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Borders of Life

Bioenergetics and cation permeability of the cytoplasmic membrane in extremophiles

Voor mijn ouders

Omslag: Berlijn, 1987

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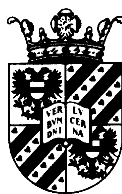

RIJKSUNIVERSITEIT GRONINGEN

BORDERS OF LIFE

bioenergetics and cation permeability of the cytoplasmic membrane in extremophiles

Proefschrift
ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. D.F.J. Bosscher,
in het openbaar te verdedigen op
maandag 28 juni 1999
om 14.15 uur

door
Jacobus Ludovicus Cornelis Marie van de Vossenberg
geboren op 22 augustus 1967
te Rosmalen

Promotores: Prof. Dr. W.N. Konings
Prof. Dr. A.J.M. Driessen

Beste allen,

Na jaren rondlopen bij Microbiologie heb ik het af, hoezee! Daar hoort traditiegetrouw een voor- of nawoord bij.

Ik begin met uiteraard Wil en Arnold. Wil, mijn onderzoek was bijna een rechtstreeks vervolg op jouw eerste stelling, en heeft deze niet weersproken. Aan jou heb ik te danken dat ik van alles wat helsch was op deez' Aard de protonpermeabiliteit kon meten, omdat je van iedere meeting terugkwam met alweer een raar & uniek beest. Arnold, nu professor: Het extremofielen-gebeuren was niet je allereerste interesse, maar het kwam toch goed. Je hebt me vaak verder geholpen en gemotiveerd als ik vast zat. Jullie beiden waren bewonderenswaardig snel en kritisch (=geduldig) met het nakijken van mijn te publiceren stukken.
Marieke, van jou heb ik de beginselen geleerd van het werken met archaea-lipiden en het doen van de protonpermeabiliteits metingen. Ook vanuit München bleef je betrokken. Deborah, jij bent mijn enige student geweest. Het was een lastig project, maar je hebt je er bewonderingswaardig doorheengeslagen. Sonja Täubchen, je hebt heel het extremofielengroepje gemotiveerd met je enthousiasme en goede ideeën. Samen met Chris, Wim en Arnold hebben we natuurlijk ook een te gekke snelle publicatie, die mij veel genoegen geeft. Sonja Koning, ik neem aan dat je mijn *Pyrococcus* resultaten binnenkort zult gebruiken. Jij hebt in ieder geval wat aan dit boekje. Querida Laurinha: Sabes, tu tens sempre razão! Tomatos! Steffen: Es war sehr nett mit Dir zu arbeiten. Auch wenn Du bei uns eigentlich nicht in ein Fettnäpfchen getreten bist, 'ne?!
Slechts een paar andere mensen zal ik noemen. Jeroen, Peter, Erik, André, Mike, Frank (bedankt voor het bier), Marleen, Esther en Liesbeth: binnen, maar ook vooral buiten het lab heb ik met jullie veel prettige uren beleefd. Dirk, Marleen en Erik, die pijp was toch wel een hoogtepunt. Gert, welke stijlfiguur of taal hebben we niet besproken? Eet dit: %: 8 %5: ; *9" 3! Rob, Girbe, Sytse en Arjen: de vele sneaken en Ameland waren erg gezellig. Sytse, als huisgenoten hebben we vele avonden gezellig gekneuterd, dat zal ik gaan missen. Ik ben erg blij dat ik mijn sportieve ei kwijt kon in deze of gene sporten met labgenoten: Het Squashteam (Peter, Jeroen, Erik, Mariken en ik hebben alvast het promoveren geoefend), het oude volleybalteam, het hockeyteam en de Lauwersloop/Brabantloop. Erg gezond (behalve voor Henk z'n enkelbanden) en gezellig. Nineke en Janny, die al die tijd knusjes met mij in het zitkamertje zaten opgescheept, jullie zullen mijn geblaat (en gepruttel) moeten missen.
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Ik hoop dat met jullie allen de contacten zo goed blijven als nu.

Jack

P.S. Leek, mocht je weinig begrijpen van de inhoud? Ik zal het ook niet toelichten in een zgn. lekenpraatje, omdat het niet is toegestaan gebruik te maken van twintigste-eeuwse visuele middelen, zoals een diaprojector of een overheadprojector. Ach, troost je: Nescire quaedam magna pars sapientiae est (Hugo de Groot).

Contents

Chapter 1: Introduction. The special composition of the cell membrane allows Archaea to live in extreme environments **1**

Chapter 2: Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of Bacteria and Archaea. **19**

Appendix: The membrane of *Pyrococcus furiosus* liposomes is as impermeable for protons as the membrane of *Sulfolobus acidocaldarius* liposomes **29**

Chapter 3: Homeostasis of the membrane proton permeability in *Bacillus subtilis* grown at different temperatures. **31**

Chapter 4: Lipid membranes from halophilic and alkali-halophilic Archaea have a low H^+ and Na^+ permeability at high salt concentration **41**

Chapter 5: Bioenergetics and cytoplasmic membrane stability of the extreme acidophilic thermophilic Archaeon *Picrophilus oshimae*. **49**

Chapter 6: Summary and concluding remarks **61**

Samenvatting **67**

References **73**

List of publications **81**

The special composition of the cell membrane allows Archaea to live in extreme environments

Jack L.C.M. van de Vossenberg, Arnold J.M. Driessen and Wil N. Konings

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Introduction

In the last three decennia, the number and variety of species that have been found to live in extreme environments has expanded tremendously (Kristjansson and Hreggvidsson, 1995). Also, many more extreme environments have been found to host microorganisms. Environments that were previously considered sterile were found to be suitable for growth of extremophiles. Most of the organisms that grow in such environments belong to a group of microorganisms with distinct characteristics. Woese *et al.* (1990) named this group '*Archaea*', and postulated the Archaea as a domain of life on Earth, separate from the previously known domains Bacteria and Eucarya (eukaryotes) (Fig. 1.).

Bacteria and Archaea are prokaryotic organisms. The membranes contain lipids which in Bacteria and Eucarya are mainly *di-esters* from glycerol and two fatty acyl chains. In contrast, Archaeal membranes contain predominantly ether lipids in which two isoprenoid chains are *ether-linked* to glycerol or another alcohol. Also the ribosomal rRNA's of Bacteria, Eucarya and Archaea differ. The division in three domains is now generally accepted, in particular after the completion of the genome sequences of many different Archaea, starting with *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum* and *Archaeoglobus fulgidus* (Bult *et al.,* 1996; Smith *et al.,* 1997) (*e.g.,* see http://www.tigr.org/). Two third of the genes found in these Archaea do not have homologues in Bacteria and Eucarya, which emphasises the genetically different position of the Archaea. Molecular phylogenetic surveys of Archaea that thrive in extreme environments shows that the evolutionary and physiological diversity in this domain is far greater than previously assumed (Smith *et al.,* 1997).

The domain of the Archaea is subdivided in the subdomains euryarchaeota and crenarchaeota. The subdomain euryarchaeota consists of methane producers (methanogens), salt loving organisms (extreme halophiles), heat-loving organisms (thermophiles), and acid-loving organisms living at high temperatures (extremely acidophilic thermophiles) (Belly and Brock, 1972; Schleper *et al.,* 1995). Methanogens grow over the whole temperature spectrum where life is found: from cold (psychrophiles) (Nichols and Franzmann, 1992) via moderate (mesophiles) (Kandler and Hippe, 1977) to extremely hot environments (extreme thermophiles) (Kurr *et al.,* 1991).

To the subdomain crenarchaeota belong the most thermophilic organism known to date, *Pyrolobus fumarii* (Blöchl *et al.,* 1997) and the intensively studied extreme thermoacidophile *Sulfolobus acidocaldarius* (Brock *et al.,* 1972) (in this thesis in Chapter 2 and 5, all other Archaea studied for this thesis are euryarchaeota). The

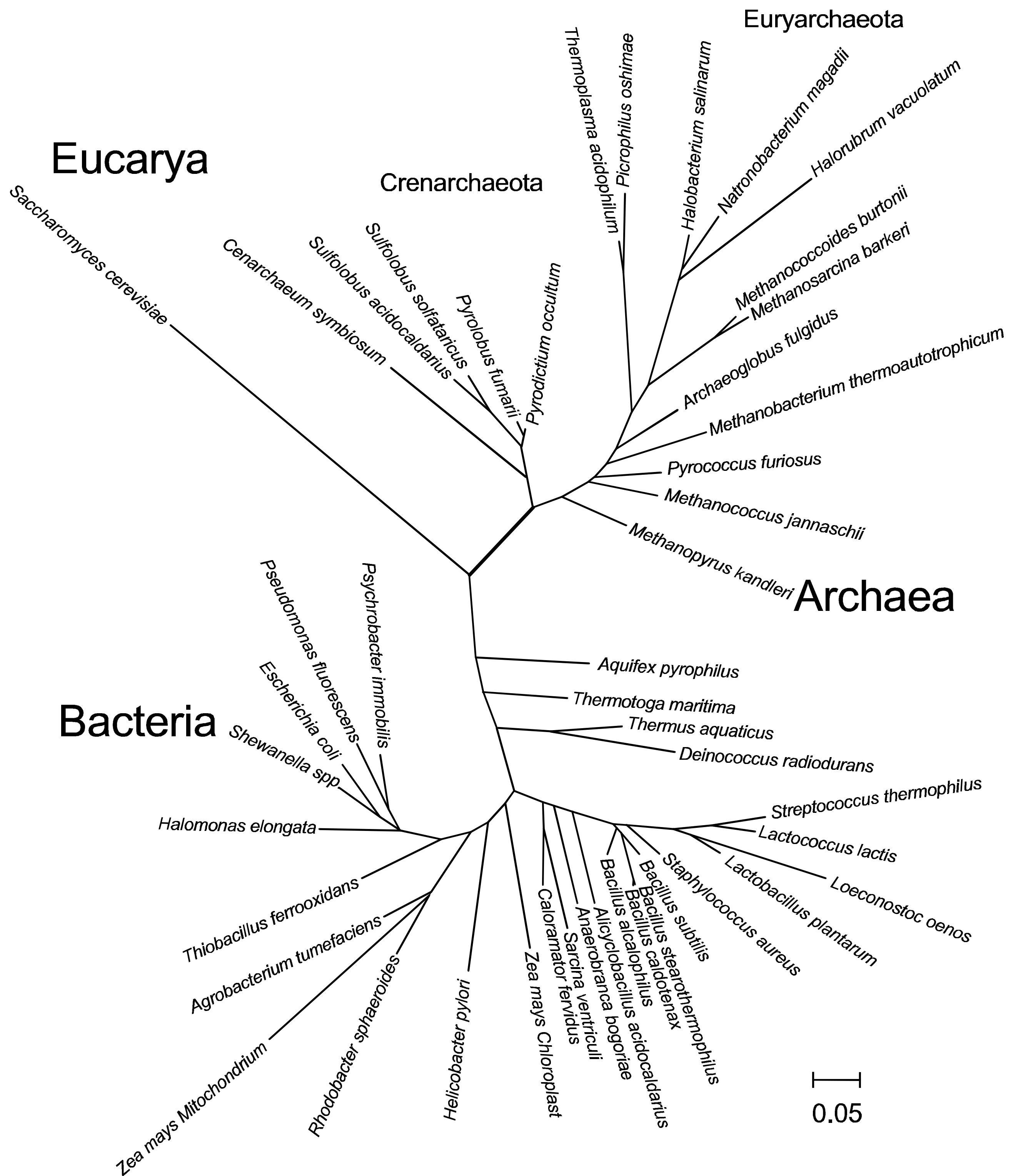

Fig. 1. Phylogenetic relation of organisms described in this thesis, organisms used in the Molecular Microbiology lab in Groningen and 'general interest' organisms. The unrooted tree is based on 16S Small Subunit rRNA sequences, using information from the Ribosomal Database Project (http://www.cme.msu.edu/RDP) (Maidak *et al.,* 1997), combined with 16S SSrRNA sequences from various on-line databases. The 18S SSrRNA sequence of yeast (Eucarya) is taken as 'outgroup'

Extreme environment	Low boundary extremophile	High boundary extremophile
Oxygen	Anaerobe (no O_2)	*'Normal'* (Aerobe, 20% O_2)
Temperature	Psychrophile (-5 to10°C)	Thermophile (55 to 110°C)
pH	Acidophile (pH 0.5 to 4)	Alcaliphile (pH 8 to11.5)
Salt	*'Normal'* (0.05 mM)	Halophile (0.5 M to saturation)
Pressure	*'Normal'* (1 bar)	Barophile (200 to >2000 bar)

Table 1. Extreme environments and what the organisms who live in these environments are called. A 'normal' organism is called mesophile with respect to temperature and neutrophile with respect to pH. The range in which extreme organisms are found is indicated between brackets.

only psychrophilic crenarchaeote discovered until now is *Cenarchaeum symbiosum* that symbiotically inhabits tissue of a temperate water sponge (Preston *et al.,* 1996). This organism grows well at 10°C, which is more than 60°C lower than the growth temperature of all other crenarchaeota studied thus far.

Despite the enormous difference in extreme and moderate environments, all organisms known so far share the same biochemical basis for metabolism and proliferation. The organization is cellular, surrounded by a lipid membrane; DNA contains the inheritable information, coding for RNA that can be translated into proteins. The autonomously replicating living organisms originate most likely from one common ancestor (Woese *et al.,* 1990), and the adaptation to the various environments must have taken place from that point. Here we present the adaptation of the membrane and bioenergetics of organisms, in particular of Archaea, that grow in extreme environments.

Extreme environments

Extreme conditions are considered those that deviate considerably from the conditions in which humans function optimally. Such conditions are described with the following physical parameters: oxygen concentration, temperature, salinity, pH, pressure, radiation (Table 1). Combinations of a number of extreme physical parameters are not uncommon. To live and survive under extreme conditions requires structural and/or physiological adaptations of the organism.

Oxygen concentration: Environments in which the oxygen concentration is low or absent (anaerobic) are found everywhere: in the gut, waste water installations, rotting materials, deep rock formations, etc. Organisms of all three domains are found in oxygen limited and oxygen free environments. Oxygen limited and anaerobic conditions are also very common in other extreme environments. The aspect of oxygen limitation will only be discussed briefly.

Temperature: About two-third of the ocean, covering about 70% of the Earth surface, has a temperature of 2-4°C. This means that a great portion of life lives at low temperatures. Organisms that can grow at temperatures below 0°C are found in arctic regions and in areas at high altitude (Baross and Morita, 1978; Morita, 1975). The lowest temperature at which growth appears to be possible is near -12°C, which is consistent with the known physical state of aqueous solutions at sub-zero temperatures. At lower temperatures, the cellular water in psychrophiles starts to freeze (see Table 1),

resulting in a concentration of intracellular salts, which has toxic effects on the organism (Mazur, 1980). In permafrost, microorganisms can survive very long periods of low temperatures.These organisms are not necessarily psychrophiles. At low temperatures, cellular metabolism stops but can be restored by warming. Even in permafrost not only psychrophiles but occasionally even thermophiles are found (Vorobyova *et al.,* 1997).

Hot environments are found all over the world but especially in volcanic areas above and below sea level, where mineralised water comes to the surface, and in deep rock formations heated by the Earth's interior. These effluents of mineralized water contain high concentrations of salts like sulphate or carbonate, which can make the effluent acidic or basic. The highest temperature at which growth of Eucarya has been found is 80°C (Cary *et al.,* 1998). Growth of Bacterial species has been detected at temperatures up to 95 °C (Huber *et al.,* 1992). Some Archaeal species can grow at temperatures up to 113 °C (Blöchl *et al.,* 1997). All the organisms that live above 90°C are found in submarine volcanic areas. Those areas are oxygen poor, mainly due to the very low solubility of oxygen at those high temperatures.

Salinity: Non-halophilic organisms can survive in media with salt concentrations up to 0.85 M NaCl. This is almost twice the salt content of sea water, which is equivalent to about 0.5 M NaCl. In these salty environments the non-halophilic organisms can only survive in the presence of suitable compounds, which are either taken up from the medium or synthesised in the cell (Lucht and Bremer, 1994; Boch *et al.,* 1994; Graham and Wilkinson, 1992). These compounds are termed osmolites. The evaporation of water has led to the development of lakes with high concentrations of dissolved salts, like the Dead Sea. Many micro-organisms can survive dehydration by reducing the water loss with a thick cell wall or by enabling a rapid recovery upon rehydration (Reed, 1986). Extreme halophiles live in environments ranging from 3 M to complete NaCl saturation (Lai *et al.,* 1991). Halophilic Archaea are so widespread in high salt environments that they almost define the hypersaline niche. Still, the most salt tolerant organism known is the Bacterium *Halomonas elongata*. It can grow between 0.05 M and saturating NaCl concentration (Vreeland, 1987).

pH: Acidic environments can be formed by geothermal processes at the Earth's surface. Volcanic sulphur is oxidised to SO_3 that reacts with water to sulphuric acid. The concentration of acid in the pool increases due to the evaporation of water. In such highly acidic environments, organisms are found that can live at pH below zero, *i.e.,* H^+ concentrations higher than 1 M (Schleper *et al.,* 1995).

Alkaline environments are also found on Earth, but only a few of these environments are stable. Most of them are soda lakes in which the sodium carbonate is the major source of alkalinity. The pH in soda lakes can be higher than 10.5. In the course of increasing alkalinity by evaporation, other salts like NaCl, also concentrate, which makes these environments very saline. Whether carbonate containing lakes become alkaline, is determined by the ratio between the concentration of carbonate on one hand and calcium plus magnesium on the other hand. If the carbonate concentration is higher than the sum of those cations, the lake is alkaline. Natural springs are generally of low salinity and the pH usually does not exceed pH 9 (Grant and Tindall, 1986).

Pressure: Environments with high pressure are found in the deep sea, in deep rock formations and oil wells. Barophilic organisms grow in these environments. High pressure has a relatively small influence on the organisms: *Escherichia coli* can easily withstand 500 bar, the pressure found at 5000 m below sea level. Mutants can

grow even at much higher pressures, and their growth is only seriously inhibited at a few thousand bar (Hauben *et al.,* 1997). Nevertheless, microorganisms from the deep-sea display high pressure adapted growth characteristics. Phylogenetic studies have established that many barophilic Bacteria currently in culture collections belong to a distinct subgroup of the genus *Shewanella* (Kato and Bartlett, 1997). Because of the difficult growth and experimental conditions, little is known about barophiles.

Radiation: High energy radiation such as X-rays or ultraviolet light (Antarctica) is damaging because it destroys large molecules such as DNA. Organisms adapted to high radiation will especially have efficient systems to repair the damaged DNA and possibly large protein-complexes. The resistance of *Deinococcus radiodurans* to extremely high levels of radiation can be transferred to *E. coli* (Dalrymple *et al.,* 1989), showing that radiation resistance is contained in only a limited number of genes. Radiation resistance will not be discussed in further detail.

The cytoplasmic membrane and bio-energetics

The cytoplasmic membrane is crucial for the generation of metabolic energy by energy transduction. In this process, the energy of an electrochemical ion gradient across the membrane is transformed into other forms of energy. Metabolic energy can also be obtained in the form of ATP and ADP by substrate level phosphorylation processes. Both metabolic energy generating processes are closely linked and together they determine the energy status of the cell. The energy transduction systems are located in the cytoplasmic membrane. Specific pumps translocate protons or sodium ions across the membrane into the external medium and this activity results in the generation of electrochemical gradients of protons or sodium ions (Lolkema *et al.,* 1994; Speelmans *et al.,* 1993a). When protons are extruded, the resulting electrochemical gradient results in a proton motive force (PMF). The PMF consists of two components that, in neutrophiles, both exert a force on the protons to pull the protons back into the cell: the ΔpH, *i.e.,* the concentration gradient of protons, and the $\Delta\psi$, the membrane potential, caused by the transport of electrical charge of the protons:

$$\mathrm{PMF} = \frac{\Delta\mu_{H^+}}{F} = \Delta\psi - 2.303\frac{RT}{F}\Delta\mathrm{pH} \quad (1)$$

expressed in mV, in which R is the gas constant, T the absolute temperature (K), and F the Faraday constant. The effect of 1 unit pH difference is 59 mV at 25°C, and 70 mV at 80°C. If the PMF is negative, the driving force on the protons is directed into the cell. In organisms that live around pH 7 (neutrophiles) both the electrical and concentration components are negative, both contributing to the PMF. In analogy with the PMF, sodium ion pumps can generate a sodium motive force (SMF).

The PMF or SMF can be used to transduce their potential energy to energy requiring processes such as ATP synthesis from ADP and phosphate and transport of specific solutes across the membrane, flagellar rotation, and maintenance of the intracellular pH and turgor (Booth, 1985). Obviously, this type of energy transduction can only operate if the transmembrane gradients of H^+ *c.q.* Na^+ can be maintained. A prerequisite for this maintenance is that the biological membranes are limited permeable for these ions.

The cytoplasmic membrane functions as a barrier between the cytoplasm and the

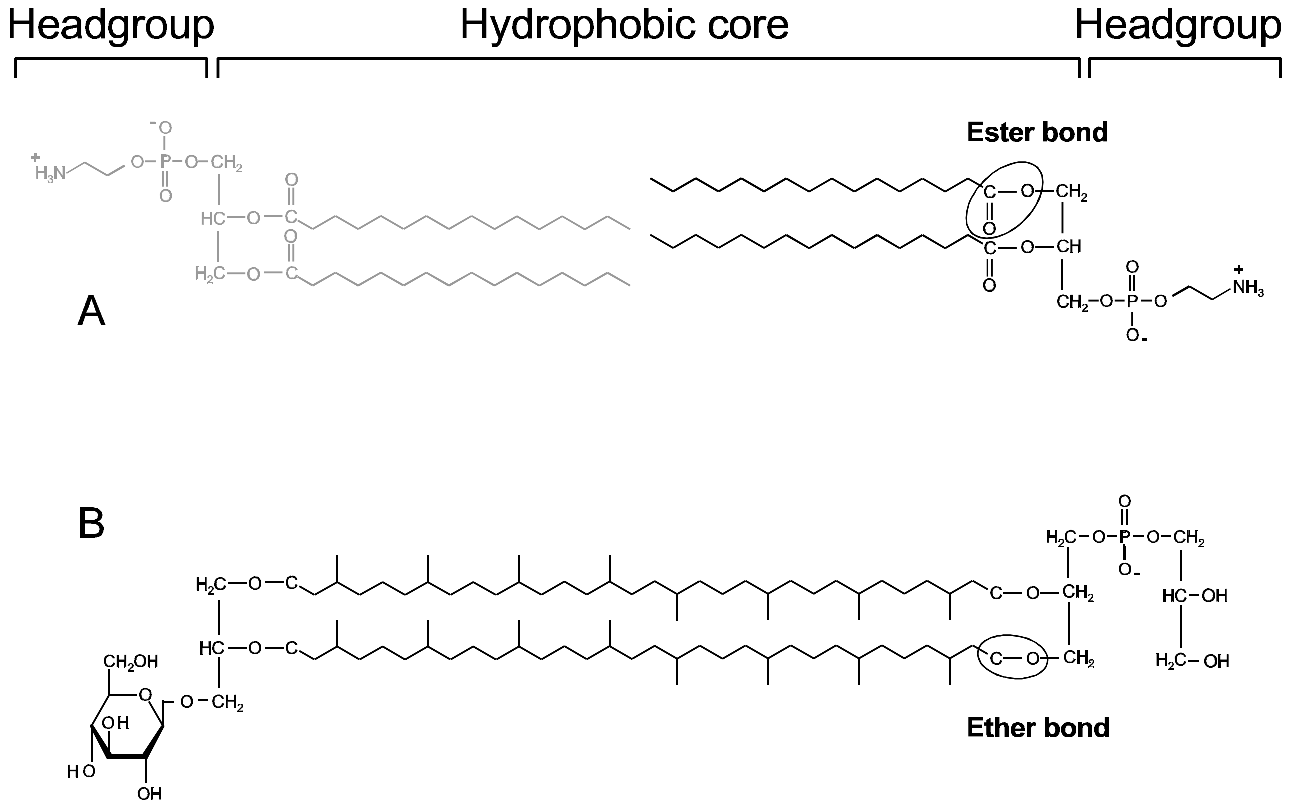

Fig. 2. Lipids from Archaea and Bacteria. **A**: bilayer forming lipids in Bacteria: Phosphatidylethanolamine (PE) from *Escherichia coli*. The acyl chain is straight (not in all cases: some Bacterial lipids have a methyl branch, or a cyclohexyl group, at the end of the acyl chain, other lipids have one or more unsaturated bonds). The connection of the acyl chain with the headgroup is an ester. **B**: Monolayer forming lipids in thermo-acidophilic Archaea: Main glycophospholipid (MPL) *Thermoplasma acidophilum*. The acyl chain contains isoprane-like branches (not all Archaeal lipids span the membrane: bilayer forming diether lipids, some acidophilic tetraethers contain cyclopentane rings), The connection of the acyl chain with the headgroup is an ester.

environment. This membrane consists of a layer of lipids in which proteins are embedded. The membrane controls the movement of solutes (ions and nutrients) into or out of the cell. From the onset of cellular life, hydrocarbon chains of at least 10 carbon atoms must have been present to form a biological membrane (Deamer, 1997). Biological membranes consist of a bi- or monolayer of lipid molecules and of proteins. In nature, an enormous diversity of lipids is found. The lipids have polar headgroups that stick into the water phase and hydrophobic hydrocarbon chains that are oriented to the interior of the membrane. At the growth temperature of a given organism, the membranes are in a liquid crystalline state (Melchior, 1982). The structure of biological membranes is mainly held together by noncovalent bonds such as Van der Waals and electric interactions, which make them highly impermeable for small ions. The barrier function of the cell membrane is critical for the functioning of the cell, as the membrane has to control the concentration of molecules and ions inside the cell. Transport of most solutes across the membrane is mediated by specific transport proteins. The permeability of membrane for small solutes and ions is restricted due to the high energy that is required for the transfer of a hydrophobic solute or ion from the aqueous phase into the apolar interior of the membrane.

The lipid layer forms a suitable matrix for proteins such as transport proteins that generate and maintain specific solute concentration gradients across the membrane. Since the permeability of the membrane is low, less energy is needed to maintain such gradients. Organisms maintain optimal conditions for membrane fluidity and permeability. The membrane is in a liquid crystalline state that allows optimal functioning of the membrane proteins. The membrane proton permeability determines the rate at which protons leak inward. The balance between proton permeability and the rate of outward proton pumping determines whether a Bacterial cell can sustain an appropriate proton motive force. Extreme environments can influence the permeability of the membrane, and some extremophiles have to adapt their membrane lipid composition in order to survive. This chapter focusses on the adaptation of the lipid composition of the membranes of different extremophiles and their permeability properties.

Permeability of the membrane for ions

Lipids in the membrane Archaea can be found in the most extreme conditions were life exists. This is probably due to the unique structure of the Archaeal membrane lipids that have some features distinct from Bacterial and Eucaryal membranes. Bacteria and Eucarya contain lipid acyl chains ester-linked to glycerol. Archaeal 'acyl chains' (phytanyl chains) are connected to glycerol (sometimes nonitol) with ether links. Usually, Bacterial and Eucaryal lipids are made from acetyl subunits, that form a straight carbon chain (Fig. 2A).

Bacteria and Archaea can respond to changes in ambient temperature through adaptations of the lipid composition of the cytoplasmic membrane (Sinensky, 1974; Gaughran, 1947). A change of temperature requires a change of the membrane lipid composition to keep the membrane in a liquid crystalline state (Russell and Fukunaga, 1990) and the proton permeation rates at a low level. At higher temperatures, this can be done by increasing the chain length of the lipid acyl chains, the ratio of *iso*/*anteiso* branching and/or by increasing the degree of saturation of the acyl chain (Prado *et al.,* 1988; Reizer *et al.,* 1985; Svobodová *et al.,* 1988). Adaptation to low temperatures in Archaea is achieved by unsaturation of lipids (Nichols and Franzmann, 1992), while an adaptation to an increase in the temperature involves cyclization of the fatty acyl chains, and/or a change from diether lipids into tetraether lipids (Sprott *et al.,* 1991; De Rosa *et al.,* 1991; Yamauchi and Kinoshita, 1995). Each species has its own specific temperature range of growth. Adaptation within one organism is limited, and for Bacteria the effective growth range is often around 30°C (Russell and Fukunaga, 1990).

Bacteria that grow at temperatures lower than 30°C have unsaturated membrane lipids with shorter acyl chains. The acyl chain usually contains a *cis* double bond. The effects of a *cis* double bond and an anteiso methyl branch are similar. However, the *cis* double bond is more effective: it places a kink in the molecule that disrupts the ordered packing of the chains in the bilayer. The kink in the chain lowers the transition temperature from gel to liquid crystalline state of the lipid (Suutari and Laakso, 1992). At low temperatures, longer and saturated lipids solidify, forming small crystals in the liquid crystalline remainder of the layer. The solid lipids render membrane proteins less functional and at the border between the crystal and liquid layer, the membrane becomes disordered and therefore more permeable for small molecules. Therefore, all organisms try to keep their lipids in a liquid

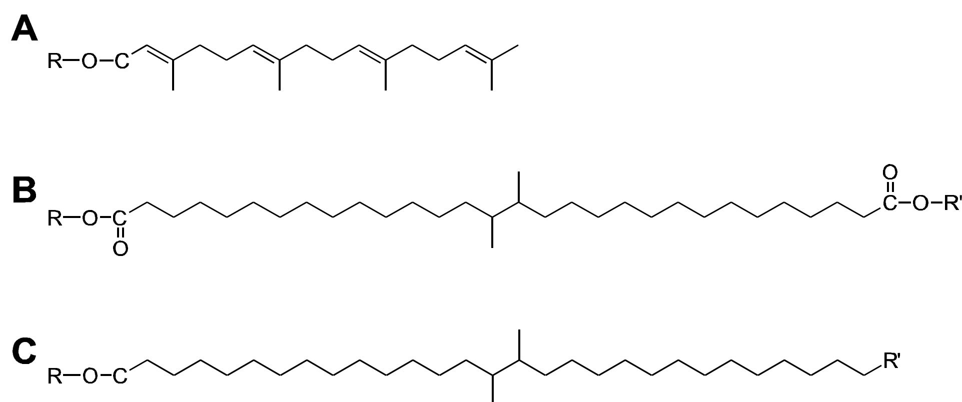

Fig. 3. Primitive lipid acyl chains as found at the crossroads of the domains Archaea, Bacteria and Eucarya. **A**: ether linked unsaturated terpenoid acyl chains as found in *Methanopyrus kandleri.* **B**: Ester linked membrane spanning acyl chain as found in *Aquifex pyrophilus* and *Thermotoga maritima.* **C**: Ether linked membrane spanning acyl chain as found in *Aquifex pyrophilus* and *Thermotoga maritima.*

crystalline state. When the temperature gets too high, the membrane lipids become too fluid, or the lipids organise in a hexagonal lipid phase. These effects result in a very leaky membrane. Many Bacteria are able to adapt the fluidity of the membrane to the temperature. This is known as 'homeoviscous adaptation' (Sinensky, 1974).

The structure and adaptation of Archaeal membrane lipids to different environments have been extensively reviewed (De Rosa *et al.,* 1991; Sprott *et al.,* 1997; Yamauchi and Kinoshita, 1995). The Archaeal lipid chain is composed of isoprene subunits (Fig. 2B). Therefore, the phytanyl chains contain methyl groups at every fourth carbon atom in the backbone. The reason for the higher stability of the phytanyl chain can be the reduced segmentary motion of tertiary carbon atoms (*i.e.,* rotation of carbon atoms that are bound to three other C-atoms, resulting in kinks in the acyl chain). The segmentary motion in the phytanyl chains is hindered due to the methyl side groups (Degani *et al.,* 1980). Hindering is particularly pronounced in the lamellar phase preventing kink formation in the phytanyl chains. The restriction in hydrocarbon chain mobility may also keep the permeability of the Archaeal membrane lower.

Most of the Archaeal lipid acyl chains are fully saturated isoprenoids that are linked with glycerol (De Rosa *et al.,* 1991; Yamauchi and Kinoshita, 1995; Kates, 1996; Koga *et al.,* 1993). Halobacterial lipids consist of a C_{20} diether lipid core (Upasani *et al.,* 1994; Kates, 1996; Kates, 1993). Membrane spanning (bolaform amphiphilic) tetraether lipids are found in extreme thermophiles and acidophiles (De Rosa *et al.,* 1991). These lipids have C_{40} isoprenoid acyl chain that spans the entire membrane (Relini *et al.,* 1996). Freeze-fracturing of these membranes reveals that cleavage between two leaflets of the membrane does not occur, which means that the water facing sides of the membrane are connected and cannot be separated (Beveridge *et al.,* 1993; Choquet *et al.,* 1992; Elferink *et al.,* 1992). Tetraether lipids from *Thermoplasma acidophilum* and *S. solfataricus* form a monolayer of a constant thickness of 2.5-3.0 nm irrespective of the type of solvent used (Stern *et al.,* 1992; Gliozzi *et al.,* 1983), another indication that tetraether lipids span the membrane. This monolayer type of organization

gives the membrane a high degree of rigidity (Elferink *et al.,* 1992; Thompson *et al.,* 1992).

Hydroxylated ether lipids have been found only in some methanogenic Archaea (Sprott *et al.,* 1990) and in the haloalkaliphilic genus *Natronobacterium* (Upasani *et al.,* 1994). Hafenbradl *et al.* (1993) reported the existence of significant amounts of unsaturated diether lipids in the hyperthermophile *Methanopyrus kandleri*. In this organism, a diether lipid was found that contained branched methyl chains with some *trans*-unsaturated bonds in its backbone (di-geranylgeranyl-glycerol, build from terpene elements) (fig. 3A) (Hafenbradl et al., 1993). *Trans*-unsaturated bonds do not introduce a kink in the acyl chain, in contrast with *cis*-unsaturated bonds. The presence of di-geranylgeranyl-glycerol is a rather primitive feature. In the biosynthetic pathway of phytanyl-glycerol, the production of geranylgeranyl-glycerol is an earlier enzymatic step in and therefore may have evolved earlier in evolution (Wächtershäuser, 1988). The first membrane could also be a mixture of lipids with Bacterial and Archaeal characteristics as in the Bacterial genera *Aquifex* and *Thermotoga*, in which the majority of the lipids are Bacterial ester linked alkyl chains. In addition, some lipids are membrane spanning C_{30} ester lipids (Fig. 3B) (Huber *et al.,* 1992; Klein *et al.,* 1979). Strikingly, *Thermotoga* and *Aquifex* contain some core lipids that have ether linked alkyl chains (Fig. 3C) (De Rosa *et al.,* 1988). The genera *Aquifex*, *Thermotoga* and *Methanopyrus* occupy a position near the division of the domains Archaea, Bacteria and Eucarya in the 16S rRNA-based phylogenetic tree (Fig. 1). This supports the above stated idea that their ‘primitive’ lipids resemble the lipids of the first organisms on Earth.

Another type of unsaturated lipid, and probably an adaptation to the low growth temperature, was found in the psychrophilic Archaeon *Methanococcoides burtonii* (Nichols and Franzmann, 1992). This lipid has a *cis* double bond that can distort the membrane order as in Bacteria that live at low temperatures. The bioenergetic and biophysical consequences of the lipid compositions in the *Mp. kandleri* and *Mc. burtonii* membrane are not known.

Salt influences the overall charge on the headgroup of lipids. Halobacteria have a very high density of negative charges on the surface of their membrane lipid (Russell, 1989). This has the advantage that the negative charges on the polar headgroups are shielded by the high cation concentration, preventing disruption of the lipid bilayers due to charge-repulsive forces and providing a charge-stabilized lipid bilayer (Kates, 1993). Moderate halophiles increase the amount of negatively charged lipids upon an increase of salt concentration in their growth medium (Russell, 1989).

Mechanism of proton permeation Water, neutral solutes and ions likely permeate the membrane by a solubility/diffusion mechanism. The mechanism for proton permeation across the membrane is not determined by simple diffusion. For protons, the rate of permeation across biological membranes is higher than the rate of Na^+ permeation (Deamer and Nichols, 1983; Deamer and Bramhall, 1986) (Chapter 2). Additionally, the proton permeability increases only one order of magnitude over a pH range of 1 to 11 (*i.e.,* ten orders of magnitude) (Nagle, 1987; Nichols and Deamer, 1980), while the permeation of other ions, such as Na^+, increases linearly with the concentration.

Three models have been proposed to explain the mechanism of proton permeation across the membrane. One model assumes that protons permeate by solubility and diffusion, but proton permeation rates predicted with that model cannot

account for the rates observed experimentally (Marrink *et al.,* 1996). High proton permeation rates have also been attributed to weakly acidic contaminants that act as proton carriers (Gutknecht, 1987). A strong acid protonophore like HCl cannot account for the permeation rates observed, because a strong acid is only present in the dissociated form (*e.g.,* H^+ and Cl^-) (Gutknecht and Walter, 1981). Lipid hydrolysis and oxidation are considered possible origins for weak-acid protonophores. Experimental data show that such protonophores indeed are carriers for protons. However, the pH dependence of protonophore mediated proton permeability differs from that of observed proton permeability (Deamer and Nichols, 1983). The clear correlation between the proton permeability without weak acids in the medium and the growth temperature of the organism (see Chapter 2 and 3) makes the contaminating weak acids mechanism less likely the major determinants of proton permeability. The third possible mechanism concerns proton permeation through a transient water wire (Nagle and Morowitz, 1978). In this model, protons can cross the membrane by hopping along a hydrogen-bounded chain of water molecules. The lifetime of a water wire is so short that only one proton can be transported. In a tetraether membrane, the probability formation of a water wire would be less because of the rigid structure of the diphytanyl chain and this would explain the lower observed permeability of tetraether lipid membranes (Elferink *et al.,* 1994) (Chapter 2 and 5).

The impact of the membrane thickness on the ion permeability characteristics indicate that the proton permeability decreases sharply upon an increase of the acyl chain length (Paula *et al.,* 1996).

Properties of Archaeal tetraether lipid membranes Liposomes composed of Archaeal tetraether lipids are more stable than Bacterial bilayer lipids and have an extremely low proton permeability (Elferink *et al.,* 1994) (Chapter 2 and 5). A study on synthetic membrane spanning lipids revealed that in particular the bulky isoprenoid core is responsible for the lowered proton permeability (Yamauchi *et al.,* 1993). Ether links are far more resistant to oxidation and high temperature than ester links. Consequently, liposomes prepared from Archaeal tetraether lipids are more thermostable (Chang, 1994). In contrast to ester links, ether links are not susceptible to degradation at alkaline pH (saponification). Ether lipids are also resistant to enzymatic degradation by phospholipases (Choquet *et al.,* 1994). The high stability of tetraether liposomes is superior to cholesterol-stabilised liposomes prepared from saturated synthetic lipids that resemble Bacterial lipids (Choquet *et al.,* 1996).

Yamauchi *et al.* (1992) compare the properties of lipids that resemble Halobacterial lipids with the typical Bacterial phospholipids. The lipids differed only in the acyl chains. The Halobacteria-like lipids were found to be stable at a wide range of salt concentrations, whereas the Bacteria-like lipids could only form liposomes at the higher salt concentrations and at very low lipid concentration. The Halobacteria-like lipids were found to be less permeable for Na^+ (at 1 M) and carboxyfluorescein than theBacteria-like lipids. It therefore appears that the exceptional properties of the halobacterial lipids are due to the Archaeal phytanyl chain. These phytanyl chains maintain the halophile lipids in a highly fluid state at the growth temperature. The higher stability of the halo(alkali)phile phytanyl chain may be due to limited segmentary motion of tertiary carbon atoms as described above. We observed that the

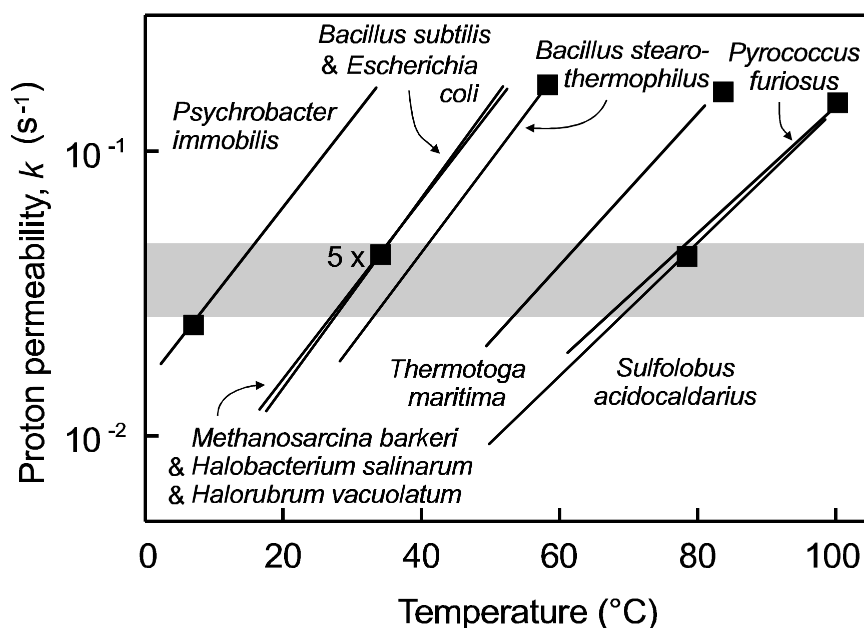

Fig. 4. Schematic representation of the proton permeability in Bacteria and Archaea that live at different temperatures. At the respective growth temperatures, the proton permeability falls within a narrow window (grey bar). The Bacteria *T. maritima* and *B. stearothermophilus* and the Archaeon *P. furiosus* have a permeability that is higher than in the other organisms. Both Bacteria overcome this problem differently.

membrane of Archaeal halophiles remained stable at high salt concentration and exhibits a similar proton permeability as other organisms that live at the same temperature (Chapter 4).

Ionophores and uncouplers can mediate ion-fluxes across membranes. For example, valinomycin (K^+-ionophore), nonactin (Na^+-ionophore), and gramicidin (H^+, K^+, Na^+-ionophore) increase the ion conductance of a black lipid membrane prepared from tetraether lipids. However, the conductance in tetraether lipids of especially gramicidin was much lower than in diether and Bacterial lipids (Stern *et al.,* 1992). The reduced effect of this ionophore in tetraether lipids is probably caused by decreased lateral diffusion of gramicidin in tetraether lipids, compared with Bacterial lipids. In halobacterial diether lipids, the Na^+/H^+ exchanging ionophore monensin could re-establish activity of the halobacterial Na^+/H^+ antiporter that was specifically blocked with DCCD (Murakami and Konishi, 1988).

Clearly, the Archaeal membrane are better adjusted to extreme environments than the membrane of Bacteria or Eucarya. This is a consequence of the lower permeability for protons, the higher stability, and the higher salt tolerance observed in Archaeal membrane lipids.

Bioenergetics of extremophiles

Temperature: Psychrophiles and Thermophiles High temperatures impose a burden on the cellular metabolism, and require a higher stability of enzymes and other macromolecules (Adams, 1993). Since the basis for membrane permeation is diffusion (the diffusion of water in case of proton permeation), the ion-permeability of the membrane increases with the temperature. When the coupling ions, *i.e.,* protons or sodium ions, permeate too fast, the organism will be unable to establish a sufficient PMF or SMF. The permeability of the cytoplasmic membrane thus is

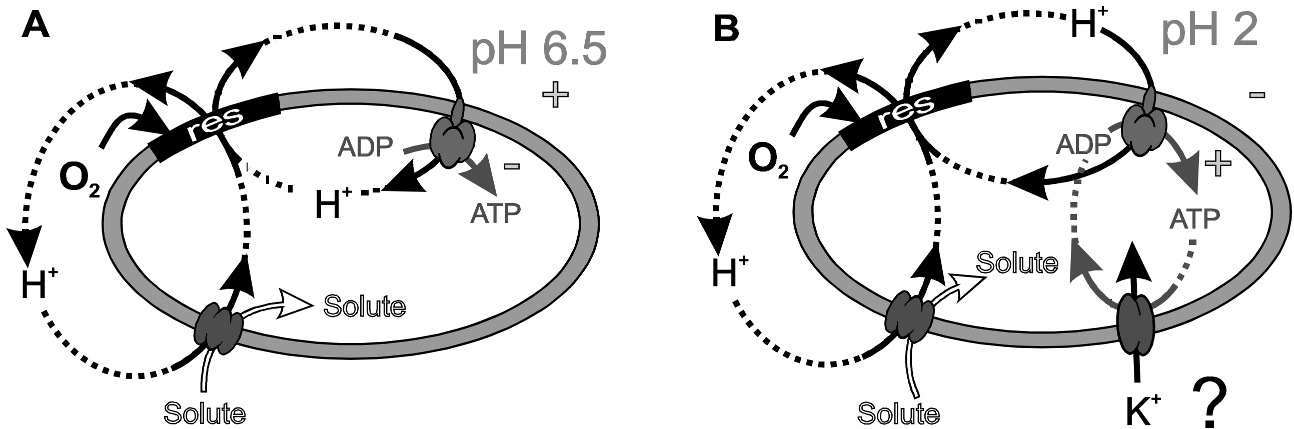

Fig. 5AB. Gradients in aerobic Bacteria and Archaea. The internal pH is around neutral (pH 7). **A**: neutral environment as for *Escherichia coli* or *Bacillus subtilis*, described in Chapter 2 and 3: The respiratory chain excretes protons, thereby generating a PMF (Δψ and ΔpH cooperate) . The protons are used to produce ATP and help to take up solutes from the environment; **B**: acid environment as for *Picrophilus oshimae*, described in Chapter 5: The respiratory chain excretes protons against a huge pH gradient. The vast ΔpH is lowered by the inversed Δψ across the membrane. The Δψ is probably effected by active uptake of K^+. Depicted are: the gradients that contribute to the PMF and the SMF. res: respiratory chain

a major factor that determines the maximum growth temperature. Liposomes have been prepared from lipids extracted from a variety of organisms that grow optimally at different temperatures. Comparison of the proton permeability of these liposomes revealed that the membrane becomes highly permeable for protons at temperatures above the growth temperature of the organism from which the lipids were derived. Membranes were found to be orders of magnitude less permeable for sodium ions. The basal sodium ion permeability depends on the temperature and barely on the composition of the membranes (Chapter 2).

The most important finding of our studies is that the proton permeability of most Bacterial and all Archaeal membranes at the temperature of growth is maintained within a narrow window (H^+-permeability coefficient near 10^{-9} cm s^{-1}) (Fig. 4 and Chapter 2). In contrast, this correlation is not observed for the permeation of sodium ions. Apparently, the lipid composition of the membrane has only a minor effect on the membrane permeability for sodium ions, and the rate of sodium ion permeation seems only to be influenced by the temperature. The homeostasis of proton permeability, termed 'homeo- proton permeability adaptation', was demonstrated in *Bacillus subtilis* grown at and within the boundaries of its growth temperature range (Chapter 3). This organism adjusts the proton permeability of the cell membrane to the growth temperature in order to be able to maintain a PMF under all growth conditions. The growth temperature dependent alterations in fatty acyl chain composition are thus mainly aimed at maintaining the proton permeability of the cytoplasmic membrane at a rather constant level. The proton permeability is an important growth limiting factor at the upper boundary of the growth temperature.

For some organisms, the proton permeability at their respective growth temperature is higher than predicted by the 'homeo- proton permeability adaptation' theory in Chapter 3 and the narrow

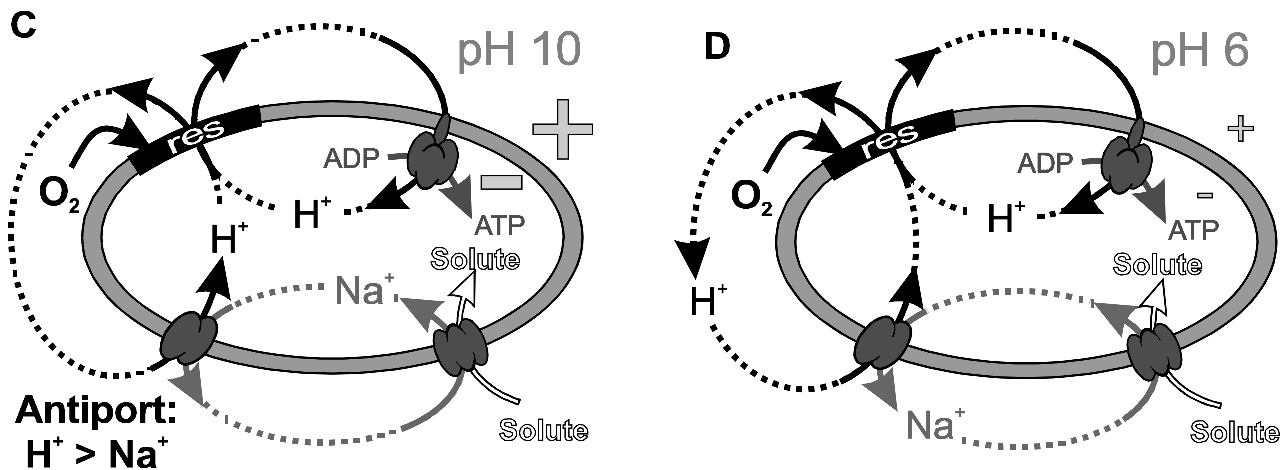

Fig. 5CD. Gradients in aerobic Bacteria and Archaea. The internal pH is around neutral (pH 7). **C**: alkalic environment as for *Bacillus alcalophilus* (Krulwich, 1995): Protons are extruded by the respiratory chain. The high Δψ imposes a PMF on the protons, who flow back into the cell via ATPase. Na^+ is extruded via an electrogenic H^+/Na^+- antiporter (more H^+ in than Na^+ out), imposing a SMF. The SMF is then used to transport solutes into the cell ; **D**: salty environment as for *Halobacterium salinarum* as described in Chapter 4: Protons are extruded by the respiratory chain. The protons are used for H^+/Na^+- antiporter, to extrude the excess of sodium ions from the cytoplasm. Sodium ions are used for transport of solutes. Depicted are: the gradients that contribute to the PMF and the SMF. res: respiratory chain

window of proton permeabilities at the growth temperature in Chapter 2. Thermophilic Bacteria, such as *Bacillus stearothermophilus* and *Thermotoga maritima* and the hyperthermophilic Archaeon *Pyrococcus furiosus* (appendix Chapter 2) are unable to maintain the proton permeability of their membrane at the high temperatures at which they grow. Thermophilic Bacteria and possibly hyperthermophilic Archaea rely on other mechanisms to maintain electrochemical gradients of the coupling ions at a viable level. This maintenance of a constant proton permeability of the cell membrane appears to be a general mechanism for psychrophilic and mesophilic bacteria and for all archaea. The only micro-organisms that are unable to exert a stringent control on the permeability properties of their membranes are the thermophilic bacteria. The proton permeability of their membranes is higher than predicted from the proton permeability of other species. Consequently in thermophilic bacteria, such as *Bacillus stearothermophilus* and *Thermotoga maritima*, ‘homeo- proton permeability adaptation' cannot be maintained at the high temperatures at which these organisms grow (Fig. 4 and Chapter 2). Some moderately thermophilic bacteria can compensate for the high proton leak by drastically increasing the respiration rate and therefore the rate of proton pumping (De Vrij *et al.,* 1988). Other thermophilic bacteria have to rely on electrochemical gradients of another ion which permeates the membrane at a much lower rate then protons. Many thermophilic bacteria shift to the less permeable sodium ion as coupling ion for energy transduction. The latter strategy is used by *Caloramator fervidus* (previously *Clostridium fervidus*) (Speelmans *et al.,* 1993a; Speelmans *et al.,* 1993b), an organism that can grow at a higher temperature than *B. stearothermophilus*, *i.e.,* at 70°C (Patel *et al.,* 1987) *versus* 65°C (Esser and Souza, 1974). Due to the high proton permeability of its membrane *C. fervidus* is unable to maintain a constant intracellular pH. Consequently, growth of *C. fervidus* is confined

to a narrow niche, *i.e.,* an environment with a pH near to neutrality.

Most psychrophilic organisms found so far belong to the Bacteria and Eucarya, and only few psychrophilic Archaea are known. Adaptation of the membrane to low temperatures is governed by membrane fluidity and proton permeability of the cytoplasmic membrane (Russell, 1990). Therefore, psychrophiles optimize their lipid composition. In psychrophiles, the proton permeability at the growth temperature is maintained at the same level as in the membranes of mesophiles at their respective growth temperature (Chapter 2).

Salt: Halophiles Halophiles, as *Halobacterium salinarum* can generate an electrochemical proton gradient across the membrane by respiration and/or the light driven proton pump bacteriorhodopsin (Michel and Oesterhelt, 1980). This organism has an H^+/Na^+ antiporter that expels the sodium ions (Murakami and Konishi, 1988). Specific potassium ion transport systems accumulate K^+, which is less harmful for the enzymes in the cytoplasm than Na^+ intracellularly at high concentration. The SMF drives the uptake of solutes by secondary transport mechanisms (Lanyi *et al.,* 1976) (Fig. 5D). Halophiles keep the cytoplasm relatively free of sodium ions, which are actively expelled. The osmotic balance is maintained by the accumulation in the cell of the less toxic potassium ions and a wide range of organic compatible solutes. Compatible solutes accumulate in the cytoplasm in order to maintain osmotic pressure in the salty environment, thereby affecting cellular function as little as possible. Compatible solutes are often zwitterionic organic molecules such as glycine betaine. Archaea, of which only methanogens can synthesize organic compatible solutes, are able to take up organic compatible solutes and ions from the environment, like Bacteria (Lai *et al.,* 1991; Robertson *et al.,* 1992). The internal potassium ion level in extreme halophiles can be more than 3 M (Lai and Gunsalus, 1992), which can be up to fivefold higher than of sodium ions (Brown, 1983; Pérez-Fillol and Rodríguez-Valera, 1986).

The proton and sodium ion permeability of halobacterial membranes does not differ from non-halophilic organisms that live at the same temperature. Membranes of halophiles and halo-alkaliphiles are mainly adjusted to the high salt concentration and to a lesser extent to pH (Chapter 4).

Acidity: Acidophiles and Alkaliphiles Alkaliphiles maintain the intracellular pH at values that are lower than the external pH. To maintain this reversed pH gradient, metabolic energy is needed. At the same time, alkaliphiles need to take up solutes from the environment. For this purpose, these organisms need an electrochemical ion gradient of protons or sodium ions. Since the ΔpH in alkaliphiles is reversed (inside more acidic than outside), a very high $\Delta\psi$ (inside negative) is needed to maintain a sufficient PMF (Fig. 5C).

Acidophiles face the opposite problem of a high ΔpH, acid outside. This high ΔpH results in a large PMF, which has to be reduced to a normal value by a reversed $\Delta\psi$ (inside positive). In spite of this compensation, the PMF of acidophiles is still high: around -200 mV (Michels and Bakker, 1985) (Fig. 5C).

Acidophiles and alkaliphiles keep their internal pH near neutrality (Matin, 1990; Booth, 1985) . Aerobic alkaliphiles use a Na^+/H^+-antiporter in combination with H^+-coupled respiration to regulate their intracellular pH (Krulwich, 1995; Speelmans *et al.,* 1995). The anaerobic thermo-alkaliphilic strain *Anaerobranca bogoriae* (*Thermoalkalibacter bogoriae*) LBS3 relies on

sodium ions for energy coupling (Prowe *et al.,* 1996). The cytoplasmic membrane of this organism seems to be very permeable for H^+ (preliminary results). Therefore, the PMF would be very low, and the protons can only be kept inside in the presence of a $\Delta\psi$.

All thermoacidophiles known are Archaea. *Picrophilus oshimae*, the most acidophilic thermophile known so far, is able to grow in 1.2 M sulphuric acid at 60°C (Schleper *et al.,* 1995). This organism maintains its intracellular pH at 4.6 at an outside pH ranging from 0.5 to 4 (Chapter 5). The lipids of *P. oshimae* cannot assemble into regular vesicular structures at pH values around neutrality. The loss of membrane integrity at neutral pH values is an intrinsic property of these lipids and likely the consequence of the adaptation of the cells to an extreme acidic environment. Consequently, the cell viability is lost in environments close to neutral. The intracellular pH is low in comparison with other extreme acidophiles that maintain intracellular pH values above 6 (Michels and Bakker, 1985; Moll and Schäfer, 1988; Peeples and Kelly, 1995) (Chapter 5). An even lower intracellular pH is observed in the Bacterium *Sarcina ventriculi*, that can grow over a broad pH range. This organism is unable to regulate its internal pH throughout the entire pH range that supports growth (Goodwin and Zeikus, 1987).

With a near-neutral cytoplasm, acidophiles have a large chemical pH gradient across the membrane that can only be maintained when the proton permeability of the membrane is very low. Liposomes prepared from lipids derived from thermoacidophilic Archaea are indeed extremely impermeable for protons, even at the elevated temperatures at which the organisms grow.

The efficient reduction of a high PMF by the reversed $\Delta\psi$ might have some unwanted side-effects. The reversed $\Delta\psi$ influences cellular processes that depend on the orientation of the $\Delta\psi$. Cationic solutes cannot be taken up with uniport uptake systems so that acidophiles are forced to use other strategies. Membrane proteins carry a net positive charge in their intracellular loops (positive inside rule) (Von Heijne, 1986). In neutrophiles this orientation has been claimed to be driven by the $\Delta\psi$ (inside negative) (Andersson and Von Heijne, 1994). The charge distribution in the membrane proteins of extreme acidophiles have been shown not to be different from neutrophiles (Van de Vossenberg *et al.,* 1998b), dispite the opposite $\Delta\psi$ (inside positive). The positive inside rule in extreme thermophiles can therefore not be responsible for the orientation of membrane proteins. The $\Delta\psi$ does not seem to orientate the membrane proteins as originally proposed.

Combinations of extremophilic traits Above we discussed the influence of one extreme parameter on the membrane and cellular physiology. Many extremophiles face more than one extreme parameter. For instance, such environments are often anaerobic, and organisms are found that manage to live under those combinations. Organisms have been discovered in alkaline soda lakes that are both highly alkaline and salty. The soda lake inhabiting organisms need not only to be alkaliphiles, but need to be haloadapted as well. These halo-alkaliphiles face a high outside sodium ion concentration, and a low external H^+ concentration. As in other alkaliphiles, the SMF is used for transport of solutes and motility. Halo-alkaliphiles contain Na^+/H^+ antiporters which exchange protons for sodium ions. The Na^+/H^+ antiporters in halo-alkaliphiles do not have a stoichiometry of 1:1 (Krulwich, 1995). A stoichiometric antiport of Na^+/H^+ would result in an increase of the internal pH and Na^+ concentration, due to the direction of the driving

force. Instead, an electrogenic Na^+/H^+ antiporter (the number of exchanged H^+ is higher than the number of Na^+) is driven by the $\Delta\psi$ and results in acidification of the cytoplasm. To date, the stoichiometry of these Na^+/H^+ antiporters has not been determined (Padan and Schuldiner, 1994).

Aim and outline of this thesis

Living cells use an electrochemical gradient of protons or sodium ions over the cytoplasmic membrane to drive transport of compounds across the membrane and to obtain metabolic energy. To maintain these gradients, the membrane needs to be sufficient impermeable for protons and sodium ions. The permeability properties change with the environmental conditions. Especially extremophiles face exceptional conditions that influence the permeability properties of the membrane. These extremophiles have to find an equilibrium in the requirement for an impermeable membrane and a suitable matrix for membrane proteins to function.

The main question in this Ph.D. study was: How do extremophiles adapt the membrane permeability for protons and sodium ions to the extreme environmental conditions?

To answer this question liposomes were used that were prepared from lipids extracted from different classes of extremophiles. A gradient of protons or sodium ions was applied and the rate of these ion fluxes across the liposomal membrane was measured. Also other properties of the membrane, like stability and fluidity, were analysed.

In **Chapter 2** lipids were isolated from organisms that grow at very different temperatures. The proton permeability was found to be dependent on the growth temperature of the organism. Organisms adapt their membrane composition so that at growth temperature the proton permeability of the liposomes, derived from different species, is comparable. The sodium ion permeability is independent from the growth temperature of the organism.

In **Chapter 3** the influence of temperature on proton permeability adaptation is tested in one single species, *Bacillus subtilis*, grown over almost its whole temperature range (from 13 to 50°C). *B. subtilis* adapts its membrane so that the proton permeability remains virtually constant at the different growth temperatures. The 'homeo-proton permeability' model is proposed. The organism keeps the membrane in a liquid crystalline state at low temperatures and reduces the proton permeability at high temperatures, thus constantly balancing between the optimal membrane fluidity and proton permeability.

In **Chapter 4** the influence of salt on the proton permeability and sodium ion permeability of halophiles and halo-alkaliphiles is described. The proton permeability and sodium ion permeability in salty environments does not differ dramatically. The main property of the membrane of halophiles is to remain stable at high salt concentrations, not to change the permeability properties.

Chapter 5 describes the bioenergetics and proton permeability of an extremely acidophilic thermophile *Picrophilus oshimae*, which lives at 60°C and pH 1. The membrane of this organism is highly adapted to low pH and high temperature. This is observed in the behaviour of the liposomes, which cannot be formed at pH 5 and higher. This correlates with the observed low intracellular pH of 4.6. The membrane potential has, as observed in other extreme acidophiles, a reversed polarity. The proton permeability at low pH is comparable with *Sulfolobus acidocaldarius* described in Chapter 2.

The work presented in this thesis is discussed in **Chapter 6**. Temperature is the parameter that

has the most pronounced effect on the lipid composition and proton permeability. The temperature dependent change in membrane lipid composition serve to keep the ion permeability of the membrane at a viable level, which is in particular essential for growth in extreme conditions.

Chapter 2

Ion permeability of the cytoplasmic membrane limits the maximum growth temperature of Bacteria and Archaea

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Summary

Protons and sodium ions are the most commonly used coupling ions in energy transduction in Bacteria and Archaea. At their growth temperature, the permeability of the cytoplasmic membrane of thermophilic Bacteria to protons is high as compared to sodium ions. In some thermophiles, sodium is the sole energy coupling ion [Speelmans, G., Poolman, B., Abee, T., and Konings, W.N. (1993) *Proc. Natl. Acad. Sci. USA* 90: 7975-7979]. To test whether sodium is the preferred coupling ion at high temperatures, the proton- and sodium ion permeability was determined in liposomes prepared from lipids isolated from various Bacterial and Archaeal species that differed in their optimal growth temperature. The proton permeability increased with the temperature, and was comparable for most species at their respective growth temperatures. Liposomes of thermophilic Bacteria are an exception in the sense that the proton permeability is already high at the growth temperature. In all liposomes the sodium ion permeability was lower than the proton permeability and increased with the temperature. The results suggest that the proton permeability of the cytoplasmic membrane is an important parameter in determining the maximum growth temperature.

Introduction

Biological membranes consist of a bi- or monolayer of lipid molecules with their polar headgroups oriented toward the aqueous phase and their hydrophobic hydrocarbon chains forming the interior of the membrane. An important feature of biological membranes is that their structure is predominantly held together by noncovalent bonds such as Van der Waals and coulombic interactions, which make them highly impermeable to small ions. The low ion permeability of membranes results from the high energy requirement for the transfer of an ion from the aqueous phase into the apolar, hydrocarbon-like interior of the membrane. This barrier is important for the functioning of the cell membrane in processes such as energy transduction. Since this permeability usually reflects a simple diffusional process, the permeability of the membrane for ions will increase with the temperature. An intriguing

	Species	T_{growth}[a]	T_{max}[a]	Acyl chain composition	Ref.[b]
		°C			
Bacteria	*Psychrobacter immobilis*	21	29	93% mono-unsaturated,	1
	Escherichia coli	37	42	32% mono-unsaturated,	
	Bacillus stearothermophilus	60	70	saturated, 80% branched acyl chains	2
	Thermotoga maritima	80	90	saturated, 7% membrane spanning lipid esters	3
Archaea	*Methanosarcina barkeri*	35	42	diether lipids, isoprenoid and hydroxy-acyl chains	4
	Sulfolobus acidocaldarius	80	83	95% membrane spanning tetraether lipids,	5

a. T_{max} and T_{growth} are the maximum and imposed growth temperatures, respectively.
b. [1](McLean *et al.,*1951); [2](Esser and Souza, 1974); [3](Huber *et al.,*1986); [4](Kandler and Hippe, 1977); [5](Brock *et al.,*1972)

Table 1. Growth characteristics and lipid composition of the cytoplasmic membrane of the Bacterial and Archaeal species used in this study.

feature of Bacteria and Archaea is that they can grow over a wide range of temperatures. Most prokaryotes are mesophiles and grow at temperatures between 20 and 55°C. A smaller group of prokaryotes can grow below -2°C, the freezing temperature of Arctic sea water (Baross and Morita, 1978). On the other hand, the highest temperature at which growth of Bacterial species has been found is 90°C, while growth of Archaeal species in marine volcanic areas has been observed up to 110°C (Huber *et al.*, 1986; Stetter *et al.*, 1983; Stetter *et al.*, 1990).

Growth at the temperature extremes requires an optimal functioning of cellular metabolism, and a high stability of enzymes and other macromolecules (Adams, 1993). Many bacterial proteins or other macromolecules have been shown to be stable and active at temperatures that are often significantly above the maximal growth temperature (Marguet and Forterre, 1994). Minor changes, sometimes even of one single amino acid, can lead to a significant increase in thermostability (Yutani *et al.*, 1977; Grütter *et al.*, 1979; Goward *et al.*, 1994; Cannio *et al.*, 1994). The reason that there is a maximum temperature of growth is, therefore, far from clear.

The cytoplasmic membrane has to retain its stability and functionality. To maintain the membrane in a liquid crystalline state, cells vary the lipid composition when they are subjected to temperature changes (Russell and Fukunaga, 1990). This homeoviscous adaptation (Sinensky, 1974) can be realized by adjusting the fatty acyl chain composition of the lipids either by varying

the degree of acyl chain saturation, branching, and/or cyclization. The headgroup composition of the lipids is hardly involved in this adaptation (Russell, 1984). Homeoviscous adaptation is thought to preserve membrane protein function, but its impact on the energy transducing properties of the membranes has hardly received any attention. Metabolic energy can be obtained at essentially two levels: at substrate level by phosphorylation processes, and by energy transducing processes in the cytoplasmic membrane. These processes are closely linked and together they determine the energy status of the cell. Energy transduction in the cytoplasmic membrane involves primary transport systems such as electron transfer systems or ATPases. These systems translocate protons or sodium ions (Speelmans *et al.*, 1993a) from the cytoplasm to the external medium, and thereby generate an electrochemical gradient of protons ($\Delta\mu_{H+}$) or of sodium ions ($\Delta\mu_{Na+}$), respectively. The resulting forces can be used to drive membrane bound processes such as ATP synthesis, uptake of solutes, flagellar rotation etc. Obviously, this type of energy transduction can only operate if the membrane has a limited permeability for the coupling ions, protons or sodium ions. In previous studies on energy transduction in Bacteria we observed that the cytoplasmic membranes of the thermophiles *Caloramator fervidus* (*Clostridium fervidus*) and *Bacillus stearothermophilus* at their respective growth temperatures are extremely permeable for protons, while the permeability for sodium ions remained low (Lolkema *et al.*, 1994). To maintain a viable metabolic energy level, these organisms use two different strategies to cope with the increased proton permeability at elevated temperatures. *B. stearothermophilus* dramatically increases the rate of proton pumping by the respiratory chain (De Vrij *et al.*, 1988), while *C. fervidus* uses sodium ions instead of protons as sole energy transducing coupling ion (Speelmans *et al.*, 1993a; Speelmans *et al.*, 1993b). A low sodium ion permeability *versus* the proton permeability would explain why *C. fervidus* can grow at much higher temperatures than *B. stearothermophilus*, at 80°C (Patel *et al.*, 1987) versus 63°C, respectively (Table 1). The latter organisms relies on protons in energy transduction.

The question arises whether the proton permeability is a limiting factor for the maximal growth temperature of psychrophilic, mesophilic and thermophilic organisms. We now report on the temperature dependency of the proton and sodium ion permeability of liposomes prepared from lipids extracted from various Bacteria and Archaea that vary in their optimum growth temperature (Table 1). The results demonstrate that the proton permeability of these liposomes becomes extremely high at temperatures above the growth temperature of the organism the membranes are derived from. For all membranes, the permeability to sodium ions was found to be lower compared to protons. However, unlike the proton permeability, the increase of the sodium ion permeability with temperature was found to be lipid independent. The data suggests that the proton permeability of the cytoplasmic membrane is a major factorthat determines the maximum growth temperature.

Results

Isolation of lipids and formation of liposomes. Lipids were isolated from a psychrophilic Bacterium and mesophilic and thermophilic Bacteria and Archaea (Table 1). With increasing growth temperature, Bacteria reduce the number of unsaturated bonds or increase the degree of branching in their lipid acyl chains. Extreme

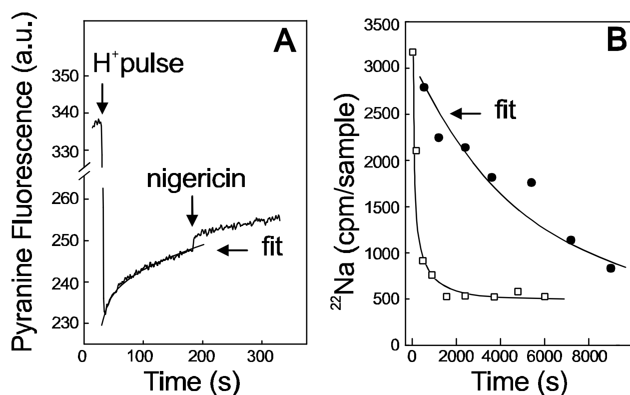

Fig. 1. Proton (A) and sodium ion (B) permeability of liposomes derived from *S. acidocaldarius* lipids. The proton permeability indicated was measured at 81.5°C and the sodium ion permeability at 60°C (**!**) and 87°C (G), respectively, as described in Materials and Methods. Solid lines indicate the data fitted to a first order kinetic equation.

thermophilic Bacteria, like *T. maritima* contain membrane spanning lipid esters. Archaeal cytoplasmic membranes consist of isoprenoid ether chains, that are either arranged in a bilayer in non-thermophiles, or form membrane spanning tetraether lipids in extreme thermophiles. Lipids were isolated from the bacterial and archaeal species listed in Table 1 as described under Experimental procedures. For comparison and quantitative purposes it was necessary to have equally sized liposomes. From each of the lipid isolates, unilamellar liposomes were formed by freeze-thawing and extrusion through a 200 nm pore size filter. This procedure yields liposomes with an average diameter close to 200 nm (Elferink *et al.*, 1994). Each liposome preparation could sustain an artificially imposed $\Delta\psi$ (data not shown) for at least several minutes, indicating the formation of sealed vesicular structures.

Proton permeability. For proton permeability measurements, liposomes prepared with a high buffer capacity on the inside were equilibrated in a solution with low buffer capacity. Valinomycin, a potassium ionophore, was added to prevent the generation of a counteracting electrical potential due to electrogenic influx of protons. The external pH, as monitored with the fluorescent pH indicator pyranine, was lowered by an H^+ pulse of 100 nmol of H^+ (Fig. 1A). The immediate drop of the external pH was followed by a slow increase in the external pH due to the influx of protons into the liposomes. Rapid proton equilibration was obtained after the addition of the ionophore nigericin (Fig. 1A) that mediates an electroneutral exchange between potassium and protons. In the presence of valinomycin, nigericin causes a complete uncoupling of the membranes. The slow increase of pyranine fluorescence after the proton pulse was fitted to a first order kinetic rate equation to yield the rate constant, k_{H+}, of H^+ influx. The proton permeability of liposomes prepared from the lipids derived from the various organisms was measured at a wide range of temperatures.

The proton permeability of the liposomes increased with temperature (Fig. 2). The higher the growth temperature of the organisms from which the lipids were extracted, the higher the temperature was at which a certain proton permeability of their liposomes was reached. Liposomes prepared from lipids derived from the psychrophile *Psychrobacter immobilis* sp. are already highly permeable to protons at a low temperature. Around 30°C, the proton permeability was found to be at least 5-fold higher than that observed for mesophiles. The proton permeability of liposomes derived from both mesophiles *M. barkeri* and *E. coli* exhibited a similar temperature dependency. Liposomes from *B. stearothermophilus* showed a slightly lower proton permeability than the liposomes from mesophiles. Liposomes derived from the extreme thermophilic Bacterium *T. maritima* and Archaeon *S. acidocaldarius* showed the lowest proton permeability at the higher temperatures. At

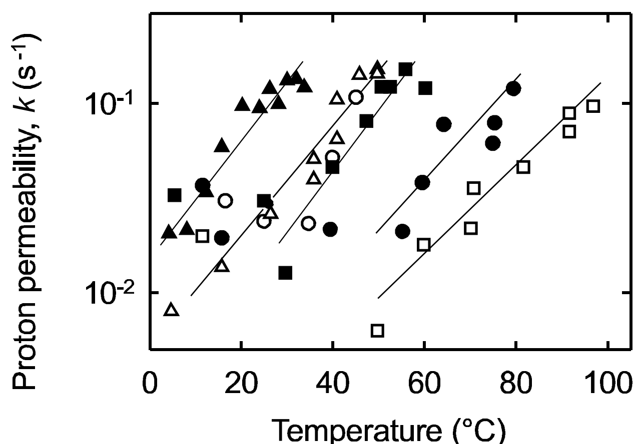

Fig. 2. Temperature dependency of the proton permeability of liposomes derived from various Bacteria and Archaea. The first order rate constant for the proton permeability (k_{H+}) was determined as described in the text for liposomes composed of lipids derived from *P. immobilis* sp (▲), *M. barkeri* (Δ), *E. coli* (F), *B. stearothermophilus* (O), *T. maritima* (M), and *S. acidocaldarius* (G).

the growth temperatures, the proton permeabilities of liposomes composed of *T. maritima* and *B. stearothermophilus* lipids were much higher than for the other organisms. We noted that at temperatures far below the growth temperature, the proton permeability of most liposomes increased again. This was not the case for *M. barkeri* liposomes, and could not be measured for *P. immobilis*. The proton permeability of each of the liposome preparations was plotted in an Arrhenius plot, and the activation energy of the proton permeability ($\Delta G'_{H+}$) was calculated from the linear part of the graph (data not shown). The $\Delta G'_{H+}$ value was in the same order for each of the liposome samples ranging from 40 to 55 kJ mol^{-1}. This suggests that the mechanism of proton permeability in these liposomes with a vastly different lipid composition is identical. However, the temperature range wherein the proton permeability varies is unique for each of the liposomes and correlates more or less with the growth temperature of the organism from which the lipids were isolated.

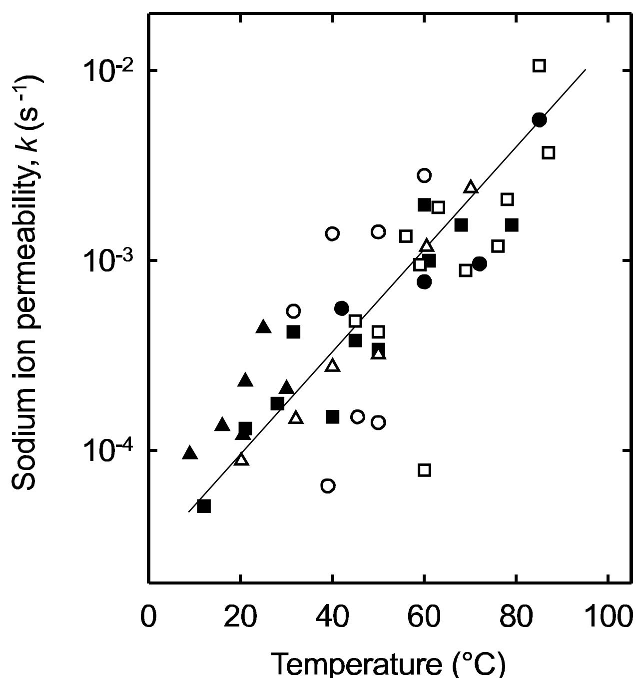

Fig. 3. Temperature dependency of the sodium permeability of liposomes derived from various Bacteria and Archaea. The first order rate constant for the sodium permeability (k_{Na+}) was determined as described in the text for liposomes composed of lipids derived from *P. immobilis* sp (▲), *M. barkeri* (Δ), *E. coli* (F), *B. stearothermophilus* (O), *T. maritima* (M), and *S. acidocaldarius*

Sodium ion permeability. The sodium ion permeability of the liposomes was measured by analysing the efflux of ^{22}Na. Liposomes were loaded with 1 mM of ^{22}Na and diluted 100 fold in a bufer of identical composition except that the ^{22}Na was replaced by the non-radioactive ^{22}Na. Under these conditions, there is no concentration gradient of sodium ions across the membrane, and only equilibration due to passive diffusion is monitored. The release of ^{22}Na by the liposomes was measured by a filtration assay. ^{22}Na release was extremely slow and followed first order rate kinetics as shown for liposomes composed of *S. acidocaldarius* lipids (Fig 1B). instantaneous equilibration of the ^{22}Na was effected by the addition of gramicidin (data not shown). The

temperature dependency of the sodium ion permeability of the membrane was measured for the different liposomes, and the first order rate constant, k_{Na+}, was used for comparison. k_{Na+} increased with the temperature (Fig. 3). The sodium ion permeability remained virtually identical for the different lipids when plotted as a function of the temperature. The activation energy of the sodium ion permeability ($\Delta G'_{Na+}$) was 47 kJ mol^{-1} which is in the same order as proton permeability. These data suggest that the lipid composition of the membrane has little effect on the membrane permeability for sodium ions. Moreover, the permeability of the membrane for sodium ions is much lower than for protons.

Discussion

In this study, we compared the proton- and sodium ion permeability of liposomes composed of lipids derived from various Bacteria and Archaea thereby covering nearly the entire range of temperatures at which these organisms can grow. For non-electrolytes, it has been shown that the passive permeability characteristics of membranes are essentially the same as those of liposomes prepared from extracted lipids (De Gier *et al.*, 1971). Liposomes, therefore, represent a good model system for a comparative study of the permeability characteristics of biological membranes. We used the first order rate constant *k* to describe the rate by which protons and sodium ions equilibrate across the membrane. The use of this parameter as a measure of the ion permeability is valid as liposomes that are obtained by extrusion are of equal average diameter. The value of *k* is independent of the amount of lipids in the sample (Elferink *et al.*, 1994). The permeability coefficient, P_T (in cm s^{-1}) can be derived from the first order rate constant *k* as described by Yamauchi et al. (1993). For protons, the values for P_T measured in this study range from 10^{-10} to 10^{-9} cm s^{-1} depending on temperature. P_T values reported in literature range from 10^{-10} to 10^{-4} cm s^{-1} depending on the lipids employed in the study (Deamer and Bramhall, 1986). For sodium ions, the experimentally determined value for P_T is much lower, *i.e.*, in the order of 10^{-13} to 10^{-11} cm s^{-1}. These values are close to those reported in literature (Gutknecht and Walter, 1981; Nozaki and Tanford, 1981). Therefore, the observed difference in proton and sodium ion permeability is 10^2 to 10^3.

An important finding of this study is that the temperature limit within k_{H+} dramatically increases is different for each of the liposomes. The relevant range coincides with the growth temperature of the organism from which the lipid was isolated. In contrast, this correlation is not observed for k_{Na+}. Rather, the lipid composition of the membrane has only a minor effect on the membrane permeability for sodium ions, and the rate of permeation seems only to be influenced by the temperature. These data support the view that protons and sodium ions permeate the membrane by distinct mechanisms (Deamer and Nichols, 1989). Various mechanisms have been proposed to explain this basal permeation of protons across the lipid bilayer. Based on theoretical considerations, it seems likely that transient water pores are involved across which the protons can permeate fast via a wire-like conductance mechanism (Nagle and Morowitz, 1978). Entry of proton into the water pore must be extremely fast to account for the experimental values for proton permeation (Marrink, 1994). The high proton permeation rates have also been attributed to weakly acidic contaminants, which act as proton carriers (Gutknecht and Walter, 1981; Gutknecht, 1987). Lipid hydrolysis and oxidation are considered to be possible origins for weak acid

protonophores. In contrast, sodium ion permeation is likely to occur according to the solubility-diffusion mechanism in which the transport rates of the permeant are proportional to the product of the solubility coefficient and diffusion constant of the permeant in the membrane. The low value of the activation energy of the permeation of sodium ions across membrane (about 50 kJ mol^{-1}) suggests that the permeating entity is the hydrated sodium ion (about 80 kJ mol^{-1}) rather than the monovalent ion (about 160 kJ mol^{-1}) (Georgallas *et al.*, 1987). The measured rates of sodium ion permeation are several orders of magnitude higher than predicted according to the solubility-diffusion model (Hauser *et al.*, 1973). To lower the energy barrier for ions to permeate the membrane, transient defects in the membrane have been implicated that would allow strands of water to penetrate into the bilayer. Although the lipid composition of the various liposomes are vastly different, the activation energy for proton permeation was found to be in the same range, *i.e.*, 40 to 55 kJ mol^{-1}. This must imply that the basal mechanism of proton permeation is similar, but that the temperature range within a steep increase in permeability is observed is dictated by other attributes of the membrane. This attribute may be, amongst others, the fluidity of the membrane. It should be emphasized, however, that in the case of *B. stearothermophilus*, the maximum growth temperature is not determined by the upper boundary of the phase transition of the membrane lipids (McElhaney and Souza, 1976).

More specific factors related to lipids have been implicated to explain the low proton permeability of liposomes composed of archaeal lipids. Unlike ester lipids such as egg yolk phosphatidylcholine, membranes composed of the archaeal model lipid diphytanyl-*sn*-glycero-3-phosphocholine are highly resistant to proton permeation. This has been attributed to the inability of the bulky isoprenoid chains to form hydrogen-bonding chains of water between the phytanyl chains of the membrane lipids (Deamer and Nichols, 1989). The membrane of *S. acidocaldarius* contains branched (isoprenoid) tetraether chains, whereas lipids from *T. maritima* are composed from ester bound acyl chains with few branches (Table 1). Both organisms were grown at the same temperature, but the proton permeability of *T. maritima* is higher than that of *S. acidocaldarius* (Fig. 2). On the other hand, this discrepancy is not apparent when liposomes composed of *M. barkeri* and *E. coli* are compared. Both organisms live at the same temperature, while the diether lipids of *M. barkeri* contain isoprenoid chains like the *S. acidocaldarius* lipids. The presence of bulky isoprenoid chains alone is clearly not sufficient to account for the observed differences in proton permeability. It may well be that the continuum of the tetraether lipids that results in a monolayer organization of the membrane presents an additional energy barrier.

What is the physiological consequence of the organism-specific temperature dependent proton permeability? The value of k_{H+} of the liposomes at the respective growth temperatures of most of the organisms used in this study is in the order of 0.02 to 0.2. The temperature dependency of k_{H+} appears to be related to the growth temperature of the microorganism. k_{H+} in *B. stearothermophilus* liposomes is, however, exceptionally high at its growth temperature. This has been noted before in membrane vesicles of *B. stearothermophilus* (De Vrij *et al.*, 1988), and it has been suggested that in this organism the high proton permeability of the membrane is compensated by a high respiration rate. Nevertheless, with increasing temperature, a point will be reached at which the microorganisms are unable to compensate for the

increase in proton permeation. Our data therefore suggests that the proton permeability is an important growth limiting factor at the upper boundary of the growth temperature.

The thermoacidophile *S. acidocaldarius* that grows at pH 2.5 and 85 °C, has to maintain a steep proton gradient in order to keep the internal pH near to neutrality, *i.e.*, pH 6.5. This can only be realised with a proton resistant membrane, and a highly efficient respiratory chain that expels the protons from the cytosol (Schäfer *et al.*, 1990). Energy transduction in this organism is proton coupled, and our data demonstrate that even at high temperatures, the membranes are highly resistant to proton permeation. For *S. acidocaldarius*, the ratio of k_{H+} to k_{Na+} is only 10-fold, and under those conditions, it is not likely that sodium would be a preferable coupling ion. For the other organisms tested, the ratio of k_{H+} to k_{Na+} becomes higher, and conditions may prevail that make it more advantageous to use sodium ions instead of protons. The rate of permeation is proportional to the concentration of the permeating ion and its diffusion constant. In addition, the proton permeability of model membranes steeply depends on the magnitude of $\Delta\psi$ (O'Shea *et al.*, 1984; Krishnamoorthy and Hinkle, 1984). Since the sodium ion permeability may be limited by its membrane solubility, it is likely that $\Delta\psi$ has less of an effect on the rate of sodium ion permeation. In the presence of a $\Delta\psi$, the ratio of k_{H+} to k_{Na+} may thus be even higher. In this respect, the thermophile *Clostridium fervidus* exclusively uses Na^+ as coupling ion (Speelmans *et al.*, 1993a). The membrane of this organism is endowed with a high proton permeability, and the payoff for *C. fervidus* is that it is unable to maintain its intracellular pH whereby it is confined to grow in an environment of neutral pH. Many microorganisms with membranes with a restricted proton permeability, however, are able to use both Na^+ and H^+ as coupling ions. The existence of both the H^+ and Na^+ cycles makes the bioenergetic system more versatile and allows the organisms to rapidly adopt to changing environmental conditions (Skulachev, 1994).

In retrospect, our data suggests that the proton permeability is an important growth limiting factor at the upper boundary of the growth temperature.

Microorganisms have developed different mechanisms to cope with the increased permeability of the cytoplasmic membrane at higher temperatures. They may either maintain the proton motive force by increasing the rate of proton pumping, or dramatically alter the membrane composition such that the membrane becomes less permeable to ions. Possibly as a last resort, cells may entirely change their energy transducing mechanism by coupling these processes to an ion that is less permeable than protons, such as sodium ions.

Materials and Methods

Strains and purification of lipids. *Sulfolobus acidocaldarius* DSM 639 was grown aerobically in a 50 l fermenter at 80°C in Brock's medium supplemented with 5.8 mM L-glutamic acid, 50 mM K_2SO_4, and 5.8 mM sucrose (Brock *et al.*, 1972). *Methanosarcina barkeri* DSM 805 (Kandler and Hippe, 1977), grown at 30°C, was kindly provided by Dr. V. Müller, University of Göttingen, Germany. *Thermotoga maritima* MSB8, DSM 3109 (Huber *et al.*, 1986), grown at 80°C was kindly provided by Dr. R. Huber, University of Regensburg. Germany. *Bacillus stearothermophilus* ATCC 7954 cells were grown aerobically in a 10 l fermenter at 60°C in 2 × Luria broth. *Psychrobacter immobilis* (*Micrococcus cryophilus* ATCC 15174) (McLean *et al.*, 1951) cells were grown in 8 g l^{-1} BBL

Nutrient broth (Becton and Dickinson) at 4°C. Cells were harvested, freeze dried, and stored at -20°C.

Freeze dried cells of *S. acidocaldarius*, *M. barkeri,* and *T. maritima* were soxhlet extracted and fractionated essentially as described by Lo and Chang (1990). Lipids were finally suspended in chloroform:methanol:water (65:25:4, v:v:v), and stored at -20°C. *B. stearothermophilus* and *P. immobilis* lipids were isolated essentially as described by Ames (1968) with some modifications (Viitanen *et al.*, 1986), and purified by acetone-ether extraction (Kagawa and Racker, 1971). *Escherichia coli* phospholipid (*E. coli* L-a-phosphatidylethanolamine type IX, Sigma) were purified by acetone-ether extraction. Lipids were stored in chloroform at -20°C.

Preparation of liposomes. Lipids were dried by vacuum rotary evaporation, and hydrated in 50 mM MOPS, pH 7.0, 75 mM KCl, and 25 mM choline to a final concentration of 40 mg ml^{-1} Liposomes were obtained by 5 consecutive freezing and thawing steps, followed by extrusion through 200 nm polycarbonate filters (Avestin Ottawa, Canada) using the Liposofast^tm (Basic, Avestin) extrusion apparatus. These liposomes (LUVETS) are unilamellar with an average size that is close to the pore size of the filter used (Elferink *et al.*, 1994).

Measurement of proton and sodium ion permeability. Proton permeability was measured essentially as described by Nichols and Deamer (1980). The external buffer of the liposomes was replaced with 0.5 mM MOPS, pH 7.0, 75 mM KCl, and 75 mM sucrose (buffer A) by chromatography over a Sephadex G-25M PD-10 (Pharmacia, Uppsala, Sweden) column to obtain a low buffering capacity on the outside. Liposomes were diluted to 1.5 mg ml^{-1} in 2 ml buffer A. The K^+ ionophore valinomycin (1 nmol mg^{-1} lipid) was added to prevent the formation of a reversed transmembrane electrical potential ($\Delta\psi$). The fluorescent pH probe pyranine (10 mM) was added to the medium to monitor changes in the external pH. Excitation and emission wavelengths used were 450 and 508 nm, respectively. After equilibration, 100 nmol H^+ (from a 50 mM H_2SO_4 stock solution) was added to lower the external pH. Influx of H^+ into the liposomes was monitored by following the partial recovery of the external pH, as measured by an increase in pyranine fluorescence. Nigericin (1 nmol mg^{-1} lipid) was finally added to equilibrate the H^+ gradient across the membrane. Aliquots of 100 nmol OH^- and 50 nmol H^+ were used for calibration purposes (Elferink *et al.*, 1994). Fluorescence measurements were performed on a Perkin-Elmer LS-50B or an SLM Aminco 4800C fluorimeter, using a thermostated, magnetically stirred sample compartment. Fluorimeter data were fitted to the first order kinetic equation:

$$f(t) = a \times (1 - e^{-k_{H^+} \times t}) + c \qquad (1)$$

in which a is the amplitude of the fluorescence signal, k_{H+} is the first order rate constant of proton influx, and c the offset. The H^+ pulse was imposed at t=0, and k_{H+} was used to compare the proton permeability of the different liposomes.

The sodium ion permeability of the liposomes was analysed by the efflux of ^{22}Na. Liposomes were prepared in 50 mM MOPS-KOH, pH 7.0, 100 mM KCl, and 0.1 mM NaCl. To this suspension (550 - 650 ml final volume), $^{22}NaCl$ (approximately 60 H 10^6 cpm; specific activity > 2200 Ci mol^{-1}, Amersham, U.K)) was added to yield a final concentration of 1 mM. Liposomes were equilibrated with ^{22}Na by incubation for

18-46 h at room temperature, or 40°C for thermophiles. To initiate ^{22}Na efflux, 100 ml of the liposomal suspension was diluted in 10 ml of 50 mM MOPS-KOH, pH 7, 100 mM KCl, and 1 mM NaCl. Since the permeability of sodium ions is low as compared to protons (Speelmans *et al.*, 1993b; Gutknecht and Walter, 1981) , it was not necessary to include valinomycin to prevent the generation of a $\Delta\psi$. Samples of 1 ml were taken and filtered over a 0.2 mm BA83 (Schleicher & Schuell) nitrocellulose filter. Filters were rinsed with 2 ml 200 mM KCl, and the amount of label retained on the filter was counted with a liquid scintillation counter. To determine the amount of ^{22}Na bound to the filters, liposomes were permeabilized for sodium ions by the addition of 1 mM of Gramicidin D (in DMSO). Sodium efflux data were fitted to Eq. (1) as described for proton influx to yield the first order rate constant for the sodium ion permeability, k_{Na+}.

Other analytical techniques. The integrity of the liposomes was tested by their ability to maintain an imposed potassium diffusion gradient in the presence of valinomycin. $\Delta\psi$ (inside negative) was measured with the fluorescent probe 3,3'-diethylthiadicarbocyanine iodide [$DiSC_3(5)$] as described (Singh *et al.*, 1985).

Acknowledgements

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The membrane of *Pyrococcus furiosus* liposomes is as impermeable for protons as the membrane of *Sulfolobus acidocaldarius* liposomes

Introduction

For chapter 2, we studied the proton permeability of liposomes, derived from organisms that cover almost the whole range of temperatures for which life is found on Earth. Testing the proton permeability in the liposomes from an organism that lives near the boiling point of water would complete the overview of the proton permeabilities in Chapter 2. To accomplish this, we tested the proton permeability in liposomes, made from the hyperthermophilic archaeon *Pyrococcus furiosus* (Fiala and Stetter, 1986).

Cells of *P. furiosus*, grown at 100°C, were kindly provided by Prof. H. Märkl, Technische Universität Hamburg-Harburg, Germany. Lipids were isolated essentially as described by Lo and Chang (1990), and the proton permeability was tested as described in Chapter 2.

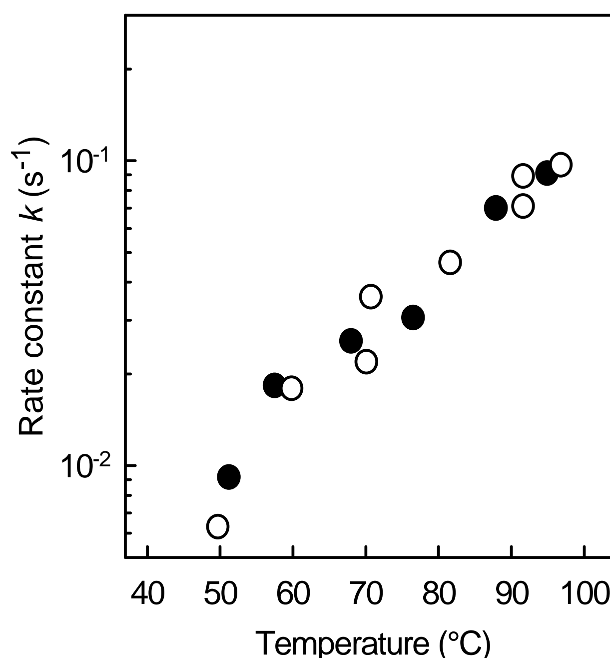

Fig. 1. Temperature dependency of the proton permeability of liposomes derived from *Sulfolobus acidocaldarius* (") and *Pyrococcus furiosus* (!). The first order rate constant for the proton permeability (k_{H+}) was determined as described in Chapter 2.

Results and Discussion

The proton permeability of the liposomes derived from *P. furiosus* was measured at different temperatures. As for the other liposome preparations, described in Chapter 2, the proton permeability of *P. furiosus* liposomes increased with increasing temperatures. The activation energy of the proton permeability is 49 kJ mol^{-1}, which is within the range of activation energies observed in the liposomes from the other organisms described in Chapter 2. The proton permeability of *P. furiosus* liposomes was comparable with the proton permeability of *Sulfolobus acidocaldarius* liposomes as observed in Chapter 2 (Fig 1). Therefore, compared with other organisms, the proton permeability of *P. furiosus* liposomes is extremely low.

The lipid composition of *P. furiosus* is not known. The close neigbours of *P. furiosus*, *P. horikoshii* and *P. abyssi*, contain mainly tetraether lipids and only a minor portion of diether lipids as in thermoacidophiles like *S. acidocaldarius* (Gonzalez *et al.*, 1998; Erauso *et al.*, 1993; Langworthy, 1977). Other hyperthermophilic archaea can contain significant amounts of diether lipids (Hafenbradl *et al.*, 1996; Kaneshiro and Clark, 1995). From the presence of diether lipids

in hyperthermophiles may be concluded that growth at high temperatures does not require a large percentage of tetraether lipids *per se*. However, even in these hyperthermophiles, the amount of diether lipids deminishes in favour of macrocyclic tetraether lipids under stress conditions as high pressure (Kaneshiro and Clark, 1995). Tetraether lipids are specially required in aerobic hot acidic environments and at high pressure because they probably can resist both conditions better than diether lipids.

The liposomes of *P. furiosus* are as impermeable as *S. acidocaldarius* tetraether liposomes, when measured at the same temperature. However, the anaerobic thermophile *P. furiosus* lives at a much higher temperature than the aerobic thermoacidophile *S. acidocaldarius*. If the membrane of *P. furiosus* would support proton coupled transport, the leak of protons at 100°C would be very high. Since anaerobes cannot compensate the high proton leak with an increased respiration as an aerobic thermophile like *Bacillus stearothermophilus* does (De Vrij *et al.*, 1988), *P. furiosus* might prefer sodium ions for energy transduction.

Acknowledgments

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Chapter 3

Homeostasis of the membrane proton permeability in *Bacillus subtilis* grown at different temperatures

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Summary

***Bacillus subtilis* was grown at its growth temperature limits and at various temperatures in between the lower and upper growth temperature boundary. Liposomes were made of the extracted membrane lipids derived from these cells. The headgroup composition of the cytoplasmic membrane lipids did not differ significantly at the lower (13°C) and upper (50°C) temperature boundary. The averaged lipid acyl chain length, degree of saturation, and ratio of iso and anteiso branched fatty acids increased with the temperature. At the temperature of growth, the membranes were in a liquid-crystalline phase, but liposomes derived from cells grown at 13°C were almost fourfold more viscous than those derived from 50°C grown cells. The temperature-dependence of the proton permeability of the liposomes was determined using the acid-pulse method with monitoring of the outside pH with the fluorescent probe pyranine. The proton permeability of each liposome preparation increased with the temperature. However, the proton permeability of the liposomes at the growth temperature of the cells from which the lipids were derived, was almost constant. These data indicate that the growth temperature dependent variation in lipid acyl chain composition permits maintenance of the proton permeability of the cytoplasmic membrane. This '*homeo- proton permeability adaptation*' precludes futile cycling of protons at higher growth temperatures and allows cells to sustain the proton motive force as a driving force for essential energy transducing processes.**

Introduction

All organisms have a specific temperature range in which they can grow. For most Bacteria the difference between lower and upper growth temperature is around 30°C (Russell and Fukunaga, 1990). They respond to changes in ambient temperature through the adaptation of the lipid composition of the cytoplasmic membrane (Gaughran, 1947). In *Escherichia coli*, this adaptation mechanism results in a constant membrane fluidity at different growth temperatures in a process that is termed 'homeoviscous adaptation' by Sinensky (1974).

Headgroup	Growth temperature	
	13°C	50°C
	% phospholipid phosphorous	
Phosphatidylglycerol and cardiolipin	54	53
Phosphatidylethanolamine	38	33
Lyso-phosphatidylethanolamine	n.d.	2.7
Phosphatidic acid	2.8	6.1
Unidentified	5	4.7

Table 1: Influence of the growth temperature on the phospholipid headgroup composition of *B. subtilis* membranes. n.d. not detectable. Each determination was performed at least twice. The maximum coefficient of variation was less than 4% for each fatty acid.

However, in *Bacillus subtilis* (Svobodová and Svoboda, 1988) and many other Bacteria (McElhaney and Souza, 1976; Reizer *et al.*, 1985; Prado *et al.*, 1990), the membrane fluidity at the growth temperature was found to increase linearly with the growth temperature, and thus does not remain constant within the growth temperature range. That urged Sinensky to modify the original homeoviscous adaptation concept into 'homeoviscous efficacy' (Cossins and Sinensky, 1984), describing the extent of homeoviscous adaptation. Growth temperature dependent changes of the lipid composition of the cytoplasmic membranes of Bacteria are mainly found in the fatty acyl chain composition of the membrane lipids, while the composition of the polar headgroups is less affected (Grau and De Mendoza, 1993; Shaw and Ingraham, 1965; Svobodová *et al.*, 1988). At higher growth temperatures, usually the number of unsaturated acyl chains declines, while the average length of the fatty acid chains, *i.e.* carbon number, increases.

Protons, and sodium ions, are the most commonly used coupling ions in energy transduction in Bacteria and Archaea. In a previous study we have investigated the influence of growth temperature on the proton permeability of liposomes prepared from membrane lipids derived from different organisms (Chapter 2). For mesophilic Bacteria and Archaea the first order rate constant of proton permeation, which is proportional to the diffusion constant, was maintained at a constant value at the respective growth temperature. These data suggest that the proton permeability of the membrane is an important parameter for viability.

These observations led us to propose the homeo- proton permeability adaptation theory that proposes that Bacteria adjust their membrane lipid composition in such a way that the proton permeability of the membrane is maintained at a low level at the growth temperature. To test this theory, the influence of the growth temperature was studied on the proton permeability of liposomes prepared from lipids of *B. subtilis* grown at and between the boundaries of its growth temperature range. The results demonstrate that the growth temperature dependent alterations in fatty acyl chain composition do not only maintain the membrane in a liquid-crystalline state. More importantly, the

Acyl chain	Growth temperature	
	13°C	50°C
	% of total	
13:0-iso	n.d.	0.5
14:0-anteiso	n.d.	1.2
15:0-iso	8.7	28.3
15:0-anteiso	50.3	21.6
16:1 ω7*c*	0.7	n.d.
16:0-iso	1.4	3.2
16:1 ω11*c*	0.6	n.d.
16:0	1	2.8
17:1 ω10*c*-iso	1.2	n.d.
17:1 ω10*c*-anteiso	7.1	n.d.
17:0-iso	2.7	29.9
17:0-anteiso	25.6	8.9
18:0	n.d.	0.6
19:0-iso	n.d.	2.2
19:0-anteiso	n.d.	0.8
unidentified	0.8	n.d.
Ratio iso:anteiso	0.17	2.0
Unsaturation, % of total	9.6	0
Averaged acyl chain/Carbon number	15.75	15.93

Table 2: Influence of the growth temperature on the fatty acyl chain composition of *B. subtilis* membranes. The main changes upon increase in temperature are the transitions from anteiso-branched to the corresponding iso-branched lipids. n.d. not detectable. Each determination was performed at least twice. The maximum coefficient of variation was less than 4% for each fatty acid..

alterations permit the cells to maintain a low proton permeability of the membrane within a wide range of growth temperatures.

Results

Phospholipid headgroup and acyl chain composition. *B. subtilis* was grown at various temperatures between its lower and upper growth temperature boundaries, 13 and 50°C. The doubling time during exponential growth was 18 h at 13°C, 6 h at 16°C and less than 1 h at the other temperatures. Phospholipids were isolated from cells grown up to the late exponential phase. The lipids from the cells, grown at the highest and lowest temperature, were used for further

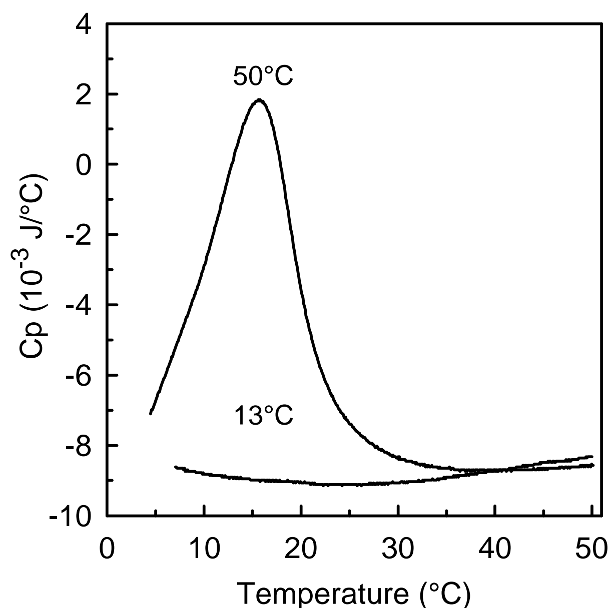

Fig. 1. DSC thermogram of liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C (a) and 50°C (b). The scan rate was 0.5 and 1°C/min. Liposomes were used at 6 mg/ml in 100 mM potassium phosphate, pH 7.0. The reproducibility of the thermograms and reversibility of the transitions were checked after each run by re-heating the sample after cooling.

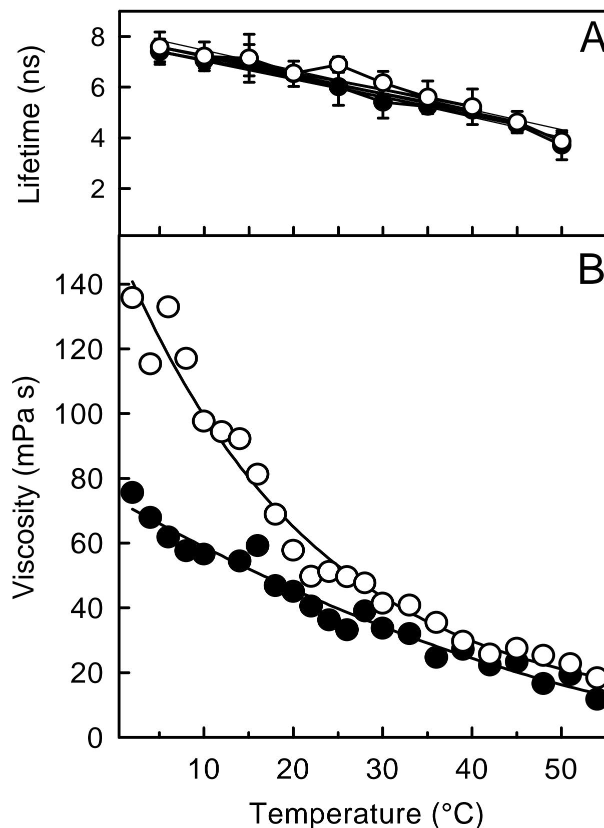

Fig. 2. Temperature dependency of the TMA-DPH fluorescence lifetime (**A**) and apparent membrane microviscosity (**B**) for liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C (**!**) and 50°C ("). Membrane microviscosity was calculated from the TMA-DPH fluorescence anisotropy and lifetime as described under Materials and Methods.

analysis. The phospholipids of cells grown at these two temperatures had similar headgroups (Table 1). Most of the lipids were cardiolipin (CL), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE). The amount of phosphatidic acid (6.4%) at 50°C was about twice as high as at 13°C (4 %). At 50°C, an increased content of *lyso*-phosphatidylethanolamine was measured, likely the result of some phospholipase activity during extraction. The growth temperature had a dramatic effect on the acyl chain composition of the phospholipids (Table 2). At 50°C, unsaturated acyl chains were completely absent. However, essentially all acyl chains were branched with twice as many iso-branched as anteiso-branched chains. The average fatty acyl chain length of the lipids of 50°C grown cells was slightly longer than in the 13°C grown cells. Cells grown at 13°C contained 9.6% unsaturated lipids, and had a 12-fold higher content of anteiso- than iso-branched lipids.

Phase transition and anisotropic behaviour of B. subtilis lipids. The phase transition behaviour of the lipids derived from cells grown at 13 and 50°C was measured by differential scanning calorimetry (DSC) in liposomes in aqueous suspension. Lipids derived from cells grown at 50°C showed a discrete phase transition at 16°C (±0.5, n = 3). The lipids derived from 13°C grown cells showed no phase transition between 5 and 50°C. The phase transition of these lipids most likely occurs at a temperature below 5°C (Fig. 1). The viscosity of the membranes was determined

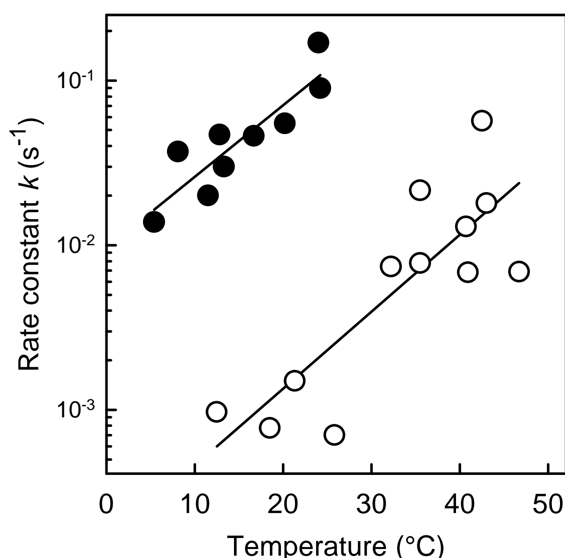

Fig. 3. Temperature dependency of the proton permeability of liposomes prepared from lipids derived from *B. subtilis* cells grown at 13°C (!) and 50°C ("). The influx of protons, as measured with the fluorescent probe pyranine, was fitted to a first order kinetic equation as described under the Materials and Methods.

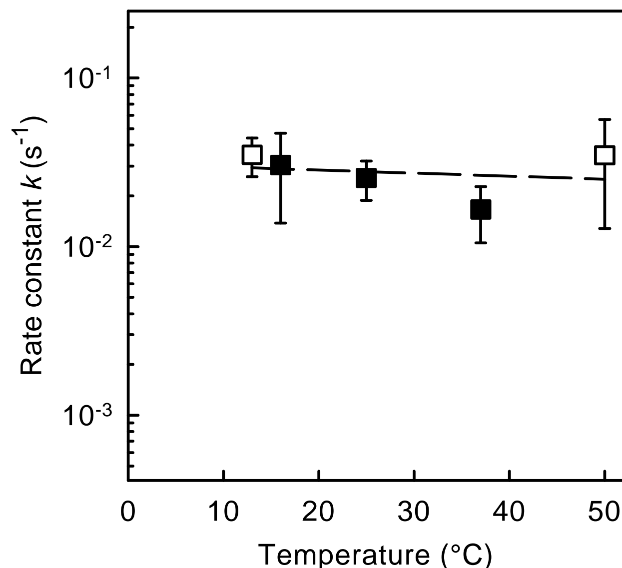

Fig. 4. Proton permeability at the growth temperature of liposomes prepared from lipids derived from *B. subtilis* cells grown at temperatures between the lower and upper temperature limit (■ ± standard deviation, n ≥ 3). Inter- and extrapolation of the exponential fit on growth temperature curves at 13 and 50 °C, shown in Fig. 3. (□ ± 95% confidence intervals). The influx of protons, as measured with the fluorescent probe pyranine, was fitted to a first order kinetic equation as described under the Materials and Methods.

by TMA-DPH fluorescence anisotropy (*r*) and lifetime (τ_ϕ) measurements (Fig. 2). The temperature dependency of τ_ϕ for the TMA-DPH fluorescence with both the liposomes prepared from lipids derived from 13 and 50°C grown cells is shown in Fig. 2A. The longest lifetime component, which accounts for more than 85% of the fluorescent signal (Heřman *et al.*, 1994), decreased from about 7.7 ns at 5°C to 3.9 ns at 50°C. By combining the anisotropy data (not shown) with the lifetimes, the apparent viscosity, η, of the membranes as a function of the temperature was calculated (Fig. 2B). At the respective growth temperatures, η is about 55 and 15 mPa s for cells grown at 13 and 50°C, respectively. The first derivative of anisotropy data of 50°C grown cells indicated a phase transition at 15°C, close to the value determined by DSC. Liposomes prepared from the lipids of 13°C grown cells showed no distinct transition.

Proton permeability of the liposomes. The temperature dependence of the proton permeability of the liposomes derived from 13 and 50°C was determined by the acid-pulse method. From the proton influx data (n ≥ 8), the first order rate constants for proton permeability, *k*, were calculated and plotted *versus* the temperature (Fig. 3). The activation energy, derived from the slope of the log fit of *k* to the temperature, was comparable for these liposome preparations, yielding an averaged value of 78 kJ/mol. These data demonstrate that the increase in proton permeability with temperature is similar for the two liposome preparations tested. However, at the higher growth temperature, the line shifted to a higher temperature range suggesting the maintenance of the proton permeability at the respective growth temperature.

To analyse this phenomenon in further detail, the proton permeability was determined of liposomes made of lipids derived from cells grown at different temperatures between 13 and 50°C. Permeability measurements (n ≥ 3) were performed only at the growth temperature of the cells from which the lipids were derived. Above 40°C, reliable measurements of the proton permeability were not possible, due to the low amplitude of the measured signal. To overcome this problem, we determined the rate constant of the exponential fits in Fig. 3 at the growth temperature. As shown in Fig. 4, the proton permeability of the different liposomes was essentially constant at the temperature that corresponds with the growth temperature of the cells from which the lipids were extracted. These data indicate that *B. subtilis* cells maintain a constant proton permeability of their membrane at various growth temperatures.

Discussion

The Bacterial membrane plays a crucial role in energy transduction of the cells. One of its essential functions is to preserve the barrier function to protons (or sodium ions) in order to keep the proton motive force (or sodium motive force) at a high value (Mitchell, 1961). Bacteria therefore must be able to control the permeability of the membrane for these energy transducing ions. So far, the proton permeability of biological membranes has hardly been studied in relation to the growth conditions or physiology of the organism.

We have previously shown that the proton permeabilities -at the growth temperature- of the membranes from different psychrophilic or mesophilic Bacteria and mesophilic or thermophilic Archaea are comparable, even when the growth temperatures of the organisms studied are ranging from 4 to 85°C (Elferink *et al.*, 1994; Chapter 2). The range of growth temperatures in that study was even wider than in the present study. The previous study suggested that Bacteria and Archaea possess mechanisms to adjust the membrane lipid composition at different growth temperatures in such a way that the proton permeability of their membranes remains constant. To test this 'homeo- proton permeability' theory we have studied the proton permeability of liposomes prepared from lipids of cytoplasmic membranes of *B. subtilis* grown at temperatures between 13 and 50°C. The result of this study indeed shows that the temperature-dependent changes of the lipid composition in *B. subtilis* result in a membrane with a nearly constant proton-permeability, irrespective of the growth temperature.

If Bacteria would not be able to adapt the proton permeability of their membrane, the temperature range of growth of Bacteria would be narrow. Without an adaptive mechanism, the proton permeability of the membrane would increase rapidly as in Fig. 3 (Russell and Fukunaga, 1990), and cells would not be able to maintain a proton motive force. Our current study shows that a Bacterial cell is able to maintain the proton permeability of the cytoplasmic membrane at a low value within a range of growth temperatures. The change in lipid composition with increasing growth temperature clearly counteracts the temperature effect on the proton permeability.

The permeability of the membrane depends on physico-chemical characteristics such as the lipid packing. In this respect, Lande *et al.* (1995) observed that the permeability of small solutes in synthetic liposomal membranes correlates exponentially with the membrane fluidity, while an apparent relationship between fluidity and permeation of protons was not observed. Possible

mechanisms for proton permeation across the membrane are summarized by Marrink *et al.* (1996), who studied the molecular dynamics of the formation of water chains across the membrane. Those studies indicate that proton permeation may occur *via* a transient water wire through the membrane.

The growth temperature results in major changes in acyl chain composition of the membrane lipids of *B. subtilis* (Prado *et al.*, 1988; Reizer *et al.*, 1985; Suutari and Laakso, 1992; Svobodová *et al.*, 1988; Clejan *et al.*, 1986; Minnikin and Abdolrahimzadeh, 1974). The most significant effect is a drastic decrease in the *anteiso* fatty acids and an increase in the *iso*-branched fatty acids with increasing growth temperature (Table 2). These variations in the lipid composition allow the cytoplasmic membrane to remain in a liquid-crystalline state at different growth temperatures (Haest *et al.*, 1974; McElhaney and Souza, 1976; Lepock *et al.*, 1990). The phase transition temperature is kept below the growth temperature due to the presence of branched lipids that distort membrane order. *Anteiso*-branches lower the transition temperature better than *iso*-branches, though neither are as efficient as the presence of a *cis* double bond in the centre of the hydrocarbon chain (Suutari and Laakso, 1992). The efficient *cis* double bonds are observed in the cells grown at 13°C.

It has been proposed that Bacteria need to maintain a constant viscosity of their cytoplasmic membrane at different growth temperatures. This 'homeoviscous adaptation' (Sinensky, 1974; Morein *et al.*, 1996; Esser and Souza, 1974) has been postulated as the maintenance of the membrane fluidity to allow membrane proteins to function optimally. In *B. subtilis* and other organisms, homeoviscous adaptation is not as strict as in *E. coli* (see introduction and Fig. 2).

Shaw and Ingraham (1965) found that the minimum growth temperature of *E. coli* is not governed by the rigidity of lipids in the membrane. When cells are shifted from 37 to 10°C, a major lag phase occurs before the cells start to grow. Starvation during this lag period did not affect the duration of the lag phase, and no changes in fatty acyl chain composition were observed. However, when the cells were supplemented with glucose, just after the lag phase, growth immediately started at 10°C while the fatty acyl chain composition was still typical for cells grown at 37°C. The reversed experiment has not been done. Taken together with the observation that the fluidity is not so strictly maintained in *B. subtilis*, we may conclude that maintenance of the proton permeability characteristics of the membrane is even more important for viability of the cells than homeostasis of the viscosity.

It has been suggested that the changes in lipid composition are needed to maintain an optimal lateral pressure in the membranes at different temperatures (Herman *et al.*, 1994). This pressure is caused by phospholipids as PE, which can form non-bilayer configurations, and depends on the type of acyl chain as the wedged shape of PE decreases with the degree of saturation of the acyl chains. In the thermophilic bacterium *B. caldotenax*, lowering of the growth temperature results in a decreased PE content, which is compensated by an increased amount of unsaturated acyl chains (Hasegawa *et al.*, 1980). However, the drop in PE content is far less dramatic than the change in acyl chain composition. In the present study and in *E. coli* (Morein *et al.*, 1996), no change in PE content is observed as a function of the growth temperature. This suggests, that for adaptation of the membrane to higher temperatures the acyl chain composition is more important than the headgroup composition.

The ability of the cell to control the permeability characteristics of the membrane may be important in determining the upper temperature limit of growth. To some extent, an increased proton permeability of the membrane at higher temperatures could be compensated by an elevated rate of proton extrusion (De Vrij *et al.*, 1988). However, without any adaptation of the membrane lipid composition upon higher temperatures, the proton permeability will become too high upon small increases of the temperature and maintenance of a viable proton motive force and homeostasis of the intracellular pH will not be possible. This study therefore adds a new dimension to the role of the growth dependent alterations in the lipid composition of cytoplasmic membranes. For future research, it will be important to elucidate the regulatory mechanisms responsible for this homeo-proton permeability adaptation of the cytoplasmic membrane.

Materials and methods

Strains and Growth Conditions. *Bacillus subtilis* DB104 (Δ*apr-684,* Δ*nprE522*) (Yang *et al.*, 1984) was grown on Luria Broth at several temperatures between 13 and 50°C, with constant high aeration. Pre-cultures were grown at 25°C. At late logarithmic phase, *i.e.* $OD_{660\,nm}$ 1.7 to 2.5, protein synthesis was blocked by the addition of 5 μg/ml chloroamphenicol, and cells were harvested by centrifugation. Cells were resuspended in 50 mM potassium phosphate pH 7, frozen as small nuggets in liquid nitrogen and stored at -80°C. Cells that were grown at 13°C were still able to grow at 50°C, and *vice versa*, indicating that growth was not due to mutation.

Lipid Extraction and Analysis. Crude lipids were chloroform/methanol extracted (Bligh and Dyer, 1959), and acetone-ether-washed (Kagawa and Racker, 1971). For the 13 and 16°C grown cells, the ether wash was conducted at 4°C. Lipids were stored in chloroform at - 20°C under a N_2 atmosphere.

The acyl chains were analysed as methyl esters by gas-liquid chromatography (Prado *et al.,* 1988). The lipid headgroup composition was determined by two-dimensional thin-layer chromatography (Broekhuyse, 1969). Chromatograms were stained with I_2 vapour, and spots were collected and analysed for inorganic phosphorous (Rouser *et al.*, 1970). Data were derived from two independent batches.

Preparation of Liposomes. Unilamellar liposomes with an averaged diameter of 200 nm were prepared by extrusion (Elferink *et al.*, 1994; Chapter 2) in 50 mM MOPS, pH 7.0, 75 mM KCl, and 25 mM choline-Cl to a final concentration of 20 mg/ml.

Time-Resolved and Anisotropy Fluorescence Spectroscopy. Lifetime and anisotropy measurements were performed with an SLM-Aminco 4800C fluorimeter (SLM-Aminco, Urbana, USA) using the fluorescent probe 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) (Prendergast *et al.,* 1981). Excitation and emission were at 360 and 430 nm, respectively. The temperature dependence of the fluorescence polarization was determined using Glan-Thompson polarizers in the excitation and emission channels. Anisotropy values, r, were derived from the polarization as described (Lakowicz, 1983).

The excited-state lifetime, τ, of TMA-DPH was determined by the phase delay technique at 18 MHz with excitation and emission bandwidth of 1 and 4 nm, respectively. The phase lifetime, τ_{ϕ}, was referenced against a glycogen scatter

reference ($\tau = 0$ ns). The relation between τ_ϕ and the temperature was fitted by iteration to the best fitting linear function (TableCurve, Jandel Scientific, San Rafael, USA). For each temperature, the rotational correlation time, φ, was calculated from the anisotropy (r) and lifetime (τ_ϕ) according to eq. 1:

$$\varphi = \frac{\tau_\phi}{(r_o/r) - 1} \qquad (1)$$

in which the zero-time anisotropy r_o was fixed to the value 0.378 (Herman *et al.*, 1994). From the rotational correlation time, the viscosity, η, was calculated according to eq. 2:

$$\eta = \frac{RT\varphi}{V} \qquad (2)$$

in which R is the gas constant, T the absolute temperature (in K), and V the total enclosed volume of one mole of TMA-DPH. A value of 959 $Å^3$ was calculated form the solvent accessible surface of TMA-DPH using the QUANTA96 software package (Molecular Simulations Inc., San Diego, USA) after energy minimalization with the CHARMm module.

Differential scanning calorimetry (DSC). The phase transition in the lipids of *B. subtilis*, grown at 13 and 50°C, was measured by differential scanning calorimetry (DSC) using the Microcal MC-2 calorimeter (Microcal Lts., Amherst, USA). Data were analysed using the ORIGIN software (Microcal Software Inc.), which involved fitting and subtraction of the instrumental base line data as described (Blandamer *et al.*, 1994).

Proton Permeability. The proton permeability of the liposomes was measured as described (Nichols and Deamer, 1980; Elferink *et al.*, 1994; Chapter 2). In short, liposomes (1.5 mg of lipid/ml) were transferred to a buffer containing 0.5 mM MOPS, pH 7.0, 75 mM KCl, 75 mM sucrose, and 10 µM of the fluorescent pH probe pyranine. The K^+ ionophore valinomycin (1 nmol/mg of lipid) was added to prevent the formation of a reversed $\Delta\psi$, and after equilibration and stabilization of the signal, 100 nmol H^+ (from a 50 mM H_2SO_4 stock solution) was added to the suspension to lower the external pH. Influx of protons into the liposomes was followed in time from the partial recovery of the external pH monitored by an increase in pyranine fluorescence (Excitation and emission wavelengths of 450 and 508 nm, respectively). After calibration of the signal in the presence of nigericine, the data were fitted to the first order kinetic equation as described (Elferink *et al.*, 1994). The first order rate constant of proton influx, k, was used for. comparison of the proton permeability of the different liposomes. Fluorescence measurements were performed on a Perkin-Elmer LS-50B (Norwalk, USA) fluorimeter, using a thermostated, magnetically stirred sample compartment.

Acknowledgements

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Chapter 4

Lipid membranes from halophilic and alkali-halophilic Archaea have a low H^+ and Na^+ permeability at high salt concentration

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Summary

We have studied the influence of pH and the salt concentration on the proton and sodium ion permeability of liposomes that were formed from lipids of the halophile *Halobacterium salinarum* and the haloalkaliphile *Halorubrum vacuolatum.* In contrast with liposomes formed from *Escherichia coli* lipids, the liposomes formed from halophilic lipids remained stable up to 4 M of NaCl and KCl. The proton permeability of the liposomes from lipids of halophiles was independent of the salt concentration and was essentially constant between pH 7 and 9. The sodium ion permeability increased with the salt concentration but was 10 to 100-fold lower than the proton permeability. It is concluded that the membranes of halophiles are stable over a wide range of salt concentrations and at elevated pH values and are well adapted to the halophilic conditions.

Introduction

Halophiles live at higher salt concentrations than found in sea water (*i.e.,* above 0.5 M). Extreme halophiles, which thrive optimally above 3 M, are mainly, but not exclusively, aerobic Archaea. Moderate halophiles are mostly Bacteria, but some are methanogens (Archaea) or algae (Eucarya). At extracellular osmolarities exceeding that of the cytoplasm, microorganisms accumulate potassium ions and/or low molecular weight organic solutes (osmolytes). These compounds enable the cell to reduce the water loss, to maintain cell turgor pressure by reducing the osmotic potential between the cell and the environment, and to protect enzymes from the low water activity (Yancey *et al.*, 1982). Polyhydric alcohols (such as sugars and glycerol), free amino acids and their derivatives, and urea associated with methylamines are the main types of osmolytes found in all water-stressed organisms except for non-methanogenic halophilic Archaea. The latter organisms mainly accumulate potassium ions, which are less toxic than sodium ions (Lai *et al.*, 1991). Although the outside potassium ion concentration may be only few millimolar, potassium ions may accumulate

to levels up to five times the internal sodium ion concentration (Brown, 1983).

Salt tolerance involves a specific adaptation of the proteins. This often concerns an enrichment in aspartate, glutamate and weakly hydrophobic residues. Many enzymes of non-methanogenic halophilic Archaea require a salt concentration of 1 M or higher for optimum activity (Yancey *et al.*, 1982). These organisms are therefore obligate halophilic. In other halophiles, proteins are not adapted to a high salt concentration, but are stabilized by organic osmolytes.

Alkaliphiles are organisms that thrive at high pH. Most cytoplasmic enzymes of alkaliphiles function optimally near neutral pH (Booth, 1985), and these organisms need to maintain the intracellular pH at or around neutrality. Alkaliphilic organisms use the electrochemical gradient of H^+ or Na^+ for energy transduction. The electrochemical gradient of H^+ exerts a proton motive force (pmf), which consists of two components, the transmembrane pH gradient (ΔpH), and the transmembrane electrical potential ($\Delta\psi$). In alkaliphiles, the pH outside is alkaline *versus* inside acid. This reversed ΔpH is compensated by a large $\Delta\psi$, inside negative, resulting in a net pmf that is directed inwards (Van de Vossenberg *et al.*, 1998a; Chapter 1).

Aerobic alkaliphiles use a Na^+/H^+-antiporter in combination with H^+-coupled respiration to regulate the intracellular pH (Krulwich, 1995; Speelmans *et al.*, 1995). Both marine and non-marine alkaliphiles require Na^+ in the growth medium (Krulwich, 1995). For most halo-alkaliphiles the outside concentration of sodium is high, and the external H^+ concentration low. In these organisms, an electrogenic Na^+/H^+ (Na^+/H^+ ratio < 1) antiport reaction drives both the necessary extrusion of sodium ions and the uptake of protons. Such a mechanism would be frustrated by an unidirectional flux of either Na^+ inward, or H^+ outward. Therefore, the membrane of halo-alkaliphiles must be highly impermeable for both protons and sodium ions.

Previous studies on the proton permeability in Archaeal and Bacterial membranes have demonstrated that most Bacteria and Archaea adjust the permeability of the cytoplasmic membranes to the growth temperature of the organism (Elferink *et al.*, 1994; Chapter 2). The proton permeability is maintained at a constant level by regulation of the phospholipid composition of the cell membrane as a function of the growth temperature (Chapter 3). The question arises how the salt concentration affects the permeability properties of the membrane of halophiles.

In this study we have analyzed the proton and sodium ion permeability of liposomes prepared from lipids extracted from two aerobic extremely halophilic Archaea: *Halobacterium salinarum* and the halo-alkaliphile *Halorubrum vacuolatum* (renamed from *Natronobacterium vacuolatum* (Mwatha and Grant, 1993)) and compared with liposomes from *Escherichia coli* lipids. *Hr. vacuolatum* grows optimally at pH 9.5. The salt- and pH-dependency of the ion permeability shows that the membrane of these organisms is very impermeant to H^+ and Na^+, even at high salt concentrations and pH values. These properties of the cytoplasmic membrane permit growth at these extreme environmental conditions.

Results

Liposome integrity and stability. Lipids were extracted from the halophilic Archaeon *Halobacterium salinarum* and the halo-alkaliphilic Archaeon *Halorubrum vacuolatum*. *Escherichia coli* Bacterial lipids were used for comparison. All species were grown around 35°C, both Archaea at high salt concentration, *Hr.*

Kingdom	Organism	Growth conditions		
		Temperature (°C)	pH	NaCl (M)
Archaea	*Halobacterium salinarum*	35	7	4
	Halorubrum vacuolatum	35	9.5	4
Bacteria	*Escherichia coli*	37	7	0.05

Table 1. Growth conditions of the organisms used in this study.

vacuolatum at high pH and both neutrophils (*E. coli* and *Hb. salinarum*) at pH 7 (Table 1). Liposomes were prepared at various salt concentrations. They were made from extracted lipids by hydration followed by freeze-thawing and extrusion through 200 nm polycarbonate filters. The size distribution of the liposomes was determined by photon correlation spectroscopy. At low salt concentration, the mean diameter of all liposome preparations was found to be 200 ± 70 nm (Fig. 1). At 0.5 M NaCl, the mean size of the *E. coli* liposomes was dramatically increased to about 550 nm with a large size distribution. At even higher NaCl concentration, the *E. coli* liposomes aggregated further and started to float. On the other hand, the size of the liposomes prepared from the lipids from *Hr. vacuolatum* and *Hb. salinarum* increased from 200 to 300 nm from 0 to 4 M NaCl (Fig. 1). The size of all liposome preparations (including *E. coli*) was not affected by the pH applied during liposome preparation between pH 7 and 9 (data not shown). The liposomes prepared from *Hr. vacuolatum* and *Hb. salinarum* lipids were stable over a wide range of NaCl concentrations (*i.e.,* up to 4 M) and retained the ability to maintain a $\Delta\psi$, applied by a valinomycin-induced potassium diffusion gradient, even after one week incubation at 20°C.

Salt dependency of the proton permeability. The proton permeability of liposomes, derived from *Hr. vacuolatum* and *Hb. salinarum*, was tested at 38°C at various NaCl or KCl concentrations with the acid-pulse method, using the fluorescent dye pyranine to monitor changes in extracellular pH. At low sodium ion concentration, *i.e.*, below 0.5 M NaCl, the proton permeability of liposomes from *Hb. salinarum* lipids was equal to that of liposomes from *E. coli* lipids (Fig. 2), while liposomes from *Hr. vacuolatum* lipids were about twice as permeable to protons. The proton permeability of liposomes from *Hr. vacuolatum* and *Hb. salinarum* lipids decreased about two-fold at higher NaCl concentration (Fig. 2). Similar observations were made when NaCl was replaced with KCl (Fig. 3). Due to the aggregation of the liposomes from *E. coli* lipids, reliable measurements of the proton permeability at NaCl concentrations above 0.5 M was impossible. The data show that the proton permeability of the liposomes prepared from lipids extracted from the halophilic Archaea is only to a minor extent affected by the salt concentration.

pH dependency of proton permeability. *Hr. vacuolatum* is a haloalkaliphile that grows optimally between pH 8.5 and 10.5, while *Hb. salinarum* grows below pH 8.5. The proton

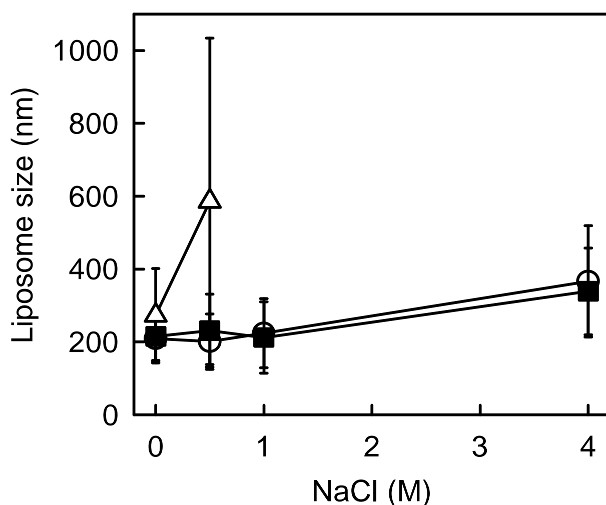

Fig. 1. Influence of the NaCl concentration on the size of the liposomes prepared from *E. coli* (△), *Hr. vacuolatum* (") and *Hb. salinarum* (#) lipids

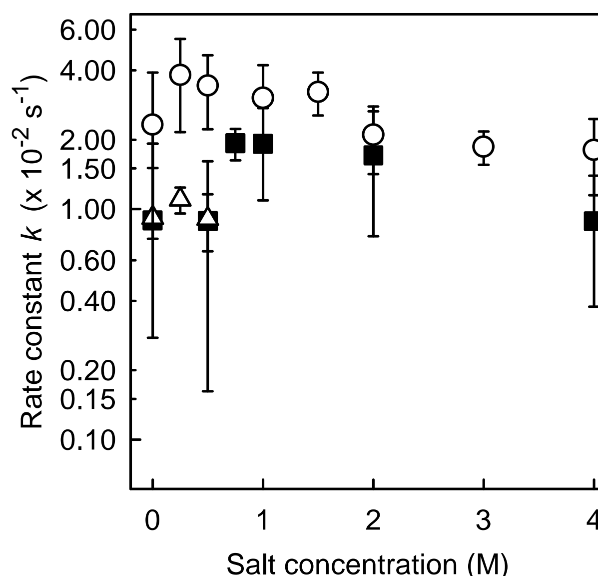

Fig. 2. Influence of the NaCl concentration on the proton permeability of liposomes prepared from *E. coli* (△), *Hr. vacuolatum* (") and *Hb. salinarum* (#) lipids

permeabilities of the liposomes, prepared from lipids of these organisms and of *E. coli*, were measured as a function of pH at a NaCl concentration of 0.1 M. The proton permeabilities of all liposomes between pH 7 and 9 varied only marginally with pH (Fig. 4). The permeabilities of both Archaeal liposome preparations were somewhat higher than in liposomes of *E. coli* lipids.

Salt dependency of sodium ion permeability. The sodium ion permeability of liposomes was measured from the efflux of ^{22}Na, preloaded into the liposomal lumen. For the liposomes from *Hb. salinarum* lipids, and to a lesser extent for liposomes from *Hr. vacuolatum* lipids, the first order rate constant for the sodium ion permeability slightly increased with increasing NaCl concentrations from 0.1 to 4 M (Fig. 5). The Na^+ permeability is about two orders of magnitude lower than the proton permeability at all salt concentrations. The instability of the liposomes from *E. coli* lipids in a highly salted medium prevented the study of these liposomes at high NaCl concentrations. At low NaCl concentration, the permeability of liposomes from *E. coli* lipids was comparable with both liposome preparations derived from both Halobacterial lipids.

Discussion

The results reported here show that stable liposomes can be prepared from the lipids derived from halophilic Archaea. Liposomes of lipids from *E. coli* do not remain intact at high salt concentrations due to extensive aggregation. Liposomes prepared from lipids derived from the halophile and haloalkaliphile behave similarly on increasing salt concentration. At increasing salt concentration the size of these liposomes increases slightly while the proton permeability remains constant. A moderate increase in the first order rate constant for sodium ion permeation was observed with increasing salt concentration. The proton permeability of the halo-alkaliphilic liposomes from *Hr. vacuolatum* lipids was not affected by the pH of the medium, and was within

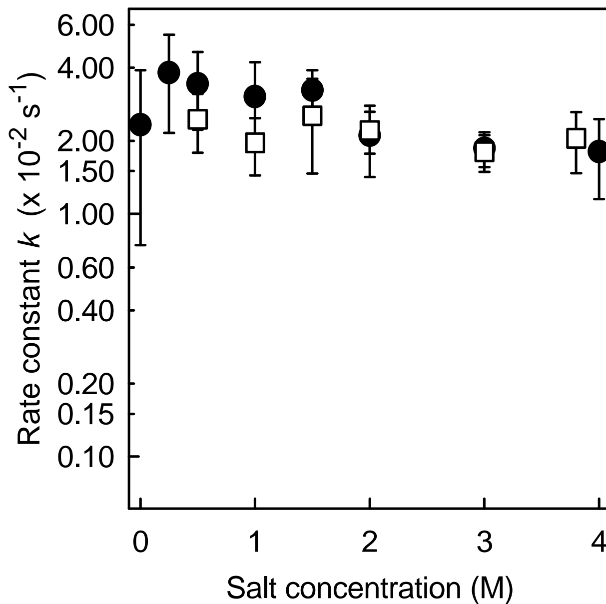

Fig. 3. Effect of the NaCl (!) and KCl (Q) concentration on the proton permeability of liposomes prepared from *Hr. vacuolatum* lipids

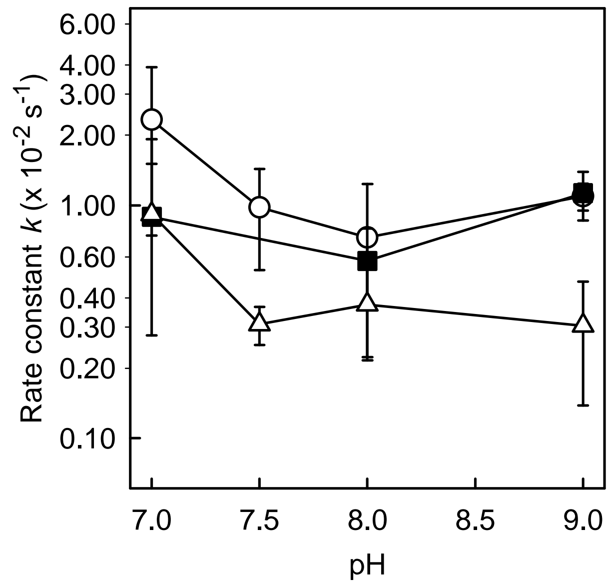

Fig. 4. pH dependency of the proton permeability of liposomes prepared from *E. coli* (△), *Hr. vacuolatum* (") and *Hb. salinarum* (#) lipids

the same range as previously observed for other organisms grown around 35°C (Elferink *et al.*, 1994; Chapter 2). It should be stressed that previous studies have shown that the proton permeability is strongly affected by the temperature (Chapter 2).

The lipid composition of membranes from only a few halo(alkali)philic Archaea has been analyzed (Upasani *et al.*, 1994; Kates, 1996). The lipid species present in the phylogenetically closely related *Hr. vacuolatum* and *Hb. salinarum* membranes are very similar (Mwatha and Grant, 1993). Archaea from the genera *Natronobacterium*/*Halorubrum* and *Halobacterium* contain 2,3-diphytanyl-*sn*-glycerol-1-phospho-3'-*sn*-glycerol-1'-methylphosphate (PGP-Me) as the main phospholipid (Kates *et al.*, 1993). *Halobacteria* have a high density of negative charges on the surface of their membranes (Russell, 1989). The advantage would be that the negative charges on the polar headgroups are shielded by the high ionic concentration, preventing disruption of the lipid bilayers due to charge-repulsive forces and providing a charge-stabilized lipid bilayer (Kates, 1993).

Halophilic lipids are known to resist high pH values (Kates, 1995). It is, therefore, not surprising that liposomes from *Hb. salinarum* and *Hr. vacuolatum* lipids behave similarly under the conditions studied. In contrast to moderate halophiles, which are mostly Bacteria, the extreme halophiles do not adapt their membrane lipid composition to fluctuations in salt concentrations. Moderate halophiles increase the amount of negatively charged lipids upon increasing the salt concentration (Russell, 1989).

Proteins in halophilic organisms are also halo-adapted. The proteins need to attract water molecules from the salty environment at their surface. Therefore, the bulk protein in halophiles is acidic in nature. The excess of glutamate and aspartate is as high as 20%. The proteins also have a lower content of hydrophobic amino acids (Lanyi, 1974).

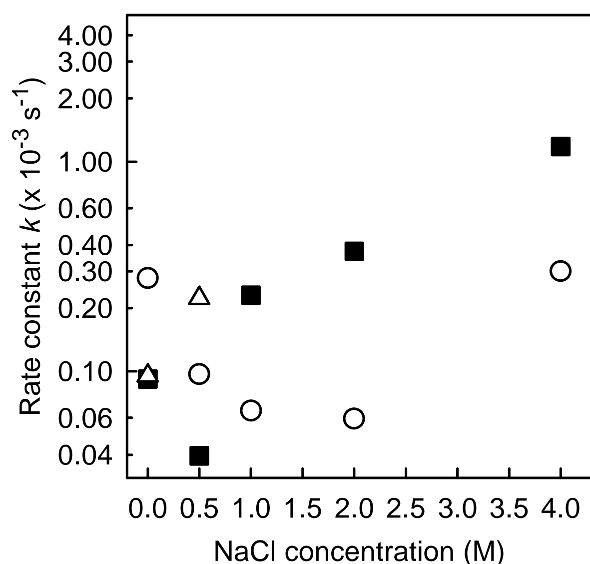

Fig. 5. Influence of the NaCl concentration on the sodium ion permeability of liposomes prepared from *E. coli* (△), *Hr. vacuolatum* (") and *Hb. salinarum* (#) lipids

Yamauchi *et al.* (1992) compared *Halobacteria*-like (Archaea-like) lipids with the typical Bacterial phospholipids. They only studied lipids that differed in the acyl chains, using 1,2-diphytanyl-*sn*-glycero-3-phosphocholine as the Archaeal model and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine as the Bacterial counterpart. The phytanyl (Archaea-like) lipids were found to be stable and could form a concentrated suspension of liposomes at a wide range of salt concentrations. In contrast, the palmitoyl (Bacteria-like) lipids could only form liposomes at the higher salt concentrations when prepared at very low lipid concentrations. Since measurements of the proton and sodium ion permeability can only be performed with dense solutions of liposomes, analysis of the permeability characteristics of the liposomes from *E. coli* lipids at high salt concentrations was not possible. The phytanyl lipids were found to be less permeable for carboxyfluorescein than the palmitoyl lipids (Yamauchi *et al.*, 1992). The exceptional properties of the Halobacterial lipids therefore appear to be due to the phytanyl chain of the lipids. The high stability of the halo(alkali)phile phytanyl chain may be due to the limited segmentary motion of tertiary carbon atoms (*i.e.,* rotation of carbon atoms that are bound to three other C-atoms) (Degani *et al.*, 1980). Hindering of the segmentary motion in the lamellar phase prevents kink formation in the Archaeal phytanyl chains. This restriction in acyl chain mobility may enhance the stability of the halophilic lipids at the high salt concentration and keep the permeability low.

Our data suggest that the membranes of halophiles and halo-alkaliphiles are mainly adjusted to the high salt concentration and to a lesser extent to pH. Irrespective of the nature of the lipids, *i.e.,* halophile versus haloalkaliphile, pH does not interfere with the membrane integrity, nor does it dramatically affect the proton permeability. In contrast, liposomes of membrane lipids of the acidophilic Archaeon *Picrophilus oshimae*, that thrives at pH 1 and 60°C, are unstable at pH values above 5 and collapse at the higher pH values (Chapter 5). Also the proton permeability of liposomes prepared from *Sulfolobus acidocaldarius* lipids was found to increase with the pH increasing from 4 to 7.

At moderate salt concentration, the Na^+ conductance of the liposomes from Archaeal lipids is similar to that found previously for Bacterial liposomes (Chapter 2). The sodium ion permeability is barely influenced by the salt concentration. Therefore, the use of sodium ions as coupling ions does not seem to be more advantageous for halophiles than for non-halophiles under the same conditions. However, since halophiles face a large Na^+ gradient, sodium ion coupled transport can be useful.

It can be concluded that neither the sodium ion permeability, nor the proton permeability characteristics of halophilic Archaea are significantly different from organisms that live at

the same temperature, albeit at low salt concentrations. The main adaptation of halo(alkali)philic membranes is their stability at high salt concentrations.

Materials and Methods

Strains and lipids. Cells of *Halorubrum vacuolatum* were grown in 10 g l^{-1} yeast extract (Difco), 7.5 g l^{-1} casamino acids, 3 g l^{-1} Na_3-citrate.$2H_2O$, 2 g l^{-1} KCl, 1 g l^{-1} $MgSO_4.7H_2O$, 0.36 mg l^{-1} $MnCl_2.4H_2O$, 50 mg l^{-1} $FeSO_4.7H_2O$, 200 g l^{-1} NaCl and 18.5 g l^{-1} Na_2CO_3 (anhydrous), pH 10-10.5. Cells of *Halobacterium salinarum* S9 were kindly provided by Dr. H. Bolhuis, Max Planck Institut für Biochemie, Martinsried, Germany. Cells were grown in 250 g l^{-1} NaCl, 20 g l^{-1} $MgSO_4.7H_2O$, 3.0 g l^{-1} Na_3-citrate, 2.0 g l^{-1} KCl and 10 g l^{-1} bactopeptone (Oxoid), pH 7.0-7.2. Lipids were isolated according to Lo and Chang (1990), and stored in chloroform/methanol/water (65/25/4, v/v/v) under a N_2 atmosphere at -20°C.

A total lipid extract of *Escherichia coli* B (ATCC 11303), grown in Kornberg minimal medium (Ashworth and Kornberg, 1966), pH 7.4 at 37°C, was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The lipids were acetone and ether washed according to Kagawa and Racker (1971) and stored in chloroform under a N_2 atmosphere at -20°C.

The lipids of *Hb. salinarum* and *Hr. vacuolatum* were analyzed by thin-layer chromatography using Kieselgel 60 (Merck, Darmstadt, D) plates and chloroform/methanol/water (65/25/4, v/v/v) as eluant. Plates were developed with iodine vapor and a molybdate reagent to reveal the presence of (phospho-)lipids (Rouser *et al.*, 1970).

Liposome preparation and integrity determination. Lipids were dried by vacuum rotary evaporation, and hydrated in 50 mM MOPS, pH 7.0, 75 mM KCl, and 25 mM choline to a final concentration of 20 mg ml^{-1}, unless indicated otherwise. NaCl or KCl was included in the buffer at the desired concentration. The pH of the medium was varied by the use of HEPES (pH 7.5), TRICINE (pH 8), or CHES (pH 9). Liposomes were sized by five consecutive freezing and thawing steps, followed by extrusion through 400 and 200 nm polycarbonate filters using the Liposofast™ (Basic, Avestin, Ottawa, Canada) extrusion apparatus (Elferink *et al.*, 1994). Liposome integrity was tested by the ability to maintain a $\Delta\psi$, formed by a potassium diffusion gradient in the presence of valinomycin. The $\Delta\psi$ (inside negative) was monitored with the fluorescent probe 3,3'-diethylthiadicarbocyanine iodide [DiSC$_3$(5)] (Singh *et al.*, 1985).

Particle sizing of liposomes. The size distribution of the liposomes was determined by photon correlation spectroscopy using a Submicron Particle Sizer model 370 (Nicomp, Santa Barbara, CA, USA) (Payne and New, 1990).

Proton and sodium ion permeability. The proton permeability of the liposomes was measured with the acid-pulse method, monitored with fluorescent probes (Molecular Probes, Leiden, The Netherlands), as described (Nichols and Deamer, 1980; Elferink *et al.*, 1994; Chapter 2). The probe HPTS (pyranine, $pK_a = 7.3$) was used for proton permeability measurements between pH 7.0 and 8.0, and SNAFL ($pK_a = 7.8$) at pH 9.0. Data were fitted to the first order kinetic equation, from which the first order rate constant k_{H+} was used to compare the permeability properties of the membranes.

The sodium ion permeability of the liposomes was estimated from the efflux of ^{22}Na. Liposomes were prepared in buffer A (50 mM MOPS·KOH, pH 7.0, and 100 mM KCl) supplemented with NaCl at the concentration indicated. To 100 µl of the liposome suspension, $^{22}NaCl$ (~ 3×10^5 cpm; specific activity > 2200 Ci/mol, Amersham, U.K) was added, and allowed to equilibrate by incubation for at least 18 h at room temperature. To initiate ^{22}Na efflux, the liposomal suspension was diluted 100 fold in 10 ml buffer A with the appropriate salt concentration. At various time intervals, samples of 1 ml were taken and filtered over a 0.2 µm nitrocellulose filter (Schleicher & Schuell). Filters were rinsed with 1 ml buffer, and the amount of label retained on the filter was determined with a liquid scintillation counter. Data was corrected for the amount of ^{22}Na bound to the filters upon the permeabilisation of the liposomes with 1 µM of Gramicidin D. Sodium ion efflux was fitted to the first order kinetic equation as described for proton influx to yield the first order rate constant for the sodium ion permeability, k_{Na+}.

Acknowledgements

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Chapter 5

Bioenergetics and cytoplasmic membrane stability of the extreme acidophilic thermophilic Archaeon *Picrophilus oshimae*

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Summary

Picrophilus oshimae **is an extremely acidophilic thermophilic Archaeon that grows optimally at 60°C and at pH 0.7. It is an obligatory acidophile that does not grow at pH values above 4.0. The proton motive force in respiring cells is composed of a large transmembrane pH gradient, inside less acid and a reversed transmembrane electrical potential, inside positive. Cells maintain an intracellular pH at around 4.6 at extracellular pH values ranging from 0.8 to 4.0. Above pH 4.0 cells lyse rapidly and lose their viability. Liposomes prepared from lipids derived from** ***P. oshimae*** **have an extremely low proton permeability at acidic pH. However, at neutral pH, the lipids are unable to assemble into regular liposomal structures. These observations suggest that the loss of viability and cell integrity above pH 4.0 be due to an impairment of the barrier function of the cytoplasmic membrane.**

Introduction

The recently described extreme acidophilic thermophile *Picrophilus oshimae* lives at an optimum pH of 0.7 at 60°C (Schleper *et al.*, 1995). It is the most acidophilic thermophile described until now. This Archaeon belongs to the order of *Thermoplasmales*, which also includes *Thermoplasma acidophilum*. *P. oshimae* is a heterotrophic organism that can only grow on yeast extract. It requires an extreme ionic environment, specifically protons, not only for growth, but also for viability (Langworthy, 1982). Acidophilic Bacteria and Archaea that grow at low pH values (pH 2 to 4) usually maintain a cytoplasmic pH close to neutrality (Michels and Bakker, 1985; Moll and Schäfer, 1988; Peeples and Kelly, 1995). Little is known about metabolism and energy transduction of extreme acidophiles. The cytoplasmic membrane is the only physical protection against an acid environment if a cytoplasmic pH close to neutrality has to be maintained. Then the membrane has to withstand an extremely steep pH gradient of up to 4 or 5 pH units. Usually this can only be realised if the membrane has a very low proton permeability at temperatures up to 60°C. In a previous study we found that

Substrate	Respiration (nmol min^{-1} (mg protein)$^{-1}$)	
endogenous	22.7	(± 5.2)
yeast extract	65.0	(± 7.2)
casamino acids	54.6	(± 11.8)
L-proline	50.7	(± 0.7)
L-glutamate	40.3	(± 3.5)
L-leucine	35.2	(± 2.5)
glucose	34.4	(± 4.3)
galactose	36.1	(± 4.6)
starch	43.6	(± 1.2)
formate	0.0	
acetate	17.6	
propionate	19.3	
lactate	0.0	

Table 1. Respiration of *P. oshimae* cells at 60°C and pH 1 in the presence of various substrates. The following substrates did not show a significant change in respiration up to a concentration of 0.2% or greater: glycerol, fructose, raffinose, alanine, arginine, asparagine, glutamine, glycine, histidine, methionine, serine, cysteate.

thermophiles have a cytoplasmic membrane that is less permeable for protons than organisms that grow at lower temperature. At the respective growth temperatures the proton permeability for most of the tested organisms is in the same range. Thermophiles alter the membrane composition such that the membrane becomes less permeable to ions (Chapter 2 and 3). For acidophiles such an impermeable membrane is even essential.

To reveal the bioenergetic features of *P. oshimae*, the magnitude and composition of the proton motive force (PMF) were studied as a function of the environmental pH. It was found that the PMF of this extreme acidophilic thermophile is equal or higher than that of neutrophilic organisms. The proton permeability of the cytoplasmic membrane was indirectly determined in liposomes prepared from *P. oshimae* lipids. These liposomes are very impermeable for protons at the pH values that fall within the growth range. However, at neutral pH values, these lipids do not assemble into regular vesicular structures. The loss of cell viability and membrane integrity at pH values above 4.0 thus appears to be an intrinsic property of the membrane lipids and is likely the consequence of the adaptation of the cell envelope to extremely acidic environments.

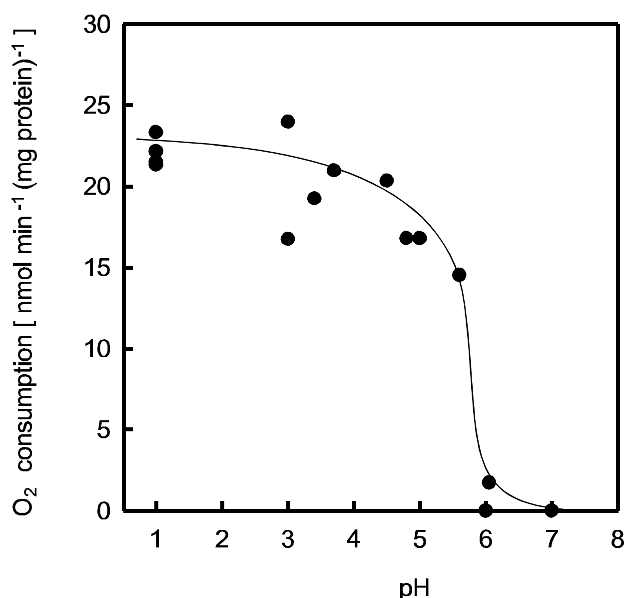

Fig. 1. pH dependency of the respiration of *P. oshimae*. Cells of *P. oshimae* were incubated in growth medium at 60°C, and energised with 0.2% yeast extract. After the experiment the exact pH values were measured. The respiration rate was calculated from the oxygen consumption as measured with a Clark electrode

Results

Respirable substrates. *Picrophilus oshimae* grows in a complex medium with yeast extract as main carbon and energy source. To identify the components that can act as energy source, the rate of respiration of cells incubated in a buffer of pH 1.0 was measured in a closed vessel with a Clark-type oxygen electrode. Cells had a high endogenous respiration rate even after starvation for several hours at 60°C. Highest respiration rates were observed in the presence of yeast extract or the mixture of amino acids, casamino acids (Table 1). Only proline, glutamate and leucine are respirable amino acids, whereas no enhancement of respiration was observed with alanine, arginine, asparagine, cysteine, glutamine, glycine, histidine, methionine and serine. From the carbohydrates, only galactose and glucose are respired whereas no activity was found with fructose, raffinose and glycerol. Although various substrates enhanced the respiration rate, none of them either individually or mixed could support growth suggesting a more complex nutritional requirement. Respiration was inhibited or even completely abolished by organic acids such as formate, acetate, propionate and lactate, but not by cysteate. The internal ATP concentration of growing cells was 2.7 ± 0.3 mM (n=3). When the medium was supplemented with starch or yeast extract, the internal ATP concentration remained equal or became slightly higher than in cells grown without substrate. When the cells were incubated with a mixture of amino acids (casamino acids), proline, glutamate or 2-deoxyglucose, the ATP concentration was about half the control. Intracellular ATP was completely depleted when the cells were incubated in the presence of 30 mM lactate.

Respiration was measured as a function of the external pH. Respiration is rather insensitive to the external pH up to pH 5, when respiration immediately ceased at pH values above 5 (Fig. 1). The formation of a precipitate at pH values above 5 suggested a major degree of cell lysis. The release of DNA, as observed from the fluorescence increase in the presence of the membrane impermeable DNA probe propidium iodide (Fig. 2) is consistent with this conclusion.

Proton motive force. The transmembrane electrical potential ($\Delta\psi$) and pH gradient (ΔpH) was measured as a function of the extracellular pH using a probe distribution assay. Cells incubated at 60°C were energised with 0.2% yeast extract. For the ΔpH measurements, the weak acids acetylsalicylic acid (aspirin) and DL-lactic acid were used with pK_a values of 3.48 and 3.08, respectively. Both acids accumulated to high levels when the external pH was around or below 1, yielding a ΔpH of more than 4 pH units (more than -275 mV, Fig. 3). The ΔpH steeply declined

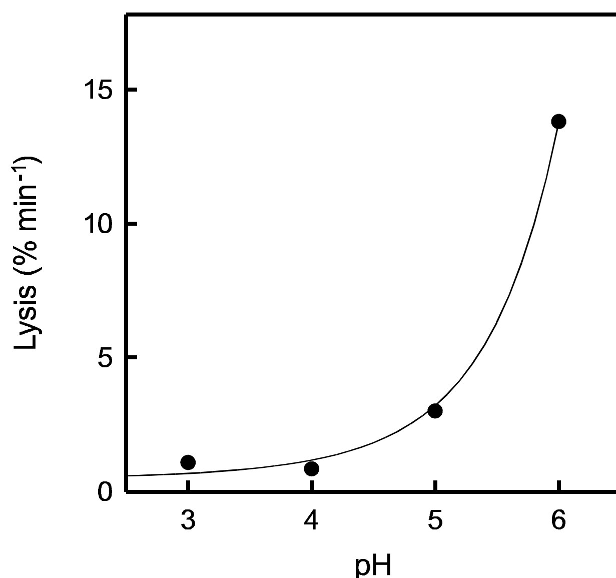

Fig. 2. pH dependent lysis of cells. Lysis was measured as described in the Materials and Methods section

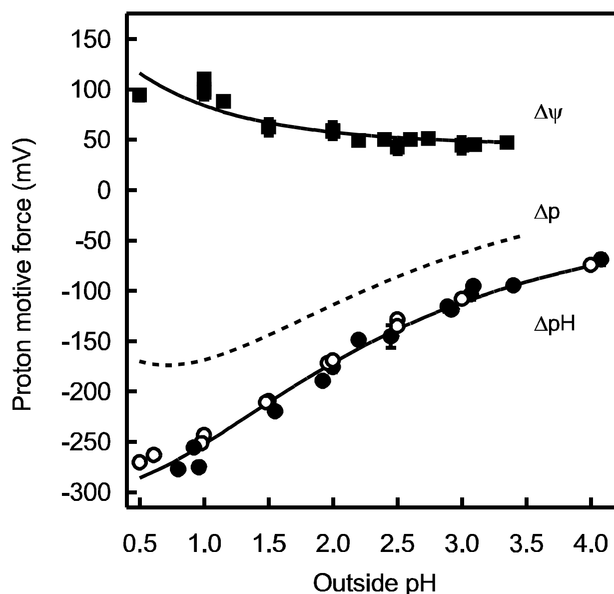

Fig. 3. pH dependency of the composition and magnitude of the proton motive force in *P. oshimae* at 60°C. Δψ (O) and . ΔpH (M,F) measurements as described in the Materials and Methods section. For the ΔpH acetylsalicylic acid (F) or lactate (M) were used as probes. (n=3)

with increasing pH, whereas the internal pH remained constant at pH 4.6 ± 0.15 (n = 25) over an external pH range from 0.5 to 3.5.

For the Δψ measurements, the anionic thiocyanate ion was used to monitor a Δψ, inside positive, and cationic tetraphenylphosphonium ions (TPP^+) for a Δψ, inside negative. Only thiocyanate was taken up by the cells (Fig. 3), while TPP^+ was extruded (data not shown), indicating a Δψ with a reversed potential (inside positive). Upon raising the extracellular pH from pH 0.7 to pH 4 the Δψ decreased sharply from +100 to +40 mV. Permeabilisation of the cells with *n*-butanol, or addition of the ionophore Gramicidin D resulted in a complete collapse of the ΔpH, whereas the Δψ was lowered to only +45 mV. The remaining Δψ presumably corresponds to a Donnan potential as binding studies with cytosolic and membrane fractions of lysed *P. oshimae* cells revealed no significant levels of thiocyanate binding (data not shown)

These studies show that *P. oshimae* at pH 1.0 and 60°C maintains a PMF of about -175 mV, composed of a large ΔpH and a reversed Δψ. The PMF drops with increasing pH.

Uptake of amino acids. Cells of *P. oshimae* energised with glucose were tested for the uptake of various amino acids at pH 1.0 and 60°C. L-Glutamate, L-histidine, L-proline, and L-serine were rapidly accumulated, whereas no uptake was detected of alanine or its nonmetabolisable analogue L-α-aminoisobutyrate (Fig. 4A). The highest levels of uptake were observed for L-glutamate. This process was therefore further investigated. L-Glutamate uptake was strongly inhibited by the ionophore nigericin that dissipates the ΔpH (electroneutral K^+/H^+ exchange). A collapse of the Δψ by valinomycin (K^+-ionophore) had no effect (Fig. 4C). L-Glutamate uptake was completely inhibited by the addition of valinomycin and nigericin at the same time or Gramicidin D. L-Glutamate uptake showed an optimum of pH 1.7 (Fig. 4B), and occurs with a K_m of 200 μM and a V_{max} of 0.92 nmol min^{-1} (mg protein)$^{-1}$. L-Glutamate uptake

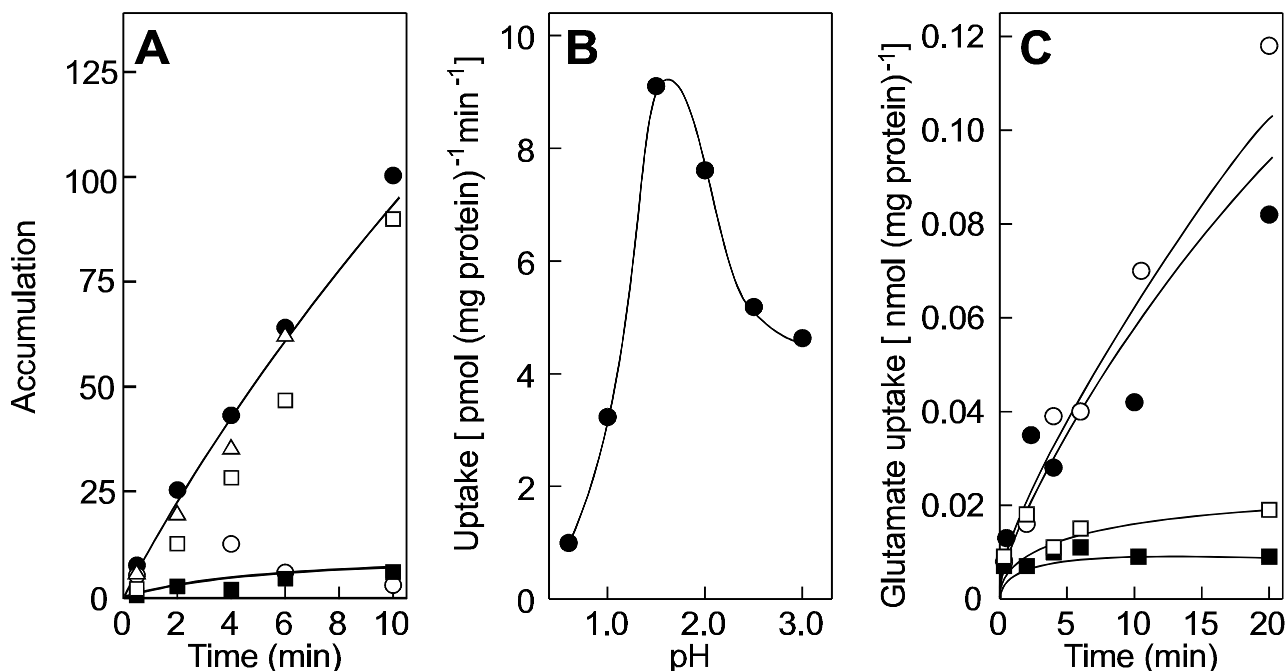

Fig. 4. A: Accumulation of amino acids by *P. oshimae* cells at 60°C and pH 1.0. Uptake of L-serine (Δ), L-glutamate (M), L-proline (G), L-histidine (O), and L-α-aminoisobutyrate (F). **B**: pH dependency of L-glutamate uptake at 60°C. **C**: L-glutamate uptake (M) and the effect of the ionophores valinomycin (F), nigericin (G), and nigericin and valinomycin (O). Concentrations of ionophores used were 1 μM

was strongly inhibited by L-aspartate and to a lesser extent by L-glutamine, while L-asparagine had no effect (data not shown). These data suggest that the uptake of L-glutamate by *P. oshimae* is dependent on the ΔpH, the main component of the PMF in respiring cells.

Membrane morphology. To study the characteristics of lipids of the cytoplasmic membrane, lipids were isolated from *P. oshimae* cells by soxhlet extraction and purified by reversed phase column chromatography. Thin layer chromatography analysis revealed the presence of one main lipid component, *i.e.* a phospholipid. Liposomes were formed from the hydrated lipids by freeze-thaw-extrusion through a 200-nm filter and examined by cryo electron microscopy. When the procedure was carried out at pH 7.0, liposomes were formed but more than 10% of the liposomes had extensive irregular substructures (Fig. 5B). At pH 4.0, a more homogenous liposome preparation was obtained with an averaged diameter of about 200 nm (Fig. 5A), while hardly any irregular shaped liposomes were evident (*i.e.* less than 2%).

Proton permeability. The proton permeability of *P. oshimae* liposomes was determined by the proton-pulse method. Liposomes with an average size around 200 nm were prepared with a high buffer capacity on the inside and dispersed in a solution with low buffer capacity. Valinomycin, a potassium ionophore, was added to prevent the generation of a counteracting Δψ due to electrogenic influx of protons. The external pH was lowered by an H^+ pulse, and monitored through the fluorescence of externally added pH dye Cl-NERF-dextran 70000. The pK_a of this dye is 4.7. Lowering of the external pH is followed by partial recovery due to the slow influx of protons into the liposomal lumen loaded with a higher buffer concentration. This phase was fitted to a

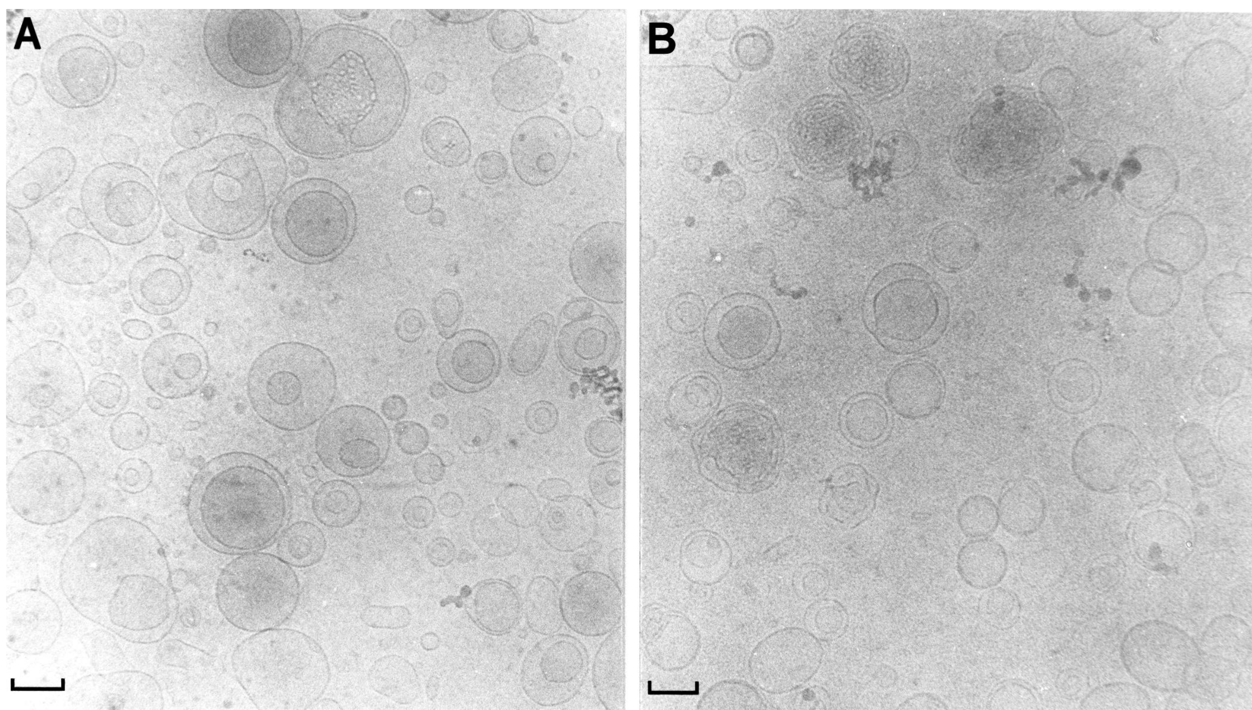

Fig. 5. Electron micrographs of liposomes of *P. oshimae* lipids at pH 4.0 (**A**) or pH 7.0 (**B**). Bar represents 100 nm

first order kinetic rate equation to yield the rate constant *k* for H^+ influx. Rapid proton equilibration was obtained after the addition of the ionophore nigericin. The proton permeability of the liposomes was measured at a function of the temperature.

At pH 4.0, the proton permeability of *P. oshimae* liposomes was nearly identical to that observed with *S. acidocaldarius* liposomes (Fig. 6). Due to the pK_a of the fluorescent dye, proton permeability measurement at lower pH values was not possible. The proton-permeability of each of the liposome preparations was plotted in an Arrhenius plot, and from the linear part of the graph the activation energy ($\Delta G'_{H+}$) was estimated of 36 kJ/mol. Liposomes prepared from *P. oshimae* lipids were completely permeable to protons at pH 7.0. The data demonstrate that liposomes prepared from *P. oshimae* lipids are highly stable and hardly permeable to protons at pH 4.0.

Discussion

Picrophilus oshimae is a thermophilic Archaeon that grows at pH values slightly lower than zero. Its optimal growth is at pH 0.7 and 60°C. Our data show that *P. oshimae* can use various sugars and amino acids as energy sources for respiration. The internal ATP concentration around 3 mM, in the presence and absence of substrate (yeast extract and starch), and even after starvation for 5 h. is comparable with the levels found in other prokaryotes (Kunji *et al.*, 1993). Cells were unable to maintain a high intracellular ATP concentration in the presence of amino acids, presumably as these can not be used as sole carbon sources for growth (Schleper *et al.*, 1995).

The bioenergetic parameters are typical for an acidophilic organism, *i.e.* a very large ΔpH and a reversed Δψ, to yield a PMF with a magnitude that is in the range found also in neutrophiles and other acidophiles (De Vrij *et al.*, 1988; Michels and Bakker, 1985; Moll and Schäfer, 1988;

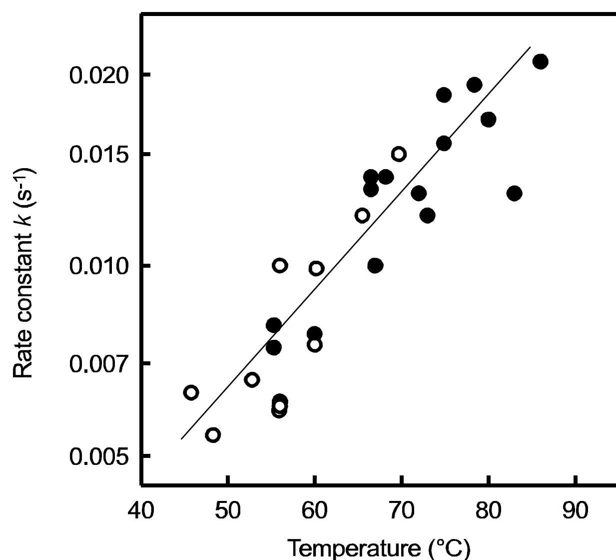

Fig. 6. Temperature dependency of the proton permeability of liposomes prepared from lipid derived from *P. oshimae* (F) and *S. acidocaldarius* (M). The influx of protons, as measured with the fluorescent probe Cl-Nerf dextran, was fitted to a first order kinetic equation as described in the Materials and Methods section

Peeples and Kelly, 1995; Matin, 1990; Booth, 1985). Both the PMF and ΔpH decline steeply with increasing pH. The ΔpH is the main constituent of the PMF at pH 1.0 and uptake of L-glutamate and presumably of most other substrates, is mainly driven by the ΔpH. This implies that *P. oshimae* is well adapted for growth at extreme acidic conditions.

The effect of ionophores and uncouplers on the transport activities has to be carefully interpreted. Valinomycin collapses the Δψ, nigericin collapses the ΔpH. Both ionophores together uncouple the cells, that can also be accomplished with Gramicidin D. Compounds as nigericin and Gramicidin D that collapse the ΔpH induce an acidification of the internal pH that results in inhibition of respiration and other cellular functions. The best system for a detailed energetic analysis of transport processes would be an isolated membrane vesicles system. Sofar preparing functional membrane vesicles of these Archaea has not been possible. The importance of pH homeostasis is evident from observations that weak acids are potent inhibitors of the growth of *P. oshimae*. At pH 1.0, 30 mM lactate and formate completely block respiration. At this pH value, these acids are almost solely present in their protonated form. Apparently, the cytoplasmic membrane of *P. oshimae* is, just like membranes of other organisms, highly permeable to the protonated weak acids. These acids not only cause an inhibition of respiration but also a complete dissipation of the ΔpH and depletion of the cellular ATP. The reason why formate and lactate are better inhibitors of respiration than acetate and propionate is not clear.

Lactate and acetyl salicylic acid were both used as internal pH indicators. The results obtained with both probes were similar. These studies demonstrated that *P. oshimae* maintains its intracellular pH around pH 4.6 when the extracellular pH is varied between pH 0.7 and 4.0. Internal pH measurements in *Thermoplasma acidophilum* have shown that this organism maintains its intracellular pH at 6.0 when the pH is varied between 0.5 and 3.0 (Michels and Bakker, 1985). The values of the internal pH are lower than those reported for non-thermophilic acidophiles (Matin, 1990). Both in *P. oshimae* and *T. acidophilum*, the ΔpH is large whereas the Δψ is reversed. The ΔpH can be completely dissipated by *n*-butanol and Gramicidin D, whereas the Δψ remains clamped at +45 mV. The latter may be due to a Donnan potential at these low pH values resulting from the immobilised charges of macromolecules in the cell (Matin, 1990; McLaggan *et al.*, 1989).

Since *P. oshimae* cells have to maintain a steep pH gradient across the membrane, we have analysed the proton permeability of liposomes prepared from lipids derived from its cytoplasmic membrane. When the liposomes were prepared at

pH 7.0, many liposomes revealed irregular substructures. These structures are possibly interlamellar attachments due to membrane fusion (Frederik *et al.*, 1991), or another form of non-bilayer structures. Liposomes, prepared at pH 3 and 4 lacked the substructures. These liposomes exhibited a very low proton permeability at pH 4.0, but became leaky when exposed to pH 7.0. The permeability coefficient, P (in cm s^{-1}) can be derived from the first order rate constant *k* (Yamauchi *et al.*, 1993). For protons, the values for P measured in this study range from 10^{-11} to 10^{-10} cm/s depending on temperature. P values reported for other lipids range from 10^{-10} to 10^{-4} cm/s depending on the lipids employed in the study (Deamer and Bramhall, 1986). The lipid composition of *P. oshimae* largely resembles that of *T. acidophilum* (Schleper *et al.*, 1995; Langworthy, 1982). Both organisms contain tetraether lipids with cyclised hydrocarbon chains, but lack the typical *Sulfolobus* nonitol ethers. The proton permeability of *S. acidocaldarius* liposomes at pH 4.0 was about 2-fold lower as compared with pH 7.0 from in a previous study (Chapter 2). Liposomes prepared from *P. oshimae* lipids were completely permeable to protons at pH 7.0.

The inability of the *P. oshimae* lipids to assemble into regular membrane structures at neutral pH may be a main reason that cells are extremely susceptible to lysis when exposed to pH values above pH 4. *S. acidocaldarius* tetraether lipids and synthetic bolaform lipids primarily adopt a membrane spanning shape upon hydration (Elferink *et al.*, 1992; Thompson *et al.*, 1992). When assayed at pH 4.0 and 60°C, liposomes prepared from *P. oshimae* or *S. acidocaldarius* lipids showed nearly identical proton permeability. This proton permeability is significantly lower than that observed for mesophilic or thermophilic organisms at their respective growth temperatures. This is required to withstand the high proton concentration at pH values below 1.0. In addition, the reversed $\Delta\psi$ will counteract the proton influx, and thus help in maintaining a ΔpH inside alkaline versus outside under extreme conditions. Our studies with the liposomes prepared from *P. oshimae* lipids are indicative for an intrinsic instability of the -cytoplasmic membrane at higher pH values. *P. oshimae* is not only well adapted to endure the extreme acidic environment, but even requires an acidic pH for membrane stability and cell integrity.

Materials and Methods

Strains, growth conditions and chemicals. Picrophilus oshimae DSM9789 (Schleper *et al.*, 1995) was grown aerobically at pH 0.8 at 60°C in Brock's medium supplemented with 0.2% yeast extract. The medium contained per litre: 1.32 g $(NH_4)_2SO_4$, 0.372 g KH_2PO_4, 247 mg $MgSO_4{\cdot}7H_2O$, 74 mg $CaCl_2{\cdot}2H_2O$, 1.93 mg $FeCl_3{\cdot}6H_2O$, 1.8 mg $MnCl_2{\cdot}4H_2O$ 4.5 mg $Na_2B_4O_7{\cdot}10H_2O$, 0.22 mg $ZnSO_4{\cdot}7H_2O$, 50 µg $CuCl_2{\cdot}2H_2O$, 30 µg $Na_2MoO_4{\cdot}2H_2O$, 35 µg $VOSO_4{\cdot}5H_2O$, 10 µg $CoSO_4{\cdot}7H_2O$ and 0.2% yeast extract. The pH was adjusted with concentrated H_2SO_4. *Sulfolobus acidocaldarius* DSM639 was grown as described (Chapter 2).

The internal volume of the cells was determined as described using ^3H_2O (1.6 GBq $mmol^{-1}$) and the membrane impermeable sugar inuline-[^{14}C-acetic acid] (163 MBq $mmol^{-1}$) (Rottenberg, 1979). The internal volume of the cells was 1.55 ± 0.11 µl per mg of protein (n=15). All radiochemicals were obtained from Amersham International Plc, Buckinghamshire, U.K., unless indicated otherwise.

Respiration measurements. Cells were de-energised by washing twice in Brock's medium without yeast extract supplemented with 35 mM KCl (assay medium), incubated for 1 h at 60°C, and again washed and resuspended in assay medium at OD_{660} 0.7. Various substrates (0.2% (w/v)) were added to the cell suspension, and the respiratory rate measured with a Clark-type oxygen electrode. Measurements were done at 60°C.

Cell lysis experiments. To test cell lysis, 2 ml fresh cell suspension (OD_{660} 0.4) was centrifuged for 30 s at 14000 rpm in an Eppendorf centrifuge. The cells were resuspended in 100 µl growth medium of the desired pH and immediately diluted into 1.9 ml growth medium of the desired pH at 55°C. Lysis was followed by the time dependant increase in fluorescence intensity, monitored with the membrane impermeable DNA probe propidium iodide (Molecular probes Inc., Eugine, OR, USA) (1 µg ml^{-1}). The fluorescence response of the probe varied drastically at different pH values. Therefore, the signal was expressed as percentage of maximum fluorescence change. Maximum fluorescence change was obtained by the addition of dodecyl maltoside (0.1% final concentration) to the cell suspension. The initial rate of lysis was calculated and plotted.

Intracellular ATP concentration measurements. For intracellular ATP measurements the cells were harvested basically according to Schönheit and Perski (1983). 1 ml Cells (OD_{660} of 5) was incubated for 2 hr at 60°C in the presence of 0.2% (w/v) substrate. From each cell suspension 250 µl was pipetted in -20°C 500 µl ethanol-KOH and immediately frozen in liquid nitrogen. Ethanol-KOH contained ethanol and an empirically determined volume of 1.0 M KOH to adjust the low pH of the cells to about 7. Subsequently the mixture was thawed on ice and frozen again in liquid nitrogen. After lyophilising, the samples were dissolved in 750 µl 20 mM Tris-acetate, 0.2 mM Na_2-EDTA, 0.05 mM DTT, 0.5 mM magnesium acetate, 0.05% BSA. pH 7.5. ATP concentrations were determined with partly purified luciferase (FLE-50, Sigma Chem. Co., St. Louis, MO, USA) (Lundin and Thore, 1975). Portions of 0.1 ml of the resulting solution were analysed with luciferine-luciferase reagent. Luminescence was measured with a Lumi-Tec A5-2021A luminescence meter (St. John Associates, Inc, Beltsville, MD, USA), using ATP solutions of known concentration as calibration.

Measurement of proton motive force. The transmembrane electrical potential, $\Delta\psi$, inside positive, and pH gradient, ΔpH, inside alkaline, were measured essentially as described by Michels and Bakker (1985). Cells (1.5-3.0 mg of protein) were suspended in 2 ml assay medium at pH 1.0. The pH was adjusted with concentrated H_2SO_4. At higher pH, buffers were used which contained either 100 mM potassium phosphate or 100 mM potassium phosphate supplemented with either 100 mM sulphuric acid or citric acid. To the suspensions, yeast extract (0.2%, w/v) was added for energisation, followed by the addition of 1.5 µM $KS^{14}CN$ (2.1 GBq $mmol^{-1}$), 1.5 µM ^{14}C-acetylsalicylic acid (2.0 GBq $mmol^{-1}$, American Radiolabeled Chemicals Inc., St. Louis, MO, USA) or 3.5 µM ^{14}C-DL-lactate (0.87 GBq $mmol^{-1}$).

Cells were incubated at 60°C and aerated with humidified air preheated at 60°C. After 10 min, three samples of 600 µl were taken and centrifuged through a 500 µl layer of silicon oil (A200/A20 ratio of 2.5/1, v/v; Wacker Chemie GmbH, München, D) into a 150 µl layer of 14% (w/v) perchloric acid and 9 mM EDTA. 100 µl

Samples were taken from both the layer above and below the silicon layer. To avoid buffer effects on counting we balanced the samples obtained from the lower and upper layer with the medium used for the upper layer and PCA/EDTA, respectively. The radioactivity was determined by liquid scintillation counting.

To determine the extent of nonspecific probe binding to cellular components, the cells (O.D.$_{660}$ = 5) were disrupted by sonication (Soniprep 150 probe sonicator, MSE, Uxbridge, Middlesex, UK), and separated into membrane and cytosol fractions by centrifugation for 15 min at 200,000*g*. From each fraction, 200 µl was transferred into the small space that is in the cap of an Eppendorf cup and subsequently covered with dialysis tubing. From the cup top only a small ring was left, used to tighten the dialysis tubing. The samples were dialysed in Brock's medium salts, supplemented with 35 mM KCl, and various radiolabelled tracers, at pH 1.5. After 2 h of incubation at 60°C, the radioactivity associated with the various fractions was determined.

Transport assays. For transport experiments, 200 µl of a concentrated cell suspension (30 mg of protein per ml) was added to 2250 µl assay medium at pH 1.0. The suspension was incubated at 60°C and aerated with humidified air preheated at 60°C. Subsequently, 0.3-5 µM of the following ^{14}C-labelled substrates was added: L-α-aminoisobutyric acid, L-glutamate, L-histidine, L-proline, L-serine and glucose with specific activities of 2.2, 9.8, 0.37, 9.6, 2.3 and 11 GBq mmol^{-1}, respectively. At various times, samples of 300 µl were taken, diluted with 2 ml 0.1 M LiCl, and filtered over a 0.45 µm BA85 (Schleicher & Schuell, Dassel, D) nitrocellulose filter. Filters were rinsed with 2 ml 0.1 M LiCl, and the radioactivity retained on the filter was counted by liquid scintillation spectrometry.

Lipid extraction and purification. Lyophilised cells of *P. oshimae* were Soxhlet extracted and fractionated essentially as described by Lo and Chang (1990). The lipids were stored in chloroform/methanol/water (65/25/4, v/v/v) at -20°C. Lipids were analysed by thin-layer chromatography using Kieselgel 60 (Merck, Darmstadt, D) plates and chloroform/methanol/water (65/25/4, v/v/v) as eluant. Plates were developed with iodine vapour and a molybdate reagent to reveal the presence of (phospho-)lipids (Rouser *et al.*, 1970).

Liposome preparation and integrity. Lipids were dried by vacuum rotary evaporation, and hydrated in 50 mM MOPS, pH 7.0, 75 mM KCl, and 25 mM choline to a final concentration of 20 mg ml^{-1}, unless indicated otherwise. Liposomes were sized to an average diameter around 200 nm by five consecutive freezing and thawing steps, followed by extrusion through 400 nm and subsequently 200 nm polycarbonate filters using the Liposofast^{TM} (Basic, Avestin, Ottawa, Canada) extrusion apparatus (Elferink *et al.*, 1994). Liposome integrity was tested by the ability to maintain an imposed potassium diffusion gradient in the presence of valinomycin by $\Delta\psi$ (inside negative) measurements using the fluorescent probe 3,3'-diethylthiadicarbocyanine iodide [DiSC$_3$(5)] (Deamer and Nichols, 1989).

Electron microscopy. The morphology of liposomes prepared at pH 7.0, 3.0 and 4.0 was studied by negative staining and cryo electron microscopy. Liposomes, 10 mg lipid ml^{-1}, were made in 50 mM potassium phosphate at the desired pH, extruded, and diluted to obtain isolated liposomes for EM. For cryo electron

microscopy, a bare sample grid was dipped in the liposome suspension and withdrawn. After withdrawal from the liposome suspension, the grid was blotted against filter paper. The remaining thin film was rapidly frozen in liquid propane at -180°C. The liposome suspension was at room temperature. The preparations were analysed at 100 kV and a sample temperature of -165°C in a Philips CM10 electron microscope.

Measurement of proton permeability. The proton permeability of the membrane was measured essentially as described by Nichols and Deamer (1980), except that the fluorescent pH dye Cl-NERF-dextran 70000 (Molecular probes Inc., Eugene, OR, USA) with an apparent pK_a of 4.7, was used for measurement at acidic pH. Liposomes were prepared in 50 mM citrate-KOH and 35 mM KCl pH 4.0. To reduce the buffer capacity of the external medium, liposomes were passed over a Sephadex G-25M PD10 column pre-equilibrated with 90 mM KCl, pH 4.0, and diluted to 1.5 mg lipid ml^{-1} in 2 ml of 90 mM KCl, pH 4.0, containing the fluorescent pH probe Cl-NERF-dextran 70000 at a concentration of 5 μg ml^{-1}. The K^+ ionophore valinomycin (1 nmol mg^{-1} lipid) was added to prevent the formation of a reversed $\Delta\psi$. The external pH was monitored from the fluorescence at 518 nm (emission 541 nm). After equilibration, the external pH was lowered by the addition of 5 μl 200 mM HCl. The proton permeability was determined from the partial recovery of the external pH due to the influx of protons into the liposomal lumen. Fluorescence measurements were done on a Perkin-Elmer LS-50B, using a thermostated, magnetically stirred sample compartment. Data were fitted to a first order kinetic rate equation as described (Chapter 2) to yield the rate constant k that was used for comparison.

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Chapter 6

SUMMARY AND CONCLUDING REMARKS

Introduction

Cells can transport solutes into and out of the cytoplasm. These transport processes are mediated by proteins embedded in the cytoplasmic membrane. Since the uptake of solutes is often coupled to proton (H^+) or sodium ion (Na^+) transport, the transmembrane electrochemical gradient of Na^+ and H^+ can be used to drive the uphill transport of solutes. Likewise, the energy in the electrochemical gradient of Na^+ and H^+ ions can be used to generate metabolic energy in the form of ATP via membrane-bound ATPases. When the coupling ions (Na^+ or H^+) would be able to diffuse freely across the membrane, the cell could not maintain the ion gradients. Consequently, solute uptake or other membrane-associated energy-requiring processes would not take place. This implies that the lipid membrane has to be impermeable for Na^+ or H^+ to enable directed transport via specialized transporters. The cytoplasmic membrane needs to meet these conditions also when cells grow in an extreme environment. High temperature intrinsically increases the permeability of the membrane for H^+ and Na^+. For instance, in an extremely acidic environment, the outside proton concentration is so high that without countermeasures, the inside of the cell would rapidly acidify as well. Likewise, in an extreme alkaline environment the cell needs to reduce proton loss to the environment. Halophiles must avoid penetration of Na^+ into the cells, and additionally maintain the integrity of the membrane in a high salt environment. Extreme environments thus require a specific adaptation of the lipid composition of the cytoplasmic membrane.

To determine the ion-permeability characteristics of membranes from extremo- and mesophilic prokaryotes, we have isolated the lipids from these organisms and used these lipids to prepare closed liposomes with an average diameter of 200 nm. These liposomes serve as a model system and can be used to obtain a deeper insight in the permeability characteristics of the biological membrane without interference by the membrane proteins. The proton permeability of the liposomes was determined by loading the liposomes with a medium with a high buffer capacity and by exposing them to an external medium with low buffer capacity. After the imposition of an acid pulse, the external pH rapidly drops but recovers due to the proton permeation into the liposomes. The rate of proton permeation can than be determined by monitoring the recovery of the external pH in time. For the determination of sodium ion permeability, efflux of radioactive sodium-ions from the liposomal lumen was monitored by means of a filter assay.

Membrane permeability adaptation to extreme environments

High and low temperature Without any particular measures, thermophiles face a dramatic increase in the membrane permeability of both H^+ and Na^+ at the elevated temperatures. Prokaryotes appear to have specific means to adapt the proton permeability of their membrane to a change in growth temperature. This phenomenon can only be observed for a single organism grown at different growth temperatures, but is also apparent when organisms are compared that have different optimum growth temperatures. For instance, *Psychrobacter immobilis*, an organism

that grows at very low temperatures (around 10°C) exhibits the same proton permeability at its respective growth temperature, as organisms that live at higher temperatures such as the mesophile *Escherichia coli* (around 37°C) or the thermophile *Sulfolobus acidocaldarius* (around 80°C) (Chapter 2). Strikingly, these organisms have a vastly different lipid composition of their cytoplasmic membrane. An adaptation of the proton permeability in response to the growth temperature was also observed within a single organism, *Bacillus subtilis*. Despite large changes in the membrane lipid composition, the proton permeability of the membrane remained nearly constant at the respective growth temperature when the cells were grown within a broad range of temperatures (13 to 50°C) (Chapter 4). We have termed this phenomenon homeo- proton permeability adaptation.

The permeability of the membrane for H^+ is several orders of magnitude higher than for Na^+. Yet, the relative increase in the Na^+ and H^+ permeability of the membrane with increasing temperature is comparable for both ions. This means that the activation energy of the permeability for Na^+ ions is comparable to that of protons. However, we did not observe a specific adaptation of the membrane to the Na^+ permeability at higher growth temperatures. For instance, when assayed at the same temperature, liposomes prepared from lipids derived from a psychrophile possess the same Na^+ ion permeability as liposomes derived from lipids of a mesophile or thermophile.

Acidic and alkaline pH Acidophiles that grow optimally at low pH need to maintain the cytoplasm near neutrality. These cells often face a huge pH gradient across their cytoplasmic membrane. Although this large ΔpH favors transport and other energy-consuming processes, its generation and maintenance require a lot of energy. Acidophiles compensate part of the large ΔpH by reversing the $\Delta\psi$ across the membrane, i.e., inside positive instead of negative. This phenomenon results in a lowering of the proton motive force to values that are normally observed with Bacteria that grown at or around neutral pH. Extreme acidophiles that live in a very acid environment (pH 0.5 to 2.5) modify their membrane lipids in order to maintain the membrane integrity. For instance, *Alicyclobacillus acidocaldarius* contains membrane lipids with a bulky hydrophobic group on the end of the acyl chain and in addition hopanoids and large glycolipids (Langworthy *et al.,* 1976; Poralla *et al.,* 1984). The membrane of acidophilic Archaea is composed of glycolipids that span the entire membrane, i.e., tetraether lipids (De Rosa *et al.,* 1983). These lipids provide a matrix that is highly impermeable to protons. Some Archaeal membrane lipids even require acid conditions for growth. Liposomes derived from lipids of *Picrophilus oshimae*, an organism that lives below pH 1 at 60°C, do not stay intact above pH 5 (Chapter 3). These organisms have an intracellular pH of 4.6. It thus appears that these cells require an acidic environment to maintain the membrane integrity. The adaptation in lipid composition of Archaeal acidophiles to acid pH is probably more effective than in Bacterial acidophiles because thermophilic Bacterial (extreme) acidophiles so far have not been found.

Alkaliphiles need to keep their cytoplasm neutral in an alkaline environment. These organisms face a reversed ΔpH, i.e., inside acid versus outside alkaline. This reversed ΔpH is compensated by a large $\Delta\psi$, inside negative. The $\Delta\psi$ prevents the loss of protons into the external environment, a process that is essential for the maintenance of the intracellular pH. Alkaliphiles might take up solutes in symport with protons.

However, the proton motive force in alkaliphiles is much lower than in organisms that live at normal or low pH. Except for the alkaliphilic halophile *Halorubrum vacuolatum*, the permeability characteristics of membranes of alkaliphiles have not been studied in detail. The H^+ permeability of liposomes prepared from *Hr. vacuolatum* lipids does not differ from that observed for halophile *Halobacterium salinarum*, an organism that lives at neutral pH (Chapter 4). In a preliminary analysis of liposomes of other alkaliphiles, we observed that the H^+ permeability is higher than for instance in *E. coli* (data not shown). Together with the presence of a low proton motive force, this suggests that protons are not the most favourable coupling ions for transport in alkaliphiles. Instead, these organisms often prefer to use Na^+ as coupling ion. This is for instance observed with the anaerobic alkaliphile *Anaerobranca bogoriae* (*Thermoalkalibacter bogoriae*) (Prowe *et al.,* 1996). The alkalitolerant *Bacillus FTU* species was found to possess two respiratory chains, i.e., one H^+ motive and one Na^+ motive. The latter respiratory chain is only present at alkaline pH. Still, some aerobic alkaliphilic *Bacillus* species utilize a proton translocating F_0F_1 ATPase for ATP synthesis.

High salt concentration Halophiles face a huge sodium ion gradient across their membrane. Potassium ions or organic solutes are used to compensate for the osmotic effect of the high outside sodium concentration. The so-called 'compatible solutes' are either taken up from the environment or synthesized. Membrane lipids of halophiles are equipped with the same H^+ and Na^+ ion permeability characteristics as non-halophilic organisms (Chapter 4). As can be expected in halophiles, their lipids can withstand a high ionic strength. This is a feature that is absent in non-halophiles. Since the H^+ and Na^+ ion permeability is hardly influenced by the salt concentration, the use of Na^+ as a coupling ion for energy transduction seems not more advantageous for halophiles than for non-halophiles under the same conditions. However, since halophiles face a large Na^+ gradient, Na^+ ion coupled transport can be useful.

Concluding remarks

Permeability mechanisms The characteristics of the Na^+ permeability differ from that found for protons. The Na^+ permeability is governed by simple diffusion of the hydrated sodium ion through the membrane (Marrink, 1994). Therefore, the Na^+ permeability increases with temperature and probably only depends on the membrane thickness and packing (Paula *et al.,* 1996).

The H^+ permeability might occur *via* a water wire mechanism in which protons are transported over a membrane spanning transient water chain. In a water wire, a chain of water molecules crosses the membrane (Gutknecht, 1987; Nagle, 1987; Marrink *et al.,* 1996). In the short period that a water wire exists, only a few ps, a proton might hop from one water molecule to the other and pass the membrane. The frequency of such an event depends on the lipid composition. As a consequence, an adaptation of the membrane lipid composition is needed to adjust to the increased permeability at higher temperatures. As shown in this thesis, the temperature is a major determinant for adjustments of the lipid composition, but any adjustment that reduces the proton permeability also results in a change in the membrane fluidity. Therefore, cells have to compromise in order to find an optimal balance between the proton permeability and the maintenance of the membrane in a liquid crystalline state.

Archaea Extreme environments are mainly occupied by Archaea. The Archaeal cytoplasmic membrane contains unique ether lipids that cannot easily be degraded, that are more stable and that are equipped with a high salt tolerance. Thermophilic and extremely acidophilic Archaea possess membrane spanning tetraether lipids that are nearly impermeable for ions and protons. These membrane lipids are extremely rigid. The low proton permeability of these membranes allows these organisms to thrive at the extreme acidic environments. The energy, otherwise lost to compensate for unwanted ion diffusion, can then be used for transport of solutes and other cellular processes. These unique properties of the Archaeal lipid membranes make Archaea better suited for life in extreme environments than the typical ester-type bilayer lipids of Bacteria or Eucarya.

Towards a better understanding of the membrane of extremophiles The H^+ and Na^+ permeability of the membranes of extremophiles has now been studied in some detail. The impact of other extreme conditions such as high pressure on the membrane permeability is less clear. Also, alkaliphiles need to be studied more extensively. The molecular mechanism of H^+ and Na^+ permeation has been approached by Yamauchi *et al.* (1993; 1992). The presence of phytanyl lipids, which resemble Archaeal lipids, results in a major reduction of the membrane permeability as compared to the typical palmitoyl lipids. The H^+ and Na^+ permeability of membranes from synthetic lipids should be analysed in a systematic manner by varying the acyl chain composition while leaving the polar head group composition constant. Brookes *et al.* (1997), who measured proton uptake as a result of an imposed K^+ diffusion potential (Brand *et al.,* 1992), did not observe a relation between liposomal H^+ permeability and the phospholipid fatty acid composition for lipids derived from mitochondria. The importance of the polar headgroup in comparison with the acyl chain, and a possible synergy between both lipid attributes should be elucidated. Furthermore, the differences between Bacterial and Archaeal acyl chains should be further studied, and their importance in salt tolerance and resistance should be elucidated. Molecular dynamic studies on proton permeation (1994) should be validated by biophysical experiments which should include Archaeal lipids as well.

A biological membrane does not only consist of lipids, but also contains membrane proteins. The presence and activity of these proteins may also influence the permeability. It is to be expected that the absolute permeability values will increase once membrane proteins have been introduced into the liposome system. Anomalies and mismatches at the border between proteins and lipids may increase the permeability. However, it is not to be expected that such effects differ greatly between organisms, and thus would affect the relationships as observed in our studies with pure lipid vesicles.

A serious problem in obtaining realistic values of the permeability of an intact membrane is the activity of membrane proteins. Reliable results can only be obtained if all ion fluxes *via* the membrane proteins can be blocked. Once the basal permeability of the membrane is understood, it can be integrated into a model that would facilitate our understanding of the chemiosmotic energy fluxes across the membrane of both extremophiles and non-extremophiles. For such a study, vesicles of *Thermoplasma acidophilum* might be good candidates. This organism lives at 60°C at pH 2 and most of the lipids are tetraether lipids (Langworthy, 1982). The membrane lacks a surface layer (S-layer), which

most probably is a major obstacle to obtain well-sealed membrane vesicles from thermo-acidophiles (Weiss, 1974; Deatherage *et al.,* 1983). The permeability of the vesicles could then be measured in an analogous system as in which liposomal fluxes are measured, eventually with the help of stopped flow techniques to detect high proton permeabilities.

The role of the permeability of coupling ions in extremophiles appears to be more important than previously realized. The necessity to adapt the membrane in order to keep the permeability at a viable level is so high that it can be regarded as a key feature for growth in extreme conditions.

Samenvatting

Bioenergetica en ion-doorlaatbaarheid van de membraan in extremofiele organismen

Inleiding

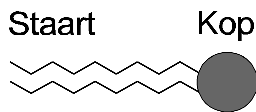
De levensvormen die we kennen kunnen worden onderverdeeld in drie categorieën: de Bacteria (bacteriën), de Eucarya (eukaryoten) en de Archaea (daar is geen Nederlandse naam voor).

De eerste twee groepen zijn vrij algemeen bekend: Bacteria zijn eencellig, en hebben geen celkern. Er zijn Bacteria die voor ons minder prettig zijn, zoals de maagzweer veroorzakende Bacterie *Helicobacter pylori*, maar er zijn ook vele nuttige Bacteria zoals de bodembacterie *Bacillus subtilis* en de melkzuurbacterie *Lactococcus lactis*. Eucarya zijn soms ééncellig, maar vaak ook meercellig; wel hebben ze allemaal een celkern. In deze groep zitten bijvoorbeeld planten, dieren (wij dus ook), schimmels en gisten. Voor de erkenning van de derde groep heeft met name Carl Woese zich jarenlang sterk gemaakt. Hij heeft in 1997 het gelijk aan zijn kant gekregen nadat de erfelijke code van een paar Archaea was ontrafeld. De Archaea hebben net als Bacteria geen celkern, zijn altijd eencellig en tot op heden is er geen ziektenverwekker van bekend. Toch zijn Archaea geen Bacteria. Sterker nog, ze lijken in sommige opzichten meer op Eucarya dan op Bacteria.. Bijvoorbeeld de manier waarop Archaea hun erfelijke informatie (DNA) aflezen lijkt erg op die van de Eucarya. Een subgroep van de Archaea is als enige groep in staat om methaan te produceren (aardgas, moerasgas, Archaea komen ook in de darm voor). Wat de Archaea vooral onderscheidt, zowel van Bacteria als van Eucarya, is de samenstelling van de membraanlipiden. Daarover later meer.

Alle cellen van de levensvormen die wij kennen, bestaan uit een druppel water-met-opgeloste-stoffen (cytoplasma) omhuld door een membraan: het celmembraan. Het celmembraan bestaat uit een film van olieachtige stoffen (lipiden) en eiwitten (membraaneiwitten); het functioneert als een barriere tegen ongewenste opname of afgifte van stoffen in het cytoplasma. Het celmembraan controleert onder meer de opname van stoffen, die de cel nodig heeft. Een tweede belangrijke functie van het membraan is de productie van energie voor de celstofwisseling. Voor deze twee processen gebruiken de cellen de eiwitten in het membraan.

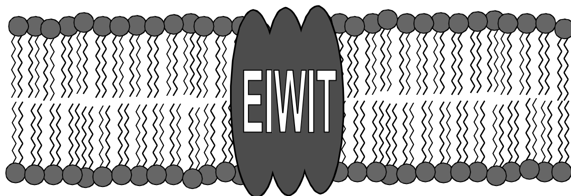
Het mechanisme voor de opwekking van stofwisselings-energie met behulp van het membraan is in 1961 ontdekt door Peter Mitchell, die daarvoor een Nobelprijs kreeg. Zijn theorie -de chemiosmotische theorie- komt hierop neer: Een cel pompt ionen naar buiten over de celmembraan, bv. met behulp van lichtenergie of energie, verkregen uit de verbranding van voedingsstoffen. Ionen zijn electrisch geladen atomen. In het algemeen worden de positief geladen natriumionen (Na^+) en/of waterstofionen (protonen of H^+) naar buiten gepompt. Met het uitscheiden van deze ionen slaat de cel twee vliegen in één klap: de cel scheidt positieve lading uit en de concentratie van de ionen binnen de cel wordt lager. Een cel kan in dit opzicht worden gezien als een IJsselmeerpolder, de uitgescheiden Na^+ of H^+ deeltjes als het water dat de polder omringt, het celmembraan als de polderdijk. Het niveau H^+ of Na^+ buiten de cel is hoger, maar de ‘dijk’ (het celmembraan) houdt ze tegen. Vervolgens kan het energieverschil, dat door het

A



B

Buiten



Binnen

Fig. 1. Het membraan bestaat uit lipiden en eiwitten. **A**: de lipiden bevatten een hydrofobe (waterafstotende) staart en een hydrofiele kop. **B**: De lipiden vormen een dubbele laag, met de hydrofobe staarten naar binnen gericht. In het membraan bevinden zich eiwitten, die verschillende functies kunnen vervullen.

hoger niveau van de buitengesloten ionen wordt veroorzaakt, worden gebruikt als in een soort plan-Lievense. Zoals bij water, dat via een generator een polder binnenstroomt, kan electriciteit worden opgewekt uit de instroom van ionen. De cel maakt echter geen electriciteit van de instroom van de ionen, maar energie-rijke cellulaire energiepakketjes, ATP, uit energie-arme pakketjes, ADP. ATP kan overal in de cel gebruikt worden waar energie nodig is. De omzetting van ATP terug in ADP levert energie op. Zo wordt via de uitscheiding van ionen de energie van verbranding of licht indirect omgezet in ATP.

De dijk-functie van het membraan wordt voornamelijk door de lipiden in het membraan vervuld. De lipiden vullen de gaten tussen de membraaneiwitten op. Lipiden beschermen de cel tegen het binnendringen van ongewenste stoffen, en ook tegen het verlies van stoffen die noodzakelijk zijn voor de celstofwisseling. De bouw van membraanlipiden is altijd volgens hetzelfde principe: de buitenkant van het membraan trekt water aan (hydrofiel) en binnenin is het membraan vettig, waterafstotend (hydrofoob). Het hydrofiele deel van het lipide-molecuul bestaat uit een kleine kopgroep, vaak fosfaat of suikers bevattend en vaak geladen. Het hydrofobe deel bestaat uit (meestal twee) lange staarten van koolwaterstof (olieachtig) (Fig. 1). De staarten worden door het water, waar ze absoluut niet van houden, naar de binnenkant van het membraan gedrukt. Aan de andere kant van het membraan gebeurt precies hetzelfde, waardoor een film wordt gevormd. Veel stoffen die opgelost zijn in het water lossen niet gemakkelijk op in vet. Daaraan ontleent het membraan zijn barrière-functie. Zeker ionen, geladen deeltjes, lossen nauwelijks op in hydrofobe omgevingen, zodat ze keurig buiten blijven. De enige manier voor ionen om in de cel te komen is via een van de membraaneiwitten. Naast de genoemde barrièrefunctie dienen lipiden ook als fundament voor de membraaneiwitten. Eiwitten moeten een beetje kunnen bewegen om optimaal te kunnen functioneren. Dat vereist dat de omgeving ook soepel kan meebewegen. De membraanlipiden moeten dus aan twee eisen voldoen: ze mogen niet lek zijn, maar moeten toch soepel blijven.

Echter, een polder werkt niet als de dijk lekt. Zo ook in een cel. Als het membraan lek (permeabel) is, vervalt de controle op het in- en uitgaan van ionen en kan er geen energie meer worden opgewekt. In extreme omstandigheden, bv. bij erg hoog water, kan een dijk het niet meer bolwerken. De dijk moet dan versterkt en verhoogd worden. Precies hetzelfde gebeurt met membranen. Onder extreme omstandigheden is een gewoon cel-membraan te permeabel. De cel kan dan worden geconfronteerd met twee problemen: interne verzuring door een overdosis H^+ (oftewel: ‘wat

staat het water hoog binnen de dijk'), en energiegebrek ('de generator werkt niet meer omdat het verschil in waterpeil tussen binnen en buiten de polder te klein is'). De cel heeft een paar strategieën om die problemen op te lossen. De strategie die in dit proefschrift wordt beschreven behelst de aanpassing van de doorlaatbaarheid (permeabiliteit) van het membraan.

De doorlaatbaarheid van het membraan werd als volgt gemeten: Van de organismen die bestudeerd zijn, werden de membraanlipiden geïsoleerd door extractie met hydrofobe oplosmiddelen. In een waterige omgeving (een gebufferde oplossing, die de H^+ concentratie constant houdt) worden de lipiden weer gedwongen zich in een membraan te rangschikken. Vele kleine stukjes membraan rangschikken zich ieder spontaan om een klein druppeltje buffer: liposomen. Om te zorgen dat al deze liposomen even groot zijn, worden ze gedrukt door een plastic folie met gaatjes van een bekende diameter (0,0002 mm). De liposomen werden hierdoor ook ongeveer 0,0002 mm groot. Voor de metingen van H^+ doorlaatbaarheid van het liposoommembraan wordt de buffer aan de buitenkant vervangen door een buffer met minder bufferwerking. Als nu aan de buitenkant H^+ wordt toegevoegd, dringt het H^+ langzaam door het membraan de liposomen in, en wordt binnenin opgevangen door de sterke buffer in de liposomen. De concentratie H^+ buiten de liposomen neemt dus gedurende het experiment af. De H^+ concentratie (pH) buiten de liposomen wordt voortdurend gemeten en de snelheid van afname van de H^+ concentratie buiten de liposomen werd gebruikt om de verschillende organismen te vergelijken.

Voor de Na^+ doorlaatbaarheid wordt radioactief natrium binnen in liposomen ingesloten. Om te meten hoeveel radioactief natrium zich in de liposomen bevindt, wordt een monster van de liposomen op een filter gebracht en nagespoeld met verse buitenbuffer. Hierdoor blijven alleen de liposomen met hun inhoud op het filter achter. Vervolgens wordt de radioactiviteit van het filter gemeten, waarna uitgerekend kan worden hoeveel natrium nog in de liposomen zit. Het nemen van een monster wordt een paar keer herhaald, waardoor de afname van de interne hoeveelheid radioactief natrium in de tijd bepaald kan worden.

Invloed van temperatuur

Organismen die liefst bij hoge temperatuur leven, worden thermofielen genoemd. Er zijn ook organismen die alleen bij lage temperatuur het beste kunnen groeien. Die worden psychrofielen genoemd. Van de lipiden, verkregen uit verschillende organismen die groeien bij temperaturen tussen 4 en 85°C, hebben we liposomen gemaakt. Bekend was al dat de Bacteria-soorten die niet thermofiel zijn, hun membraan tot een zekere temperatuur intact kunnen houden. Boven die - temperatuur 'smelten' de lipiden en gaat het membraan kapot. Voor dit proefschrift is de invloed van temperatuur op de Na^+ en H^+ doorlaatbaarheid bestudeerd. Uit dit onderzoek zijn twee dingen duidelijk geworden. Ten eerste gaan alle liposomen meer ionen lekken (neemt de doorlaatbaarheid toe) als de metingen bij een hogere temperatuur werden verricht. Ten tweede hangt de doorlaatbaarheid heel erg af van de groeitemperatuur van het organisme (Hoofdstuk 2). De doorlaatbaarheid van het membraan van een organisme dat leeft bij 4°C is bij zijn groeitemperatuur (rond 4°C dus) gelijk aan de doorlaatbaarheid van het membraan van een organisme dat leeft bij 80°C bij zijn 80°C. Dat is een erg interessante waarneming, en daarom hebben we deze studie uitgebreid naar één enkel organisme, *Bacillus subtilis* (Hoofdstuk 3). We hebben *B. subtilis*

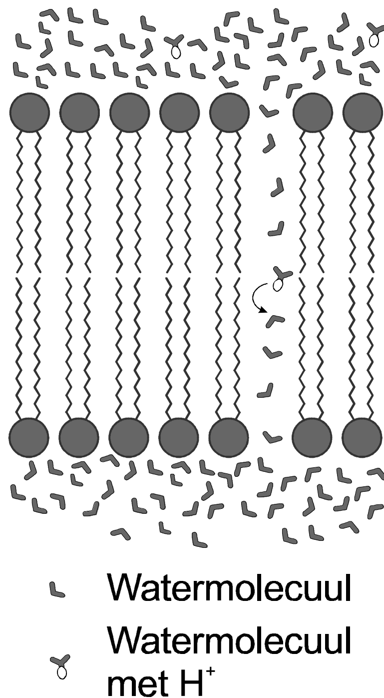


Fig. 2. Water-kanaaltje. Gedurende zeer korte tijd vormt zich een keten van watermoleculen in het membraan. Die tijd is net lang genoeg om een H^+ deeltje van buiten naar binnen te laten gaan. Het H^+ deeltje springt van het ene naar het andere watermolecuul in de keten (pijl).

laten groeien bij zijn laagst mogelijke (13°C) en hoogst mogelijke groeitemperatuur (50°C) en enkele temperaturen daar tussenin. In *B. subtilis* bleek de doorlaatbaarheid voor H^+ bij de diverse groeitemperaturen constant te zijn. De H^+ doorlaatbaarheid wordt dus sterk gereguleerd door de groeitemperatuur.

De Na^+ doorlaatbaarheid is ook gemeten. Die doorlaatbaarheid was steeds veel lager (minstens 100 keer) dan de H^+ doorlaatbaarheid. De Na^+ doorlaatbaarheid neemt toe met de temperatuur, maar de temperatuur-afhankelijkheid van de Na^+ doorlaatbaarheid is, in tegenstelling tot de H^+ doorlaatbaarheid, voor alle liposomen gelijk. Een organisme dat leeft bij hoge temperatuur, heeft gewoon een hogere doorlaatbaarheid van het membraan voor Na^+, maar die doorlaatbaarheid is altijd nog vele malen lager dan voor H^+. Sommige organismen, zoals *Caloramator fervidus*, gebruiken daarom de gemakkelijker te onderhouden gradiënt van Na^+ om ATP te maken en voedingsstoffen uit het medium op te nemen.

Het fundamentele verschil in de doorlaatbaarheid tussen Na^+ en H^+ ligt waarschijnlijk in de mechanismen van H^+ en Na^+ verplaatsing over het membraan. Na^+ doet dat via 'normale' diffusie: het komt soms van één kant midden in het membraan terecht en gaat van daar vervolgens eventueel naar de andere kant. H^+ deeltjes bewegen waarschijnlijk via een keten van watermoleculen (water wire), die soms het hele membraan overspant. Deze keten blijft heel erg kort bestaan, slechts 5 picoseconde (= 0,000000000005 = 5×10^{-12} seconde). Die tijd is net lang genoeg voor één H^+ deeltje om via de keten naar binnen te gaan. Het H^+ deeltje springt dan van het ene watermolecuul op het volgende watermolecuul in de keten (Fig. 2). Als een dergelijk water-draadje vaak voorkomt, kunnen de H^+ deeltjes in groten getale het membraan passeren. Het membraan is dan lek.

Invloed van zuurgraad

Organismen die in een zure omgeving (bij lage pH) leven, heten "acidofielen". Hun tegenhangers aan de alkalische kant (of basische kant, bij hoge pH), heten "alkalifielen". Acidofielen hebben het probleem dat er enorm veel H^+ deeltjes in hun omgeving zitten, alsof het water buiten de dijk heel erg hoog staat. Dat levert twee problemen op: de H^+ deeltjes kunnen moeilijk naar buiten worden gepompt en als het membraan lek is zijn ze ook zo weer binnen. Daarvoor heeft de acidofiel onder andere de volgende truc. Zoals boven beschreven is de energie in de H^+ energiegradiënt vastgelegd in de vorm van een gradiënt van positieve lading en een concentratie-gradiënt.

De concentratie H^+ buiten de cel is hoog; daar kan de cel niets aan veranderen. Wat de cel wel kan aanpassen is de lading binnen de cel. In normale cellen staat er een electrische spanning van enige tientallen milliVolt over het membraan, intern negatief ten opzichte van de omgeving. Met andere woorden, in normale cellen willen de positief geladen H^+ (en Na^+) deeltjes graag naar binnen. In acidofielen echter is de lading *binnen* het membraan positief ten opzichte van buiten. De H^+ deeltjes buiten hebben dan wel een enorm hoge concentratie, maar hun neiging om naar de positief geladen binnenkant van de cel te vloeien wordt behoorlijk geremd volgens het principe dat gelijkgerichte ladingen elkaar afstoten. In het onderzoek in dit proefschrift zijn twee acidofielen onderzocht: *Sulfolobus acidocaldarius* en *Picrophilus oshimae* (resp. in Hoofdstuk 2 en 5). Beide organismen hebben deze omgekeerde lading over het membraan (Hoofdstuk 5, en referenties daarin). Beide organismen hebben bovendien een extreem impermeabele membraan. Hun membraan is bij de lage pH van het medium veel minder permeabel dan van alle andere geteste organismen, waardoor de H^+ lek veel lager is. *Picrophilus oshimae* leeft bij een heel lage pH (1 en lager, zuurder dan maagzuur) en bij 60°C. Er zijn tot nu toe nog geen andere organismen bekend, die onder deze omstandigheden kunnen groeien. De lipiden van *Picrophilus oshimae* kunnen een pH, hoger dan 5 (pH 7 is neutraal), niet eens verdragen (Hoofdstuk 5). Het membraan van *Sulfolobus acidocaldarius*, een organisme dat leeft bij 80°C en pH 2,5, kan nog wel een neutrale pH weerstaan.

Alkalifielen hebben het probleem dat de H^+ concentratie buiten de cel lager is dan in de cel. Zij kunnen geen nòg lagere interne H^+ concentratie aanleggen. Alkalifielen lossen dit probleem op met een *verhoging* van de lading (buiten positief) die over het membraan staat. Het ladingsverschil over het membraan van alkalifielen is meer dan het dubbele van wat voorkomt in organismen die leven in een pH-neutrale omgeving. Op die manier worden H^+ deeltjes extra aangetrokken om naar binnen te gaan. In mijn onderzoek heb ik de proton-doorlaatbaarheid in verschillende alkalifielen getest, waaronder een alkalifiel die in zoute omstandigheden leeft (combinaties van extreme omstandigheden komen ook vaak voor). De doorlaatbaarheids-metingen in de membraanlipiden van een alkalifiel die leeft in een zoute omgeving, gaf geen verschil met het membraan van organismen die leven bij neutrale zuurgraad. Het lijkt er op dat alkalifielen, anders dan acidofielen, de doorlaatbaarheid van het membraan niet aanpassen.

Invloed van zout

Organismen die in een zoute omgeving leven heten halofielen. Het grootste probleem van een halofiel is de hoge concentratie Na^+ in de omgeving. Veel Na^+ is niet alleen slecht voor de werking van enzymen, maar veroorzaakt ook osmotische problemen. Het zout in de omgeving trekt water aan uit de cel, waardoor deze zou verschrompelen en uitdrogen. Om dat te voorkomen heeft de cel maatregelen genomen. De cel hoopt intern een ander ion op: K^+ (kalium). K^+ is minder schadelijk dan Na^+ voor het functioneren van vele eiwitten. Bovendien kunnen halofiele Bacteria en sommige halofiele Archaea nog organische stoffen in de cel concentreren. De stoffen die de cel tegen uitdroging beschermen heten osmolyten. De osmolyten zorgen ervoor dat de cel niet verschrompelt.

Halofielen gebruiken, net als de meeste andere organismen, de gradiënt van H^+ deeltjes. De concentratie Na^+ buiten de cel is veel hoger dan binnen, maar de Na^+ deeltjes die onverhoopt de

cel binnenkomen kunnen via een membraaneiwit naar buiten worden getransporteerd door uitwisseling tegen H^+ deeltjes. Zo blijft de interne Na^+ concentratie laag. De doorlaatbaarheid van het membraan van halofielen verschilt niet van die van niet-halofielen die bij dezelfde temperatuur leven (Hoofdstuk 4). De belangrijkste aanpassing van het membraan van halofielen is het vermogen stabiel te blijven bij hoge zoutconcentraties. Zelfs tot aan een concentratie waarbij het zout uitkristalliseert (verzadiging), blijven liposomen, gemaakt van lipiden van halofielen, intact en functioneel.

Archaea zijn geschikt voor een extreme omgeving

De hiervoor besproken Archaea leven vaak in de meest extreme omstandigheden. Ze hebben ook een echt voordeel ten opzichte van Bacteria en Eucarya: hun membraanlipiden zijn speciaal aangepast aan extreme omstandigheden. Bacteria en Eucarya hebben een esterverbinding tussen kopgroep en hydrofobe staart (Zie Hoofdstuk 1, Fig. 2A). Archaea hebben echter een etherverbinding (Zie Hoofdstuk 1, Fig. 2B). Etherverbindingen zijn veel stabieler dan esterverbindingen. Ze blijven ook goed intact in hoge zoutconcentraties (Hoofdstuk 4). Bovendien zijn er geen enzymen bekend die etherverbindingen in de lipiden kapot maken. Ook hebben alle Archaea CH_3-zijtakken aan de hydrofobe staarten van hun membraanlipiden, waardoor de lipiden stabieler worden. Sommige Archaea, met name degene die zowel bij hoge temperatuur als in een zure omgeving leven, hebben nog een extra troef in handen. Ze hebben de uiteinden van de hydrofobe staarten aan elkaar geplakt, waardoor het membraan nog steviger en ondoorlaatbaarder wordt (Zie Hoofdstuk 1, Fig. 2B). Van deze maatregel zou het membraan wat minder soepel worden bij lage temperatuur, maar omdat de organismen met dit minder soepele membraan bij hoge temperatuur leven is dat geen serieus probleem. Leven in extreme omstandigheden is voor Archaea gemakkelijker door de bijzondere samenstelling van de membraanlipiden.

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