growing optimally at high ionic strength; (3) barophiles, living at high pressure; and (4) psycho- and thermo-philes, growing optimally at low and high temperatures, respectively. Furthermore, some microorganisms can even grow optimally in milieus in which two or more of these extreme physical conditions exist simultaneously.

All living cells have a cytoplasmic membrane that functions as the major barrier between the cytoplasm and the environment. The barrier properties of this membrane are mainly determined by the lipid molecules, which expose their polar headgroups to the water phases and their apolar fatty acid tails to each other. The cytoplasmic membrane is responsible for the selective passage of compounds from the cytoplasm to the environment and vice versa. It forms a matrix in which the energy-transducing enzymes and carrier proteins are embedded and by which specific solute concentration gradients can be generated and maintained (Poolman et al. 1994). The cytoplasmic membrane therefore plays a crucial role in maintaining the energy status of the cell (Hellinger & Konings 1985), and regulating the intracellular milieu (Booth 1985; Abee & Konings 1990), the turgor pressure (Garcia & Booth 1987) and other energy-transducing/consuming processes.

Usually, the ion concentrations of the environment differ considerably from those of the intracellular milieu. Various ion pumps in the cytoplasmic membrane are used to generate ion concentration gradients in order to maintain an optimal intracellular ion composition (Booth 1985; Skalinev 1987; Walderhaug et al. 1987; Abee & Konings 1990). Only under certain conditions, when parameters of the external milieu are similar to the internal ones, e.g. pH 7.5, low Na⁺ concentrations and high concentrations of K⁺ and other nutrients and with ATP being formed by substrate-level phosphorylation, are bacteria able to grow without ion homeostasis and without a proton motive force (Harold & Van Brunt 1977; Kinosita et al. 1984).

In this review we describe the energy-transducing mechanisms in extremophilic microorganisms. Special attention is...
paid to the nature of the coupling ion in energy transduction and the possible advantages of having a H⁺ cycle, Na⁺ cycle or both are discussed. The important features of enzyme systems that use Na⁺ as coupling ion are described.

**Energy Transduction can Occur with H⁺ and Na⁺ as Coupling Ion**

Light or chemical energy can be converted by energy-transducing enzymes into an electrochemical transmembrane H⁺ gradient (inside alkaline and negative). This gradient exerts a force on the protons, the proton motive force (Δp); which in turn can be used to drive energy-consuming processes. This mechanism of energy coupling as a means to drive endergonic reactions at the cytoplasmic membrane has been termed the chemiosmotic hypothesis (Mitchell 1961). The proton motive force generated upon H⁺ extrusion is composed of an electrical potential (Δψ) and a chemical gradient of H⁺ (the pH gradient, ΔpH):

\[ Δp = Δψ - ZΔpH (mV) \]

where Z = 2.3(RT/F), R is the gas constant, T the absolute temperature, and F the Faraday constant.

Until 1980, H⁺ was considered to be the primary coupling ion in chemiosmotic processes in bacteria (Dimroth 1987). However, it is now well established that energy transduction in bacteria is not uniquely dependent on H⁺ cycling. Electrochemical gradients of Na⁺, which are generated/consumed by similar types of transport mechanism as those that form and consume a Δp, are frequently encountered in microorganisms. These organisms use a sodium motive force (Δs) exclusively or in addition to a proton motive force for their vectorial energy-transducing machinery (Skulachev 1985, 1987, 1989; Dimroth 1992a, b). The sodium motive force is also composed of an electrical and chemical component, given by the equation:

\[ Δs = Δψ - ZΔpNa (mV) \]

Interconversion of the different ion motive forces is achieved by secondary transport mechanisms, such as Na⁺/H⁺ antiporters (see below).

**H⁺ and Na⁺ Cycles in Prokaryotes**

Energy transduction in most bacteria is performed by combining a primary H⁺ pump with a secondary transport system for the generation of a Na⁺ gradient. The contribution of the Na⁺ gradient to energy transduction can vary from marginal to essential. Organisms such as *Lactococcus lactis* rely completely on H⁺ as coupling ion while others, like the enterobacterium *Escherichia coli*, take an intermediate position (Poolman 1993; Poolman & Konings 1993). In *E. coli* the Δp is generated by a primary pump (respiration-linked or ATP-driven H⁺ translocation) but a Na⁺/H⁺ antiporter is needed when the Na⁺ concentration is high and the medium is relatively alkaline. Some solute transport systems in *E. coli* are Na⁺-coupled, but usually more than one mechanism exists (or can be expressed) for the same solute, so that the organism is not strictly dependent on a Na⁺ gradient (Dibrov 1991).

The presence of a Na⁺ cycle is essential in extremely alkaliphilic bacteria, and an electrogenic Na⁺/H⁺ antiporter is used to keep the intracellular pH below the extracellular one (Ivey et al. 1992). In the alkaliphiles, all secondary symport systems studied to date are Na⁺-coupled (see below). *Clostridium sevariae* is perhaps the most extreme in this regard, since its energy transduction at the membrane is exclusively dependent on Na⁺ (Speelmans et al. 1993a).

**Primary and Secondary Na⁺-transport Systems in Bacteria and Archaea**

In Bacteria and Archaea the proton and sodium gradients can be formed by:

1. Redox-linked primary ion pumps (cyclic and linear electron transfer chains);
2. The light-driven proton pump (bacteriorhodopsin in halophilic bacteria);
3. Membrane-bound ATPases;
4. Membrane-bound pyrophosphatases;
5. Membrane-bound decarboxylases;
6. Methyltransferases in methanogens (and acetogens);
7. Secondary transport processes.

Upon re-uptake of H⁺ and/or Na⁺ the proton and/or sodium cycle is completed. This uptake of Na⁺ has occasionally been shown to be rate-determining in the Na⁺ cycle (Krilwich et al. 1985). The downhill flux of H⁺ and/or Na⁺ can be used to drive endergonic reactions that take place at the cytoplasmic membrane. In most cases, a particular enzyme uses either H⁺ or Na⁺ as coupling ion. Occasionally, however, enzymes (transporters) can choose between H⁺ and Na⁺ (Li⁺) (see below). The following Δp- and Δs-consuming processes have been described.

1. Secondary solute transport, uptake of substrates, such as amino acids, sugars, nucleotides and ions, and excretion of metabolic end products, toxic compounds etc. can be coupled to Δp and/or Δs. A special case is the Na⁺/H⁺ antiport system, which interlinks the H⁺ and Na⁺ cycles.
2. ATP synthesis: ATP formed from ADP and P, at the expense of Δp or Δs.
3. Motility; the flagellar motor has been shown to be driven either by the Δp or Δs.
(4) Other membrane-linked endergonic reactions, such as protein translocation, DNA uptake during transformation, conjugation and phage infection, cell wall synthesis, N₂-fixation, regulation of cell volume and cell turgor, heat production and transhydrogenases, have only been described as Δp-dependent processes.

**Redox-linked Na⁺ Pumps**

Respiration-driven Na⁺ pumps can be found in marine and halotolerant/alkalitolerant bacteria. In Vibrio alginolyticus, two NADH:quinone reductases are present: NQR₁ and NQR₂. NQR₁ is specifically stimulated by Na⁺; while NQR₂ is Na⁺-independent (for a recent review see Unemoto & Hayashi (1993)). Na⁺-dependent, NADH:quinone reductases have also been described for other bacteria. Most of the organisms are Gram-negative, marine and moderately halophilic or moderately alkaliphilic bacteria. With the exception of the alkalitolerant Bacillus FTU (Se-meykina & Skulachev 1992), NADH-quinone reductases in Gram-positive, moderately halophilic bacteria translocate H⁺ across the membrane (Unemoto et al. 1992).

Other redox-linked primary Na⁺ pumps comprise the terminal cytochrome o oxidase from Vitreoscilla sp. This enzyme complex has been purified and reconstituted into liposomes and has clearly been shown to be a primary Na⁺ pump (Efiok & Webster 1990). Bacillus FTU and E. coli grown under conditions of low Δp have also been reported to contain terminal oxidases that extrude Na⁺ (Avetisyan et al. 1991). In E. coli a cytochrome d oxidase and in Bacillus FTU an o-type oxidase have been claimed to be Na⁺ pumps (Aveytisian et al. 1992) (Muntyan & Skripnikova 1993), but the data have not yet been obtained with purified enzymes or well-defined membrane systems.

In methanogenic microorganisms (belonging to the Archaea), some steps in the reduction of CO₂ to methane are dependent on Na⁺, i.e. an endergonic reaction in the pathway is driven by Δs whereas an exergonic reaction generates a Δs (Bechtes et al. 1992; Müller & Gottschalk, 1992; Schönheit 1992). In Acetobacterium woodii the conversion/reduction of methylene-tetrahydrofolate to methyl-Co(III)-corrinoid is probably involved in the generation of a Δs (Heise et al. 1989, 1993).

Na⁺-pumping ATPases and Δs-driven ATPsynthases

ATP-driven Na⁺ and H⁺ pumps can be categorized into: (1) F-type: (2) V-type (vacuolar ATPase); and (3) P-type (or E₆E₇ATPase). F-type ATPases comprise the F₅F₆-ATPase of bacteria, chloroplasts and mitochondria. In respiratory and phototrophic bacteria, the F₅F₆-ATPase normally functions in the synthesis mode (i.e. the enzyme utilizes the Δp and/or Δs to synthesize ATP). In fermentative bacteria, on the other hand, the enzyme usually works in the opposite direction (a Δp and/or Δs is generated upon hydrolysis of ATP).

V-type ATPases have been defined as ion (proton) motive ATPases associated with membranes of eukaryotic organelles other than mitochondria and chloroplasts. On the basis of sequence similarities, archaeal H⁺-ATPases of Sulfolobus acidocaldarius (Lübben & Schäfer 1987), Methanosa-rcinae barkeri (Inatomi 1986) and Halobacterium halobium (Mukohata & Isoyama 1987) have been categorized in this family. V-type ATPases have also been recognized in bacteria such as Enterococcus hirae (Kakinuma & Igarashi 1990; Takase et al. 1993), and possibly Thermus thermophilus (Yokoyama et al. 1990) and Clostridium fervidus (Speelmans et al. 1993a). With the exception of the V-type ATPase of E. hirae and the putative V-type ATPase of C. fervidus, which have been shown to transport Na⁺, the other examples of V-type ATPases translocate H⁺ rather than Na⁺ (see below). V-type ATPases are specifically inhibited by NO₃⁻, in contrast to F₅F₆-ATPases for which N₃N'-dicyclohexylcarbodiimide (DCCD) is often used as diagnostic inhibitor.

P-type ATPases form two covalent acyl phosphate intermediates as part of their reaction cycle (E₁ and E₂), which differ in conformation. The P-type ATPases are quite specifically inhibited by micromolar concentrations of ortho-vanadate. P-type ATPases have been described for the translocation of H⁺ or Na⁺ but members of the same family of enzymes have also been shown to transport various other compounds (Siebers & Altendorf 1992).

Na⁺-ATP synthases have frequently been detected in organisms that use primary Na⁺ translocation for Δs generation (Dimroth 1992b). Solid biochemical evidence for the ion specificity of translocation catalysis by the enzymes is limited and some controversies exist. For instance, it has been suggested that ATP synthase in marine bacteria, such as Vibrio alginolyticus, is driven by Δs (Skulachev 1987). However, the purified and reconstituted ATPase of V. alginolyticus exhibits H⁺- rather than Na⁺-pumping activity (Krausholz et al. 1990; Dmietriev et al. 1991). The most thoroughly characterized Na⁺-linked ATP synthase is from the strictly anaerobic, marine bacterium Propionigenium modestum, which is a typical member of the F₅F₆ family (Laubinger & Dimroth 1987). The F₅F₆-ATPase complex is activated by sodium ions (Laubinger & Dimroth 1988a), but the enzyme not only pumps Na⁺ but also H⁺, i.e. when the Na⁺ concentration is low (Laubinger & Dimroth 1988b). The specific activation of the ATPase activity by Na⁺ is lost upon dissociation of the F₁ moiety from the membrane-bound F₅ subunits, and the activation by sodium can be restored by reconstitution of the enzyme complex in liposomes (Laubinger & Dimroth 1988b). Interestingly, a functional hybrid ATPase composed of F₁ from E. coli and F₅ from P. modestum generates a Na⁺ gradient (Laubinger et al. 1990), or can synthesize ATP when a Δs is applied (Dmietriev et al. 1993). These experiments strongly indicate that the ion specificity is conferred by the F₁ moiety.
Figure 1. Different types of secondary transport in bacteria. The driving force on solute A is indicated for each transport mechanism. For transport systems that use Na+, the chemical Na\textsuperscript{+} gradient (ΔNa\textsuperscript{+}) provides the driving force instead of the H\textsuperscript{+} gradient (ΔpH).

**Na\textsuperscript{+} Extrusion by Membrane-bound Decarboxylases**

The membrane-bound, oxaloacetate decarboxylase of *Klebsiella pneumoniae* was the first primary Na\textsuperscript{+} pump to be discovered in bacteria (Dimroth 1980). Later, methylmalonyl-CoA and glutaconyl-CoA decarboxylases were also found to be Na\textsuperscript{+}-translocating enzymes (Dimroth 1987, 1991). The species in which these enzymes are found are not closely related phylogenetically. Decarboxylases are membrane-associated enzymes that are composed of three or four different polypeptides (Dimroth 1992a): (I) a hydrophilic α-subunit of 60 to 65 kDa, which performs the decarboxylation reaction and in which a H\textsuperscript{+} is consumed and CO\textsubscript{2} is formed independent of Na\textsuperscript{+}; (2) a membrane-bound β-subunit of 33 to 34 kDa, which possesses the Na\textsuperscript{+}-binding/translocation site; (3) a membrane-bound γ-subunit of either 9 kDa (in the case of oxaloacetate decarboxylases) or 19 to 24 kDa (in the case of methylmalonyl-CoA and glutaryl-CoA decarboxylases). The biotin moiety is either bound to the α-subunit (in the case of oxaloacetate decarboxylases) or to the γ-subunit. In the latter case a δ-subunit of 14 to 16 kDa, with unknown function, is also present.

**Secondary Na\textsuperscript{+}-coupled Transport Systems**

Three categories of secondary transport can be distinguished in bacteria (Mitchell 1968; Poolman & Konings 1993): (1) Uniport systems, which function independent of any coupling ion; (2) Symport systems, in which solutes move in the same direction [the (electro)chemical gradient of one solute is used to drive the uphill transport of the other]; and (3) Antiport systems, in which solutes move in opposite directions. The solutes transported by secondary systems can be neutral or negatively or positively charged and different numbers of solutes may be co- or counter-transported. The driving forces of these processes therefore vary considerably [see Figure 1; for a review of Poolman & Konings (1993; Poolman et al. 1994)]. In the examples 3 to 9 in Figure 1, protons are shown as coupling ion but, in most cases, similar systems have been described which use Na\textsuperscript{+} as co- or counter-transported species. In general, Na\textsuperscript{+}-coupled secondary transport is found more frequently in extremophilic Bacteria and Archaea growing at high temperatures, alkaline pH or high salinity, whereas H\textsuperscript{+} coupled transport is more common in acidophiles and non-extremophilic Bacteria. However, several exceptions to this rule have been reported (see below), and in many cases Na\textsuperscript{+}- and H\textsuperscript{+}-coupled transport systems can be expressed in one and the same organism. For an overview of Na\textsuperscript{+}-dependent cotransport the reader is referred to the review of Maloy (1990), updated by Speelmans (1993).

**Na\textsuperscript{+}/H\textsuperscript{+} Antiport**

Padan & Schuldiner (1992) stated that all bacteria have Na\textsuperscript{+}/H\textsuperscript{+} antiporter(s). The anaerobic thermophile *C. fervidus* appears to be an exception to this rule, since a functional Na\textsuperscript{+}/H\textsuperscript{+} antiporter could not be demonstrated in intact cells of or membrane vesicles derived from this organism (Speelmans et al. 1993a, b). In addition to a role in the Na\textsuperscript{+} cycle, necessary for Δs-consuming processes or in creating an energy buffer for Δp (Skulachev 1987), the Na\textsuperscript{+}/H\textsuperscript{+} antiporters also serve important roles in control mechanisms and homeostasis of the cell, e.g. by lowering the intracellular Na\textsuperscript{+} concentration to prevent Na\textsuperscript{+} toxicity and regulating the internal pH (Booth 1985; Ivey et al. 1992; Schuldiner & Padan 1992). In methanogens the Na\textsuperscript{+}/H\textsuperscript{+} antiporter has been suggested, in some circumstances, to work in the opposite direction and to generate Δp at the expense of Δs (Kaesler & Schönheit 1989).

**Motility**

Cell mobility is achieved by the action of flagella. In most organisms studied to date, rotation of the flagellum is driven by the proton motive force (Khan 1990). In marine bacteria (Tokuda 1992) and alkaliphilic bacteria (Hirota & Imae 1983; Sugiyama et al. 1985; Bogachev & Skulachev 1993) flagellar motility is Na\textsuperscript{+}-dependent at alkaline pH. In the marine bacterium, *V. alginolyticus*, flagellar rotation is Δp-driven at neutral pH. *Vibrio parahaemolyticus* possesses two types of flagella. The single polar flagellum, suited for
swimming in liquid medium, is driven by the Δψ, whereas the lateral flagella, suited for swimming over viscous surfaces, are Δp-driven (Atsumi et al. 1992).

**Molecular Mechanism of Na⁺ Translocation**

An intriguing phenomenon is the interconvertability of Na⁺ and H⁺ as coupling ion in some Na⁺-, H⁺-translocating enzymes. The F₆,F₆'-ATPase of *Propionigenium modestum* (Laubinger & Dimroth 1988b), the alanine carrier of PS3 (Hirata et al. 1984), and the melibiose carrier (MelB) of *E. coli* (Tsuchiya & Wilson 1978) can use either Na⁺ (Li⁺) or H⁺ as coupling ions. The observation that a number of enzymes involved in energy transduction use either Na⁺ or H⁺ as coupling ion could be reflected in the actual catalysis of cation transport. Boyer (1988) suggested that transport systems that choose between Na⁺ and H⁺ as coupling ion may actually use H₂O⁺ (the steric analogue of Na⁺) instead of H⁺ as transported species. Such a mechanism would account for competition between protons and sodium ions for a single cation-binding site. Also, an evolution from proton to sodium-ion coupling and vice versa can be easily envisaged when protons and sodium ions use a similar translocation mechanism.

H⁺ translocation in the electron transfer chain of respiring and phototrophic organisms can occur by the proton motive Q cycle, which involves flavosemiquinone and/or ubiquinone radicals as intermediates (Mitchell 1976; Sone 1990; Trumpower 1990). It is unlikely that the quinols can function as Na⁺ carriers. Therefore, in redox-linked Na⁺ translocation one has to invoke a coupling mechanism in which Na⁺ transport is enzyme mediated, e.g. as proposed for the Na⁺ motive NADH-quinone reductase in *Vibrio alginolyticus* (Tokuda 1992). It has also been suggested that terminal oxidases, such as the cytochrome o oxidase of *Vitreoscilla* (Efok & Webster 1990) and Bacillus FTU (Muntyan & Skripnikova 1993) and the cytochrome d oxidase of *E. coli* (Avetisyan et al. 1993), pump Na⁺.

Some details of the coupling mechanism of the Na⁺ pump methylmalonyl-CoA decarboxylase have been elucidated by following the flux of Na⁺ ions using the isotope **2Na⁺**. It has been shown that the enzyme pumps two Na⁺ out per decarboxylation reaction. The enzyme also catalyses exchange of external and internal Na⁺ (Hilpert & Dimroth 1991), but there is no evidence that the enzyme can translocate 11⁺ (11O⁺).

A strong phylogenetic relationship between the Na⁺(H⁺)-translocating ATPase of *P. modestum* and the H⁺-translocating ATPases of other organisms has been found (Dimroth 1992b). This has important implications for the mechanism of ATP synthesis and hydrolysis via F₆,F₆'-type enzymes. All models in which a specific role for H⁺ has been proposed that cannot be performed by another cation are likely to be incorrect. Studies on the Na⁺ (H⁺)-ATPase of *P. modestum* indicate that Na⁺ (or H⁺) binding at the F₆ moiety triggers a conformational change which brings about ATP synthesis in the F₆ moiety (Dimroth 1992b). The observation that purified F₆ of the ATPase of *P. modestum* catalyses Na⁺/Na⁺ counterflow (Kluge & Dimroth 1992) indicates that distinct cation-ion binding site(s) are present on F₆. This cation conduction through F₆ is unlikely to proceed via a network of hydrogen-bonded chains, at least not when Na⁺ is translocated.

In secondary transport systems, the residues that participate in Na⁺ or H⁺ transport across the membrane are often not known but, with the aid of mutagenesis techniques and kinetic analysis of the mutant proteins, some amino acids essential for ion translocation have been identified (Poolman & Konings 1993). Similar to the situation with the ATPase, the observation that some homologous proteins can either translocate H⁺ and/or Na⁺ argues against the operation of a H⁺ relay network in these carrier proteins as has been proposed for the lactose carrier of *E. coli* (Kaback 1990). For the melibiose carrier protein (MelB) of *E. coli*, a number of aspartic acids present in transmembrane segments I, II, and IV have been implicated in Na⁺-coupling of transport (Pourcher et al. 1993; Zani et al. 1993). Although these studies showed that the N-terminal portion of the MelB protein is important for cation recognition, no universal or easily recognizable Na⁺-binding site can be identified. Further support for a role of the amino-terminal transmembrane segments in cation recognition has come from studies in which various portions of the MelB protein of *E. coli* were exchanged with the corresponding domains of the H⁺-(Li⁺)-dependent MelB protein of *Klebsiella pneumoniae* (Hama & Wilson 1993). However, the analysis of chimeric MelB proteins has also indicated that, for different sugar substrates, different parts of the carrier participate in cation recognition/transport, which makes it virtually impossible to define the Na⁺-binding site without further structural information on the protein.

**Ion Homeostasis**

The ion composition of the cytoplasm is kept within certain limits for optimal metabolic activity (Booth 1985; Skulachev 1987; Walderhaugh et al. 1987; Abee & Konings 1990). Since the ion composition usually differs from that of the environment, active pumps are needed in the membrane to maintain this concentration difference. Usually, the internal pH is kept near neutrality (Booth 1985; Abee & Konings 1990) and the K⁺ concentration inside is maintained at a higher concentration (Walderhaugh et al. 1987) and the Na⁺ concentration at a lower concentration (Skulachev 1987) than in the surrounding medium.

**pH Homeostasis**

Since most biomolecules (including proteins and nucleic
acids) have groups that undergo protonation/deprotonation by which conformations and thus biological (physiological) functions will be affected, regulation of cytoplasmic pH serves an important physiological function. The intracellular pH of neutrophilic bacteria is usually set between 7.6 and 7.8 (Schuldiner & Padan 1992) whereas for acidophiles it is around pH 6.5 (Matin 1990b) and for alkaliphiles around 8.2 (Ivey et al. 1992). In order to maintain a constant pH under conditions that a primary proton pump is active, the rate of proton extrusion has to be regulated and/or compensated by an appropriate rate of H⁺ uptake. Na⁺/H⁺ and K⁺/H⁺ antiporters as well as the F₂F₁-ATPase have been implicated in pH homeostasis of the bacterial cell (Kroll & Booth 1981; Kobayashi et al. 1984; Nakamura et al. 1984; Schuldiner & Padan 1992). An important factor of this regulation is the modulation of the activities and/or expression levels of the antiporters and the F₂F₁-ATPase as a function of the intracellular pH.

**Na⁺ Homeostasis**

In marked contrast to the homeostasis of the cytoplasmic pH, the intracellular Na⁺ concentration is not constant in *E. coli* (Castle et al. 1986a, b). The Δs is constant at a given pH, but the intracellular Na⁺ concentration varies with pH and the external Na⁺ concentration. These observations indicate that the extracellular pH affects the intracellular Na⁺ concentration. At neutral pH and at 400 to 500 mM extracellular Na⁺, the intracellular Na⁺ concentration of *E. coli* has been estimated to be 40 to 50 mM (Padan & Schuldiner 1992; Schuldiner & Padan 1992). These intracellular Na⁺ concentrations can apparently be tolerated without bacteriostatic effects (Schuldiner & Padan 1992). *Escherichia coli* cells react to a high Na⁺ load, i.e. when the intracellular concentration approaches the threshold concentration of 40 to 50 mM, by swimming away from the toxic ion and at the same time by increasing the expression of NhaA, one of the Na⁺/H⁺ antiporters of the cell (Padan & Schuldiner 1992; Schuldiner & Padan 1992).

**Bioenergetics and Ion Homeostasis in Extremophiles**

Important insights into the bioenergetics of microorganisms have originated from studies on Bacteria and Archaea that were isolated from extreme environments. For instance, the high stability of some integral membrane protein complexes in thermophiles has facilitated the purification and subsequent biochemical analysis of the enzymes (Yoshida et al. 1977). In this section, the bioenergetics and ion homeostasis of extremophiles will be reviewed and compared with that of non-extremophiles for which some general properties have already been outlined above. In Table 1, an overview of various types of extremophiles, their definition, and their distribution among the domains of Bacteria and Archaea are given.

**Acidophiles**

Obligate acidophiles have an optimum pH of growth which is extremely low (pH 1 to 4). To shield the intracellular enzymes and other components from a low medium pH, the organisms maintain a large pH gradient across the membrane. Special lipids are present in the acidophiles which may minimize leakage of H⁺ down the pH gradient and stabilize protein complexes at low pH values (Russell & Fukunaga 1990). For instance, ω-cyclohexyl groups in lipids of the thermophilic bacterium *Bacillus acidocaldarius*, membrane-spanning, tetraether lipids containing cyclopentane rings in the thermophilic Archaea such as *Sulfolobus acidocaldarius*, and other unusual fatty acids have been implicated as special adaptations to growth at extremely low pH values (Russell & Fukunaga 1990).

Acidophiles maintain the cytoplasmic pH at around 6.5, which is at least one to two pH units lower than the intracellular pH of neutrophiles and alkaliphiles (Krulwich & Ivey 1990; Matin 1990b). The large ΔpH (3 to 4 pH units) in (aerobic) acidophiles is partly compensated by a Δψ, positive inside; this polarity of the Δψ is opposite to that in neutrophiles (Bakker 1990; Matin 1990a). The reversed Δψ is formed by electrogenic K⁺ uptake which allows the cells to extrude more H⁺ and thus to maintain the internal pH at a higher level (Skulachev 1987; Walderhaugh et al. 1987; Bakker 1990). The large ΔpH formed in acidophiles has only been observed in intact cells. In membrane vesicles at low external pH, a smaller ΔpH and Δψ of ‘normal’ magnitude and polarity (negative inside) is formed upon addition of respiratory substrates (Guffanti et al. 1984).

In *S. acidocaldarius* H⁺ is pumped out by the respiratory chain (Schäfer & Moll 1988) and the Δp is used to drive ATP synthesis (Moll & Schäfer 1988). Also, amino acid uptake in *B. acidocaldarius* (W. De Vrij, unpublished work) and ATP synthesis in *Thiobacillus ferrooxidans* (Apel et al. 1980) are H⁺-coupled processes. A scheme for energy transduction in aerobic acidophiles is presented in Figure 2A. As with the other schemes presented below, generalizations of energy transduction have been made on the basis of the best-studied examples of a particular class of organisms.

**Alkaliphiles**

Obligate alkaliphiles have an optimum pH of growth which is high, and these organisms are not able to grow at or near neutral pH. The best studied obligate alkaliphiles belong to the genus *Bacillus*. These organisms contain highly branched lipids and lipids that are more unsaturated than those of facultative alkaliphiles (Clejan & Krulwich 1988; Dunkley et al. 1991). The internal pH of obligate alkaliphiles is kept at 8.2, which is at least two units more acidic than the external pH (Krulwich & Ivey 1990; Ivey et al. 1992) and somewhat higher than the cytoplasmic pH of neutrophiles. In alkaliphilic bacilli H⁺ is pumped out by the
Table 1. Definition and classification of extremophiles.

<table>
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<th>Extremophile</th>
<th>Growth definition</th>
<th>Aerobe/anaerobe†</th>
<th>Archaea/Bacteria</th>
<th>Other possible extremophilic properties</th>
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<td>Halophile</td>
<td>&gt; 7.5 M [NaCl]</td>
<td>Aerobe/anaerobe</td>
<td>Archaea</td>
<td>Alkaliphilic</td>
</tr>
<tr>
<td>Marine</td>
<td>0.5 M [NaCl]</td>
<td>Aerobe/anaerobe</td>
<td>Archaea/Bacteria</td>
<td>Alkaliphilic, psychrophilic, thermophilic, barophilic</td>
</tr>
<tr>
<td>Barophile</td>
<td>&gt; 1 atm</td>
<td>Aerobe/anaerobe</td>
<td>Archaea/Bacteria</td>
<td>Marine, thermophilic, psychrophilic</td>
</tr>
<tr>
<td>Psychrophile</td>
<td>Topt &lt; 15°C</td>
<td>Aerobe/anaerobe</td>
<td>Bacteria</td>
<td>Marine, barophilic</td>
</tr>
<tr>
<td>Thermophile</td>
<td>Topt &gt; 85°C</td>
<td>Aerobe/anaerobe</td>
<td>Archaea/Bacteria</td>
<td>Barophilic, marine, acidophilic</td>
</tr>
</tbody>
</table>

* Physical condition which determines whether a particular organism is classified as extremophilic.
† Classification based on the microorganisms isolated to date.

Topt — Temperature at which optimal growth occurs.

respiratory chain, thereby alkalifying the cytoplasm. Acidification of the cytoplasm is achieved by an electrogenic Na⁺/H⁺ antiporter, which imports two H⁺ upon export of one Na⁺. Since for every two H⁺ taken up one Na⁺ is exported, a significant uptake (or leak) of Na⁺ is needed to regulate the internal pH. Evidence has been presented for an involvement of Na⁺-coupled nutrient uptake in this regulation (Kruilwich et al. 1985). The relatively large reversed ΔpH, together with a Δp of normal polarity, results in a Δp that is low (−25 to −50 mV). The organism, however, can maintain a large ΔpNa and Δs to drive various membrane-linked processes. Motility (Hirotà & Imae 1983; Sugiyama et al. 1985; Kruilwich et al. 1990) and substrate transport (Kruilwich et al. 1990) are coupled to the sodium motive force, but ATP synthesis, on the other hand, is H⁺-coupled (Hicks & Kruilwich 1990; Hoffman & Dimroth 1990). The energetics of 'oxidative phosphorylation' has still not been solved satisfactorily. Non-chemiosmotic phenomena (Guffanti et al. 1984; Guffanti & Kruilwich 1992) and unusual H⁺/ATP stoichiometries have been proposed to explain the observed Δp-driven ATP synthesis (Hoffman & Dimroth 1991; Dimroth 1992c). The vectorial energy-transducing processes in an aerobic alkaliophile are depicted in Figure 2D.

The way in which the internal pH is regulated in anaerobic alkaliophiles is far from clear but a Na⁺/H⁺ antiporter seems to play a crucial role in pH homeostasis in these organisms (Koyama et al. 1988).

Halophiles

Extreme halophilic microorganisms grow at high ionic strength. The membrane lipids of these Archaea are composed of diphthanylglycerol diether analogues of glycerophospholipids (Russell 1989; Gambacorta et al. 1995). The extreme halophiles contain high intracellular concentrations of Na⁺ (up to 1 M) and K⁺ (4 to 5 M), and their proteins seem to have adapted to this high salt concentration by having a higher fraction of acidic amino-acid residues and a more compact packing of the polypeptide chain than proteins from non-halophilic bacteria (Zaccari & Eisenberg 1991).

In the halophiles studied to date, the Δp is formed by a respiratory chain or, in some cases by the light-driven H⁺ pump bacteriorhodopsin. A Na⁺/H⁺ antiporter is used to pump Na⁺ outwards (Konishi et al. 1992), and solute uptake has been shown to be Na⁺-coupled in several halobacterial species (Lanyi 1979). As in the obligate alkaliophiles, ATP synthesis in obligate halophiles is H⁺-coupled (Mukohata & Isoyama 1987). A scheme for the energy transduction in an extreme halophile is shown in Figure 2C.

Halotolerant, Alkalitolerant and Marine Bacteria

Moderate halophiles need salt for growth and can tolerate salt up to 3.5 M. At increasing salt concentration the relative amount of negatively charged phospholipids is increased (Russell 1989). In contrast to the halophiles, halotolerant/alkalitolerant and marine bacteria keep their internal Na⁺ concentration low. At neutral pH and under aerobic conditions these organisms generate a Δp by respiration, and use a secondary Na⁺/H⁺ antiporter to generate a Na⁺ gradient. In addition, respiratory-linked primary Na⁺ pumps and Na⁺-extruding decarboxylases can be expressed under conditions of a low Δp (Dimroth 1992a; Tokuda 1992). The ability to switch between secondary and primary Na⁺ pumps has been proposed as an adaptation to the fluctuating physical conditions in, for instance, an algal mat (see below). Solute uptake and motility have been shown to be Na⁺-coupled processes in several Vibrio spp. (Tokuda 1992); in most cases the nature of the coupling ion of the membrane-bound ATPase has not been established. However, for the strictly anaerobic, marine bacterium Pr. modestum, a Na⁺-coupled ATP synthase has been demonstrated (Laubinger & Dimroth 1988a; Dimroth 1992b, c). Energy transduction in alkali tolerant/halotolerant and marine organisms is shown in Figure 2D.
Barophiles
Little is known about the physiology of barophiles. Barophiles grow optimally at high pressures, and this physical parameter can be important for psychrophiles (living in the deep ocean) and extreme thermophiles with an optimum temperature of growth > 100°C (growing below the sea level). For the bacterium NPT3, the proportion of unsaturated fatty acids is increased in order to maintain the membrane in a liquid-crystalline state at elevated pressure (DeLong & Yayanos 1985). It is not known whether H⁺, Na⁺ or both are used as coupling ions in energy transduction in this type of organism.

Psychrophiles
Psychrophiles have a low temperature of growth (below 20°C). The phospholipid composition of the cell membrane is such that it is in a liquid-crystalline phase at the low growth temperatures (Russell & Fukunaga 1990), thereby enabling the membrane-embedded enzymes to function properly at low temperatures. Little is known about the coupling ion(s) involved in energy-transduction, although for the marine bacterium Vibrio ABE-1 a Na⁺-ATPase has been suggested (Harashima et al. 1993); this organism probably exhibits the same principles of energy transduction as mesophilic Vibrio spp. belonging to the class of marine bacteria.

Thermophiles
Thermophiles have an optimum temperature of growth above 65°C. The cytoplasmic membrane is in the liquid-crystalline phase at the optimum temperature of growth because it accommodates a high percentage of lipids with relatively long fatty-acid chains. Usually the membranes of thermophilic bacteria also contain a high amount of glycolipids (Russell & Fukunaga 1990). For an individual organism, an increase in growth temperature mainly affects fatty acyl chain composition; the affects on lipid content and headgroup composition are relatively minor. In general, with increasing temperature of growth an increase in degree of saturation, chain length and/or iso-branching of the acyl chains is observed (Russell & Fukunaga 1990). Sometimes special lipids (the sterol-like hopanoids) are present in thermophiles; these may also reflect an adaptation to life at higher temperatures, by making the membrane more rigid (Ourisson et al. 1987). The membranes of thermophilic Archaea can contain tetraether lipids or mixtures of di- and tetra-ether lipids which stabilize the membrane and lower the...
proton (ion) leakage relative to that of phospholipid bilayer membranes (Russell & Fukunaga 1990; Van De Vossenberg, S., Driessen, A.J.M. and Konings, W.N. unpublished work).

* Bacillus steathermophilus* and PS3 contain a H⁺-pumping respiratory chain (Fee et al. 1986; De Vrij et al. 1988). De Vrij et al. (1988) showed that although the membranes of *B. steathermophilus* have a relatively high H⁺ permeability at their optimal growth temperature, an active H⁺-pumping respiratory chain compensates for this. As far as amino-acid transport has been studied in thermophilic bacteria, a clear preference for Na⁺ as coupling ion is observed (Hirata et al. 1984; De Vrij et al. 1990; Heyne et al. 1991; Konings et al. 1992). To generate the Δψ, PS3 uses a Na⁺/H⁺ antiporter (Goto 1980). ATP synthesis in this organism is H⁺-coupled (Yoshida et al. 1977). Similarly, *Thermus thermophilus* and the archaeon *Sulfolobus acidocaldarius* contain a H⁺-extruding respiratory chain and a V-type, H⁺-coupled ATP synthase (Fee et al. 1986; Lübben & Schäfer 1987; Schäfer & Moll 1988; Yokoyama et al. 1990). Energy transduction in aerobic thermophiles is schematically shown in Figure 2E.

The acetogenic, anaerobic *Clostridium thermosautrophilicum* contains a H⁺-extruding electron transfer chain (Hugenholtz et al. 1987), a H⁺-coupled ATP synthase (Ivey & Ljungdahl 1986) and Δp-driven amino-acid uptake systems (Hugenholtz & Ljungdahl 1990). In the related *C. thermocellum*, electrogenic Na⁺/H⁺ antiport activity, which allows the cell to generate a Δψ, has been demonstrated (Tarciano et al. 1987).

*Clostridium feroeidus* is an anaerobic, peptidolytic thermophile (Speelmans et al. 1989) that generates a Δψ by using a F/V-type Na⁺-extruding ATPase (Speelmans et al. 1993a). In this bacterium, all amino-acid uptake systems studied to date are Na⁺-coupled (Speelmans et al. 1989, 1993b). Surprisingly, however, Na⁺/H⁺ antiport activity could not be detected in the organism (Speelmans et al. 1993a). Since other H⁺-pumping mechanisms are also lacking, the organism is unable to regulate its internal pH, and, consequently, is only able to grow in a narrow pH range (Speelmans et al. 1993a). As yet, *C. feroeidus* is the only species for which there is solid evidence that Na⁺/H⁺ antiport activity is absent. The energy-transducing processes in this anaerobic thermophile are depicted in Figure 2F.

**Advantages and Disadvantages of Na⁺-coupled Energy Transduction in Extremophiles**

Extremophiles other than Thermophiles

The possibility of switching between primary and secondary H⁺ and Na⁺ transport allows marine bacteria to adapt readily to conditions fluctuating in pH and salinity. In a marine algal mat the pH is neutral in the morning, and alkaline in the evening (Skulachev 1987). At alkaline pH, the Δp is low and lowering of the internal Na⁺ concentration via a secondary Na⁺/H⁺ antiporter will not be very effective. Furthermore, if H⁺ (or H₂O⁺) and Na⁺ (or Li⁺) compete for a similar cationic binding site on transport enzymes and the pH is high (H⁺ < 10⁻⁸), the enzymes may not be specific enough for H⁺ (or H₂O⁺) and may use Na⁺ instead (at 0.5 M). This hypothesis implies that the use of a primary Na⁺ pump at alkaline and high salt conditions has distinct advantages over a primary H⁺ pump.

The secondary ΔpNa-generating system in extreme alkaliphiles is essential for adaptation at high pH (moderate salinity, non-marine). The low Δp (−25 to −50 mV) is due to the electrogenic Na⁺/H⁺ antiporter activity, which generates a reversed ΔpH (up to three pH units). The Na⁺/H⁺ antiporter has a crucial role in the homeostasis of the cytoplasmic pH and allows the cells to face extracellular pH values as high as 11 (Ivey et al. 1992). Since Δp is low and Δs is high in the alkaliphiles the use of Na⁺ as coupling ion in, for instance, solute transport would be favourable (as large concentration gradients can be maintained).

Extreme halophiles are adapted to high intracellular Na⁺ concentrations (up to 1 M) (Grant 1990). The organisms use a primary H⁺ pump and use a Δp-dependent Na⁺/H⁺ antiporter to extrude Na⁺ against a high extracellular Na⁺ concentration (3.5 M NaCl). The Na⁺ gradient (Na⁺/Na⁺) which can be generated by the Na⁺/H⁺ antiporter (Konishi et al. 1992) in the extreme halophiles is less than 10-fold (ΔpNa < −60 mV) and, therefore, the use of H⁺ as dominant coupling ion in energy transduction could be advantageous.

A primary Na⁺ pump can generate a Δψ which allows anaerobic organisms to cope with a low Δp, provided membrane-bound chemiosmotic reactions can use Na⁺ as coupling ion. Since Na⁺/H⁺ antiporters require a high Δp to maintain a large Na⁺ concentration gradient, this form of Na⁺ export may not be sufficient for ΔpNa generation and/or Na⁺ homeostasis at low Δp. Furthermore, the absence of a ΔpH allows a more efficient excretion of acidic end-products by passive efflux and/or H⁺-symport mechanisms. If the excretion of acidic end-products together with protons has to proceed against a large Δp, the end-product concentrations inside the cell may increase to inhibitory levels.

**Thermophiles**

Thermophilic bacteria have to invest more energy to maintain a Δp than mesophiles, due to the relatively high ion (proton) permeability of the membrane at the optimal growth temperatures (Kuhn et al. 1980; De Vrij et al. 1988). Especially in thermophilic anaerobes, this may cause problems due to the limited amount of ATP that can be
supplied by the fermentative pathways. The permeability of the cytoplasmic membrane for Na\(^+\) ions is much less than for H\(^+\) ions. The permeability coefficient for Na\(^+\) is approx. 10\(^{-14}\) cm/s at 25°C, whereas the permeability coefficients for H\(^+\) vary between 10\(^{-4}\) and 10\(^{-6}\) cm/s at 25°C (Gennis 1989). Between 18 and 38°C, the rate of Na\(^+\) influx was found to be 7 to 8 orders of magnitude lower than the rate of H\(^+\) influx into liposomes (Speelmans 1993). At increasing temperatures, both the Na\(^+\) and H\(^+\) fluxes into the liposomes increase, but the absolute H\(^+\) flux increases by much more than the Na\(^+\) flux (Speelmans 1993). On the basis of these differences in ion permeability, the use of a ΔpNa is of considerable bioenergetic advantage for microorganisms, and in particular for thermophiles.

In the thermophilic anaerobe, C. fervidus the yield of metabolic energy per molecule of substrate is much less than in aerobes. Furthermore, ΔpH dissipating end-products (CO\(_2\), NH\(_3\), and acetic acid) are formed. Such a fermentative organism has to develop special mechanisms in order to cope with life at high temperature. The H\(^+\) permeability of liposomes prepared from C. fervidus lipids does not differ significantly from that of liposomes prepared from E. coli phosphatidylethanolamine/egg phosphatidylycholine (Speelmans 1993). Hence, at its growth temperature (68°C) C. fervidus will be confronted with higher rates of passive H\(^+\) diffusion across the membrane than mesophilic bacteria. Therefore, in thermophilic anaerobes, a relatively higher fraction of the metabolic energy formed needs to be spent on maintaining a high Δp than in aerobic thermophiles or mesophilic anaerobes. Since Na\(^+\) is less membrane permeable than H\(^+\) less metabolic energy has to be invested to maintain high driving forces for energy-transducing processes when Na\(^+\) is used as coupling ion. This advantage will only be valid if no H\(^+\) cycling is present at the same time. This turns out to be the case in C. fervidus. The organism, however, has to pay a price for using Na\(^+\) as the sole energy-coupling ion, since the pH range in which growth is possible becomes narrow. When no mechanisms are available for pH homeostasis, and the external pH changes as a result of bacterial metabolism (or otherwise) and the buffering capacity of the intracellular milieu is exceeded, the activity of pH-sensitive metabolic processes will decrease and ultimately affect growth. By fermenting amino acids C. fervidus produces both acidic and alkaline end-products, and this may keep the external pH relatively constant even at high cell densities. Furthermore, the organism can survive less favourable circumstances (e.g. a low or high pH) by forming spores.

**Concluding Remarks**

In recent years a wealth of data has become available about the bioenergetics of some extremophiles, such as extreme halophiles, aerobic alkaliphiles and aerobic thermophiles. The information, however, is less complete for energy-transducing processes in other types of extremophiles, such as acidophiles, barophiles, psychrophiles and various types of anaerobic extremophiles.

From the information currently available it can be concluded that prokaryotes have very versatile Na\(^+\)- and H\(^+\)-extrusion systems and very versatile ways to cope with high concentrations of internal Na\(^+\), varying concentrations of H\(^+\) or combinations of these parameters. In extremophiles in particular, growing under circumstances considered to be a stress condition for 'normal' bacteria, and often dealing with a low proton motive force, the role of Na\(^+\) in energy transduction is crucial. The most extreme representative is the anaerobic thermophile *Clostridium fervidus*, the energy transducing processes of which are completely dependent on Na\(^+\) as coupling ion.

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