This is to certify that the

dissertation entitled

GENERAL PRINCIPLES OF MEMBRANE FUNCTIONS
IN ADAPTATION AND ADAPTABILITY OF BACTERIA
TO EXTREME ENVIRONMENTS

presented by

Carol Ann Mindock

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 01/27/98

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GENERAL PRINCIPLES OF MEMBRANE FUNCTIONS IN ADAPTATION AND ADAPTABILITY OF BACTERIA TO EXTREME ENVIRONMENTS

By

Carol Ann Mindock

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1998

ABSTRACT

GENERAL PRINCIPLES OF MEMBRANE FUNCTIONS IN ADAPTATION AND ADAPTABILITY OF BACTERIA TO EXTREME ENVIRONMENTS

By

Carol Ann Mindock

Based on the hypothesis that membranes function to propagate the available thermal energy spectrum to the cell interior, using an Arctic permafrost bacterium we further confirm that there is a regular (mathematical) relationship between the available thermal energy spectrum and the membrane acyl chain length distributions. These acyl chain length distribution curves are Gaussian, in which the mean and variance are determined by temperature, indicating that at least for Gram-positive organisms the acyl chain length selection process is random. The relationship between the variances of the distribution curves and their respective growth temperatures are shown to be linear over the broad permissible growth range of the organism with deviation from linearity at the extremes of its growth temperature limit. We show that this relationship is general for Gram-positive organisms using data from the literature. This suggests that knowing the temperature, one can predict the fatty acid composition over a temperature range for a particular bacterium.

We also show that effects of chemical perturbants, such as solvents and antibiotics, alter the Gaussian acyl chain distribution curves in the same manner as increasing or decreasing temperatures depending on whether they have a fluidizing or rigidifying effect on the membrane, indicating that a temperature could be used to allow the bacterium to grow in the presence of normally unfavorable solvent concentrations.

Since heat is the driving force for all cellular chemical processes, it was of interest to determine whether temperature affects the balance of metabolic processes in the cell and how this relates to membrane structure. NMR and ^{14}C-labeling experiments show that the Arctic permafrost bacterium utilizes glucose much more slowly at 4°C than at 24°C. This bacterium makes primarily branched chain fatty acids, however, an increase in straight chains (in particular *n*-C_{16}) is seen as the growth temperature increases from 4°C to 30°C. This is consistent with the increased utilization of glucose at higher temperatures.

Here we show that Arctic permafrost strain 45-3 does not respond to near freezing conditions by synthesizing so called compatible metabolites. Rather the adaptations seen are changes in cell surface compositions, namely alterations in membrane acyl chains, the change to nonpolar headgroups, and a reduction in the amount of polysaccharide capsule.

At the other extreme of temperature, we further characterize the ability of *Sarcina ventriculi* to form transmembrane fatty acids in response to any fluidizing effect on its membrane. We show that exogenously added fatty acids can be joined tail-to-tail at their ω-1 positions to fatty acids in *S. ventriculi* membrane preparations in the presence of a low molecular weight ketone. This coupling is independent of the acyl chain headgroup as shown by the coupling of exogenously added n-heptane and hexadecylpyridinium chloride. The process is suppressed if cells are cultured in deuterium oxide indicating that the trigger is a kinetic factor related to motional dynamics. These results and results from NMR spectroscopy indicate that the process is regulated by motional dynamics and occurs via a radical mechanism in which a hydrogen atom is extracted from each acyl chain and the two acyl radicals join to form a new carbon-carbon bond. Further work is being done to isolate possible enzymes involved in this mechanism.

Dedicated to my parents for all of their love and support.

ACKNOWLEDGMENTS

In the many years it has taken me to accomplish this goal, I have had a great deal of support and encouragement from a lot of wonderful people whom I wish to thank.

First and foremost, I wish to thank my mentor and friend Dr. Rawle I. Hollingsworth. I feel privileged to have learned science and research under his guidance. He has taught me to think beyond the current views of science by applying fundamental concepts of math, physics, and chemistry to living systems. His continual support and encouragement as well as his friendship have been instrumental in helping me reach this goal.

I also want to thank the members of my committee: Dr. William Wells, Dr. Estelle McGroarty, Dr. J. Gregory Zeikus, and Dr. Melvin Yokoyama for their helpful advice and suggestions regarding this dissertation project.

I want to thank past and present members of the Hollingsworth group for all of their help, advice, support, and for making the lab such a fun place to work. I have alsoenjoyed both the scientific and cultural discussions we have had.

I would like to thank Beverly Chamberlin in the Mass Spectrometry Facility for her help in running the 505 GC/MS. I would also like to thank Dr. Long Le and Dr. Kermit Johnson in the NMR Facility for their assistance in setting up numerous NMR experiments.

In my years here I have been fortunate to have met some very great friends. I would especially like to thank Carla, Brian, and Casey Swan; Scott Herr; Kim and Greg Katch; and

Upal and Tracy Sengupta. Thank you so much for all of the fun times. Because of you I have many special memories of my years here.

Most of all I wish to express my sincerest love, thanks, and appreciation to my family: my parents, Jean and Jim; and Jim, Brenda, Joe, Katurah, Daniel, Stephanie, and Jennifer; and my grandparents, Jean and Del Hahn. My accomplishments are a result of your continual unconditional love, support, and encouragement.

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
DGDG	Diglucosyldiglyceride
DPG	Diphosphatidylglycerol (cardiolipin)
DPPC	DL-α-phosphatidylcholine, dipalmitoyl
DQF-COSY	Double quantum filtered-correlated spectroscopy
DSC	Differential scanning calorimetry
DTA	Differential thermal analysis
ESR	Electron spin resonance spectroscopy
FAME	Fatty acid methyl esters
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
LAM	Longitudinal accordian modes
MeOH	Methanol
MGDG	Monoglucosyldiglyceride
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine

PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phophatidylserine
RITC	Rhodamine *iso*-thiocyanate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TAG	Triacylglycerol
TLC	Thin layer chromatography
TOCSY	Total correlated spectroscopy
TSB	Trypticase soy broth

CHAPTER I

OVERVIEW OF GENERAL BACTERIAL ADAPTATIVE MECHANISMS

Bacteria, as a class of organisms, occupy almost all the environmental niches known on earth including many that are man-made, from extremes of temperature, pH, pressure, salinity, and solvent concentrations. A key to adaptation in the face of such vastly different circumstances is the maintenance of a functioning biochemical machinery and structural integrity. What are the most important physical properties bacteria must maintain to survive? Are there common adaptative mechanisms the various species have developed to survive more than one type of environmental condition? Or are there different mechanisms used even among species adapted to the same types of environments?

One characteristic bacteria must maintain is membrane integrity. However, it is not enough just to keep the membrane intact to ensure survival to extreme environments or to variations in those niches. It must also function under the new conditions. As single-celled organisms, the membrane in bacteria is in constant contact with its environment, and as such may serve as the primary sensor to environmental fluctuations. The membrane may also serve to transmit the available thermal energy (determined by temperature) into the cell via certain vibrations of the acyl chains [1]. Membrane fluidity is another important consideration. It is commonly known that bacteria alter their lipid and fatty acid composition as a result of various environmental perturbations. One theory for the purpose of these alterations is to maintain the same degree of membrane fluidity, termed "homeoviscous adaptation" [2]. Others argue that maintaining a certain amount of the necessary bilayer type phase is more important, termed "homeophasic adaptation" [3].

Other characteristics are also important to cell survival such as maintaining protein/enzyme stability and function; and altering the balance of metabolic pathways to utilize available nutrients and generate needed energy. These factors also play a role in determining membrane structure and composition. This literature review will focus on theories of membrane functions in adaptation and adaptability of bacteria to extreme environments as well as common molecular mechanisms of adaptation used by various types of extremophiles. Since much of the work in this dissertation was done using a Siberian permafrost bacterium, a section of this review focuses on special issues in subzero temperature adaptation.

Membrane Structure and Organization

All cells require at least one membrane to contain the cytoplasm and all cellular functions. Procaryotes, the simplest of all organisms, contain membranes only in their cell envelope. The peripheral membrane(s) is not only a boundary for the cell, but also serves as a semipermeable barrier to the outer environment as well as a location for many cell processes to take place. While the composition of membranes is complex and varies greatly between organisms, the basic structure is still considered to be the fluid mosaic system described by Singer and Nicolson [4]. This model depicts membranes as containing integral and peripheral proteins embedded in a fluid-like matrix consisting of a bilayer of lipids.

In addition to the cell envelope membrane(s), procaryotes usually also contain a rigid cell wall composed of the macromolecule peptidoglycan. In Gram-positive bacteria this cell

wall lies outside the cytoplasmic membrane. In Gram-negative bacteria the cell wall lies between the inner (cytoplasmic) and outer membrane (Figure 1.1). Peptidoglycan is a polymer composed of glycan strands cross-linked by peptides. The glycan strands are usually made of alternating units of N-acetylglucosamine and N-acetylmuramic acid which are β-1,4-linked. The short peptide chain is linked by its amino terminus to the carboxyl group of muramic acid and contains L and D amino acids. These peptide chains are cross-linked by interpeptide bridges enabling the formation of a rigid macromolecule that surrounds the cell. While there is a high degree of variability in the peptidoglycan structures among various procaryotes especially in the peptide structures, there are relatively few cases of phenotypic alterations when a bacterium is cultured under different growth conditions. When structural alterations do occur, it is usually when certain nutrients are limiting factors for growth [5].

The lipid composition of membranes, however, is highly variable not only among cell types but also due to numerous environmental perturbations. This diversity is due to variations in both the polar head groups and the fatty acyl chains. The most common membrane lipids are shown in Figure 1.2. The membranes of eucaryotes primarily consist of glycerol-based phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin. Another major class of eucaryotic membrane lipids are the sphingosine-based lipids, including sphingomyelin and glycosphingolipids. Glycolipids, both glycerol- and sphingosine-based, often function as cell-surface associated antigens and recognition factors in eucaryotes. Eucaryotic membranes, especially mammalian plasma membranes, contain

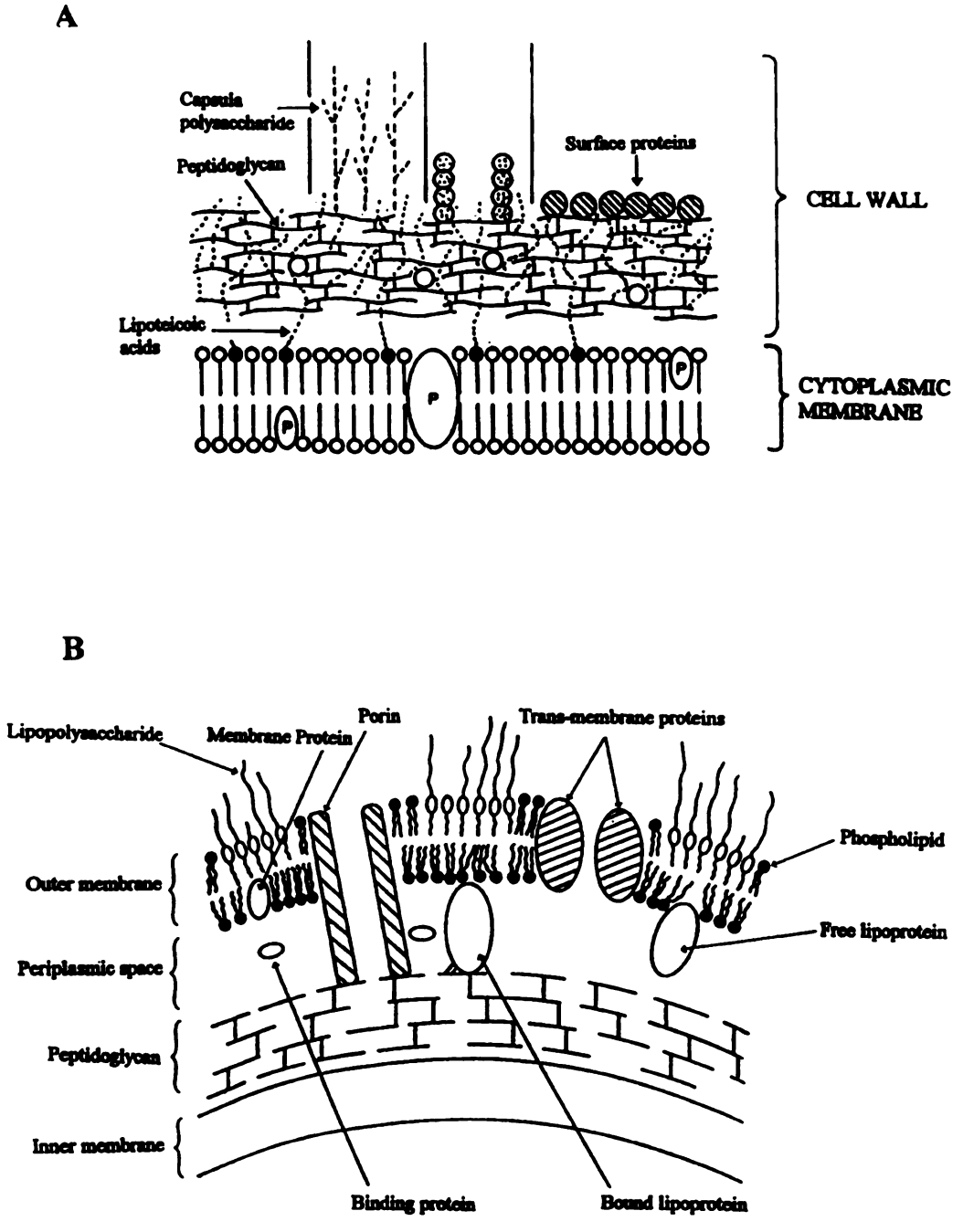


Figure 1.1: Representation of the cell envelopes of (A) Gram-positive bacteria and (B) Gram-negative bacteria. (adapted from ref. 94)

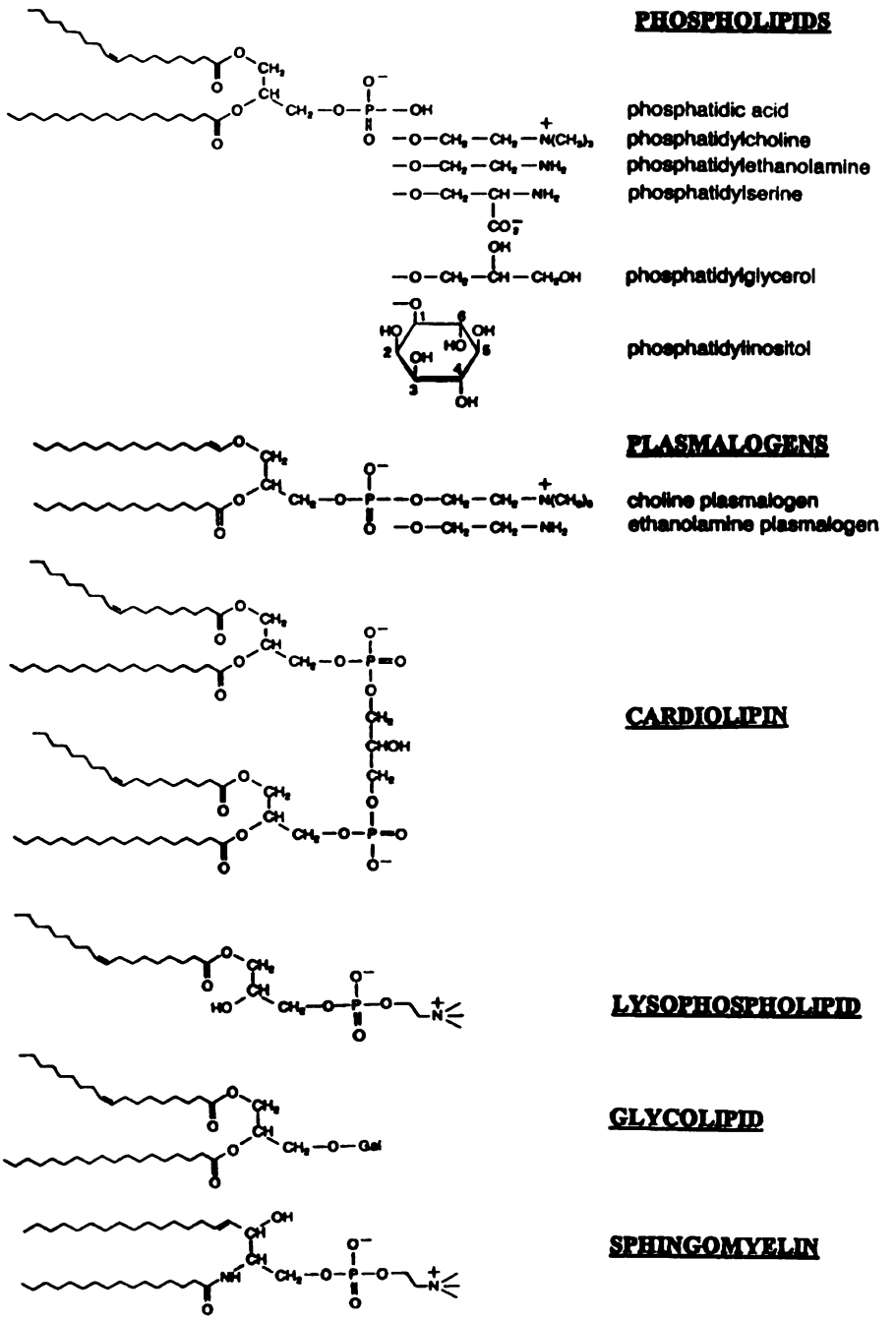


Figure 1.2: Structures of some common classes of membrane lipids.

cholesterol as well. While the eubacteria, cyanobacteria, and the archaea are typically distinguishable by their lipid composition, a common feature among them (with few exceptions) is that they all lack sterols. Among the eubacterial class phosphatidylglycerol (PG), PE, and cardiolipin are the major membrane lipids. In addition, Gram-positive bacteria also contain mono- and diglycosyldiacylglycerols. Cyanobacteria (which are photosynthetic) typically contain PG and glycolipids. The archaea, which seem to lie phylogenetically between procaryotes and eucaryotes, contain a special class of lipids in which the usual ester-linked fatty acids of the above mentioned lipids are replaced with ether linked phytanyl alkyl chains. The most common types are analogues of PG, phosphatidylglycerol phosphate (PGP), and phosphatidylglycerol sulphate (PGS). Another degree of membrane lipid diversity lies in the fatty acyl and alkyl structures. These structures vary in length, unsaturation, and branching. Some have carbocyclic rings. The possible combinations between lipid headgroups and hydrocarbon tails easily allow for hundreds of different lipid molecules to make up the membrane lipid matrix. The lipid species mentioned above are just the most common lipids found in membranes. Many organisms make other unusual lipids, especially those organisms that live in extreme environments.

Another feature of biological membranes that may serve an important function in adaptation and adaptability is the high degree of lateral organization. Experiments using several techniques show that membrane lipids (and proteins) partition into many phases or domains. These domains are either gel or liquid-crystalline in nature [6]. The driving forces for phase formation are not well understood, though some possibilities are the minimization

of free energy and geometric packing constraints. Free energy minimization would be achieved by organizational features that minimize the void spaces, the interaction of acyl chains with the aqueous phase, and the gauche:trans ratio of the carbon-carbon bonds in the acyl chains for the lipid molecules in a domain. Free energy minimization would also be achieved by maximizing the interchain interactions and by specific molecular associations that give rise to phases of different symmetry [7].

The molecular geometric packing of lipids may also be a driving force for phase separation. The size of the head group and degree of unsaturation of the acyl chains leads to three dimensional lipid structures that are cylindrical, conical, or inverted conical shaped [8]. In addition the 2-fatty acid chain of phospholipids initially extends perpendicularly to the glycerol backbone, but it bends back at the second carbon atom to become parallel to the first acyl chain. This nonequivalence of the acyl chains leads to tilting of some phospholipids [9]. As a result phospholipids with different tilts and/or different acyl chain lengths are more prone to separate into phases such that packing defects are plated out to the domain boundaries [7].

The enormous membrane lipid diversity and the organization of membrane lipids into different domains are two characteristics of membranes that enable procaryotes (and simple eucaryotes) to survive in constantly changing environments. As will be discussed further, bacteria alter their membrane lipid composition in response to numerous perturbations. The sensory mechanism of these perturbations, though, may be triggered by

disruptions in the membrane phases.

General Theories on the Roles of Membranes in Adaption

Biological membranes exist in a phase transition between a gel state, in which the lipid hydrocarbon tails are primarily in an all-*trans* configuration, and a liquid-crystalline state, in which the hydrocarbon tails contain a number of *gauche* conformers. The motional dynamics (fluidity) of these lipids is critical to the numerous functions carried out by the membrane. A large proportion of membrane lipids needs to be in the liquid-crystalline phase to maintain transport and certain enzymatic functions. Studies with *Escherichia coli* and *Acholeplasma laidlawii* indicate that growth of these organisms is severely impaired when more than 50% of their membrane lipids are in the gel phase [10]. Perturbations that alter membrane fluidity often result in a rapid alteration of lipid acyl chains and/or the modification of the lipid head groups. Typically shorter and more unsaturated acyl chains are incorporated when perturbations cause the membrane to become more gel-like; while longer and more saturated acyl chains are made in response to fluidizing effects. Other modifications include acyl chain branching and cyclization. All of these modifications affect membrane fluidity.

One theory for the alteration of membrane lipids is to maintain the same degree of fluidity regardless of growth temperature (and possibly due to other variations in the environment). This theory, termed homeoviscous adaptation, was first described for *E. coli* using electron spin resonance spectroscopy (ESR) [2]. ESR was also used to demonstrate

homeoviscous adaption in two other organisms, *A. laidlawii* [11] and *Bacillus stearothermophilus* [12]. All three organisms are able to alter their membrane lipid composition in response to temperature changes to maintain a virtually constant membrane fluidity. Other organisms also conserve membrane fluidity, though it does not always seem to be a complete compensation over the growth temperature range for particular organisms. Cossins and Sinensky proposed a coefficient of homeoviscous efficacy to quantify the compensatory response. The efficacy was calculated as the ratio of the change in membrane order to the difference in growth conditions (temperature, pH, etc.). An efficacy value of 1.0 indicates complete compensation of membrane fluidity, while values between 0 and 1.0 indicate partial fluidity compensation for the different conditions. The efficacy for procaryote membranes varies between 0.2 and 1.0, while the efficacy for all eucaryotes is less than 0.5 [13]. The large variation in efficacy values may be due in part to the techniques and/or probes used.

Membrane fluidity is related to the properties of its composite lipids. The lipid bilayer, however, is not a typical liquid and as such does not behave like a bulk fluid. For typical liquids, like water, fluidity is defined as the reciprocal of viscosity. Membranes have different characteristics which also must be taken into consideration when trying to determine fluidity. For instance membranes are microscopic fluids with stringent boundary conditions and are essentially two-dimensional. Also, while typical fluids have distinct phases with regards to temperature (solid, liquid, or gas), membranes have numerous phases that can exist simultaneously (solid and liquid-crystalline).

Due to these special characteristics of bilayers, "membrane fluidity" is not very well defined and thus is hard to quantify. Typically membrane fluidity refers to the disorder of the acyl chains (structure) and/or their ease of movement (dynamics). Therefore, quantization of membrane fluidity encompasses several parameters such as diffusion (translational and rotational), order, packing, and permeability. These parameters are related, though how is not clearly understood [14]. As a result different techniques and different spectroscopic probes may measure different membrane parameters--yielding widely varied results in the measurement of membrane fluidity. Differential scanning calorimetry (DSC) and differential thermal analysis (DTA) are nonperturbing methods used to measure biomembrane phase transitions, but are not capable of direct measurement of fluidity within a given phase state. ESR and fluorescence spectroscopy are often used to directly measure lipid phase states and fluidity, but the probes used may partition into certain nonrepresentative domains as well as perturb the membranes. Nuclear magnetic resonance (NMR) techniques, such as T_1 relaxation measurements, are capable of accurately monitoring phase transitions and providing orientations and rates of motion of the lipid molecules [10]. NMR techniques are likely to provide the most accurate measurements of membrane lipid fluidity, though currently they are not widely used. Despite the different techniques used, it is clear that most organisms do alter their membrane compositions to maintain a constant degree of membrane fluidity.

Another theory has been proposed for membrane lipid composition alterations based on the highly variable homeoviscous efficacy coefficients. This theory, termed homeophasic

adaption [15], suggests that it is more important for organisms to maintain a particular ratio of bilayer to non-bilayer forming lipids. A large fraction of non-bilayer forming phases would decrease the stability of the bilayer. The three dimensional structure of lipid molecules depends on the size and shape of its headgroups and acyl chains. Lipids that have a cylindrical shape (like PC, PS, and PG) would favor the typical bilayer phase. PE containing unsaturated acyl chains, and cardiolipin or PA complexed with calcium form conical molecular structures, which favor inverted hexagonal or cubic phases (Figure 1.3). Lysophospholipids form inverted cones and would favor micelle type structures [8, 16]. Non-bilayer structures have been implicated in such physiological functions as adhesion, joining, and fusion [16, 17].

In support of this theory Wieslander and coworkers proposed that the regulation of lipid composition in *A. laidlawii* is governed by molecular shape. The dominate lipids in this organism are monoglucosyldiglyceride (MGDG) and diglucosyldiglyceride (DGDG), which have wedge and rodlike molecular shapes, respectively. The organism regulates the level of these two lipids to maintain a balance of lamellar and hexagonal phases [18-20]. While this theory is interesting, evidence supporting the formation of non-bilayer phases primarily comes from experiments with single lipids, or a mixture of only a few types of lipids. Membranes are extremely complex mixtures, and the fact that simple (few components) lipid mixtures assume non-bilayer phases in water does not mean they will form the same structures in a complex mixture.

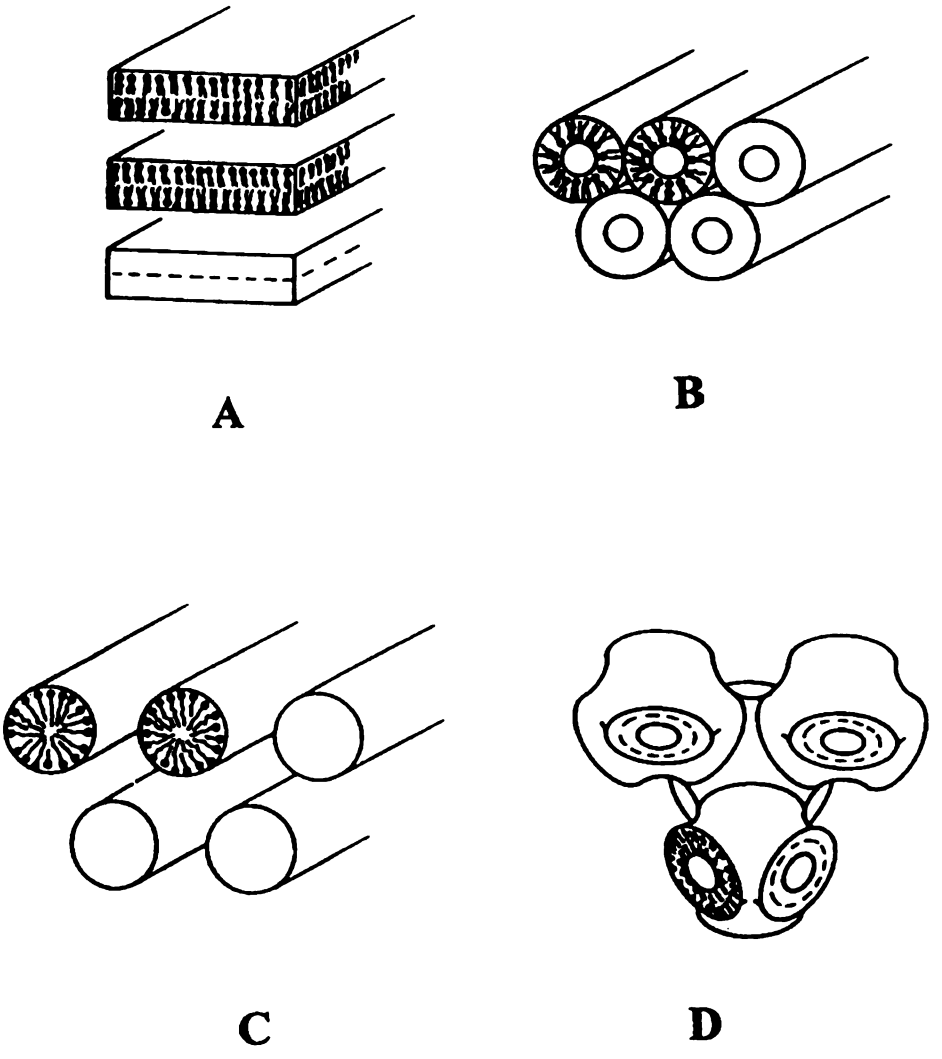


Figure 1.3: Lipid polymorphism in the liquid crystalline state. (A) Lamellar phase (L_α); (B) Hexagonal inverted phase (H_{II}); (C) Hexagonal phase (H_I); and (D) Cubic phase. (adapted from ref. 95)

Membrane acyl chain heterogeneity also serves another function in the adaptability of organisms to their niches. As membranes completely surround cells, they are capable of transferring the available thermal energy (which are propagated by collisions) to the interior of cells. The heat or infrared quanta from the environment (of the magnitude "kT") is transferred by molecular vibrations. These quanta are passed via water in biological systems by collisions with other molecules. The acyl chains of membrane lipids serve as oscillators to focus these quanta to the cell interior. The quanta are transferred by specific acyl chain vibrations called longitudinal accordian modes (LAMs). By changing the lengths of the acyl chains, the membrane is tailored to propagate the available thermal energy spectrum. Evidence that membranes transform the available energy is shown in experiments in which the probability distributions of the main acyl chain length series are plotted from organisms at different growth temperatures and in the presence of D_2O. Probabilities of the energies per chain are plotted. The graphs indicate a Gaussian distribution of the acyl chain lengths and their energies, and there is a direct correlation between the variance (σ^2) of the Gaussian distributions and kT. As temperature decreases, kT decreases as well. The distribution of available energies becomes more narrow as indicated by an increase in the height of the Gaussian curve. Therefore, low temperature effects (including D_2O effects) indicate cells have a more limited set of available energies which can be utilized. This study indicates that organisms are able to "tune" their membranes to the value of kT by adjusting the acyl chain distributions of the membrane lipids [1].

Molecular mechanisms of adaptation

Procaryotes and the archaea, unlike most eucaryotes, have the ability to adapt to many environmental extremes. Procaryotes and the archaea colonize most of the harshest environments on earth, from extremes in temperature (hot and cold), pH (acidic and alkaline), salinity, osmotic pressure, and they survive in the presence of many detrimental chemical substances. To survive in all of these environmental conditions organisms must maintain certain functions, including membrane structural and functional integrity, osmotic balance, protein stability and function, and the ability to carry out numerous metabolic processes. In adapting to the environment (and changes within) these organisms have certain molecular mechanisms which they have altered (or can alter) to maintain critical cellular functions. These alterations include changes in membrane and other cell surface structures, osmolyte changes, protein stability and expression, and the ability to balance metabolic processes.

Membrane alterations

Bacteria can alter their membrane structure in many ways. One way is to alter the lipid composition, though it is not always affected by environmental changes--the most commonly studied of which is temperature. In one study psycrophiles, and not psycrotrophs, were found to alter their lipid composition in response to temperature [21]. In another study, four psychrophilic *Vibrio* species were found to have thermosensitive conversion of PS to PE or of PG to DPG, though the direction of the temperaure change that induced these conversions was not the same in the four organisms [22]. On the other hand, several

psychrotropic organisms like *Pseudomonas spp.* [23] and *Micrococcus cryophilus* [24] do not alter there phospholipid composition in response to temperature changes. It has been observed for several organisms, including *Tetrahymena* [25], *Mycobacterium smegmatis* [26], and several temperature-sensitive mutants of *E. coli* [27, 28], that the ratio of cardiolipin to phosphatidylglycerol increases with temperature. The increase in cardiolipin effectively cross-links the lipid headgroups to counteract increased membrane fluidity with the increase in temperature. Similarly, *Sarcina ventriculi* also cross-links its lipid headgroups in response to numerous perturbants that increase membrane fluidity [29]. One strain of *Bacillus stearothermophilus* increases its phosphoglycolipid content when the growth temperature is raised from 45 to 71 °C [30] This result is in agreement with the trend towards a general increase in the amount of glycolipids with increasing temperature observed in organisms ranging from psychrophiles to moderate and extreme thermophiles [3]. In Tetrahymena the alterations in lipid headgroups is much slower, and is suggested to be a secondary adaptive mechanism to fine tune membrane dynamics after altering acyl chain composition [31].

While bacteria may or may not alter their lipid headgroup composition, most eubacteria and cyanobacteria alter their fatty acid composition in response to perturbations in their environments. Eubacteria can alter the membrane fatty acid chain length, unsaturation, branching, and cyclization. Cyanobacteria can only alter the chain length and unsaturation of their acyl chains [3]. Typically changes to the membrane acyl chains occur rapidly in response to perturbations. Shorter, more unsaturated or branched acyl chains occur

when bacteria are exposed to cold, high pressures, and substances that reduce the membrane fluidity. Fatty acid cyclization may also decrease on lowering growth temperatures in Gram-negative bacteria. This is seen in such organisms as *E. coli* [32, 33], *P. fluorescens* [34, 35], *P. halosaccharolytica* [36, 37], *Proteus mirabilis* [38], and *Serratia marcescens* [39]. In response to temperature changes the amount of branched chain fatty acids and/or the relative proportion of *anteiso* versus *iso*-branched fatty acids is altered in Gram-positive bacteria. Low temperatures result in an increase in the lower melting *anteiso*-fatty acids and decreasing amounts of higher melting, odd-carbon, *iso*-fatty acids [40]. The reduction of cyclopropyl fatty acids seen in Gram-negative bacteria as temperatures drops is probably due to the synthetic mechanism for forming these fatty acids. The formation of these fatty acid involves methylation by S-adenosylmethionine (SAM). The synthesis of SAM requires a lot of energy (ATP). As will be discussed in more detail, the available thermal energy which drives cellular processes is much less at low temperatures causing the formation of SAM to be thermodynamically unfavorable, such that less SAM would be available at lower temperatures. The increase in branched fatty acids in Gram-positive organisms also is most likely due to thermodynamic alterations in the balance of cellular metabolic pathways. A shift in metabolism at low temperatures to favor amino acid metabolism over carbohydrate metabolism would increase the branched acyl-CoA primers available for fatty acid synthesis causing an increase in the amount of branched lipid acyl chains.

In aquatic environments organisms must contend with hydrostatic pressure. On average hydrostatic pressures below the ocean continental shelf are 200 atmospheres and

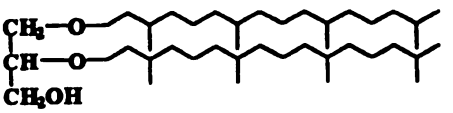
reach as high as 1100 atmospheres at the deepest part of the ocean. In addition temperatures below 1000 m are usually less than 4°C. Hydrostatic pressure compresses lipid bilayer causing an ordering effect on the acyl chains increasing the gel state of membranes. Hence pressure acts similarly to low temperatures in causing a decrease in membrane fluidity and as a result similar alterations in membrane acyl chains are observed [41]. The barophilic marine bacterium CNPT3, most likely in the *Vibrio* genus, increases it's proportion of unsaturated acyl chains particulary $C_{16:1}$ and to a lesser extent $C_{18:1}$ at the expense of $C_{14:0}$, $C_{16:0}$, and $C_{14:1}$. While the amount of $C_{14:1}$ decreases with increasing pressure this bacterium still contains a larger percent of $C_{14:1}$ than most marine vibrios [42].

In addition to temperature and pressure, the chemical composition of the aqueous environment surrounding cells influences cellular structures and functions. In regards to cell membranes, a major portion of the lipids are anionic resulting in a negative surface potential which is stabilized by cations in the aqueous phase. As a result the salinity and pH of the surrounding aqueous phase effects the membrane surface potential and ionization of the anionic phospholipids [41]. It has been observed that an increase in salinity results in similar acyl chain changes cause by low temperatures and increased pressures. Namely in Gram-negative bacteria cyclopropyl and unsaturated fatty acids increase while in Gram-positive bacteria an increase in branched chain fatty acids is observed. The increase in cyclopropyl fatty acids of Gram-negative bacteria must be interpreted with caution since in some strains the major change is from unsaturated to cyclopropyl acyl chains, which would not alter the fluidity greatly [43]. In the halotolerant (meaning no specific salt requirements for growth)

bacterium *Staphylococcus epidermidis* a large increase in *anteiso*-15:0 fatty acids occurs in response to salt concentrations of 25%, which is again a similar response to low temperature [44]. The increase in salinity would affect the polar headgroups of lipids, and cellular membranes in general have an overall negative charge. In fact, an increase in the anionic membrane lipid content relative to zwitterionic lipid has been observed with increased salinity. In Gram-negative bacteria the major change is an increase in PG over PE, and sometimes an increase in DPG as well. In Gram-positive bacteria, which usually have a more complex lipid composition, an increase in phosphoglycolipids, PG, and/or DPG is seen with increased salinity [43]. These changes in headgroups, however, are similar to the fluidizing effects caused by increased temperatures. Some aspects of these trends in acyl chain versus headgroup alterations are confusing and may arise from differences between the systems studied and the ranges of salt concentrations used in the studies. For instance the cations of the salts at low to medium concentrations may stabilize the negative charges of the lipid headgroups through electrostatic interactions causing an ordering effect on the acyl chains and rigidifying the membrane. As a result more unsaturated, cyclopropyl, and/or branched acyl chains would offset this rigidifying effect. However, at high salt concentrations the abundance of ions would have a chaotropic effect on the membrane. To counteract this fluidizing effect increases in DPG (cardiolipin) would cross-link the lipid headgroups and stabilize the membrane.

Longer, saturated and straight acyl chains occur in membranes when bacteria are exposed to high temperatures, extremes of pH, and substances that increase membrane

fluidity. In addition to these general acyl chain structural changes, many bacteria have special types of membrane lipids that allow these organisms to thrive in harsh environments or adapt to habitats that undergo many extremes. These include, in particular, thermophilic bacteria or bacteria that can adapt to high temperatures. Some of these specialized lipids are shown in Figure 1.4. These lipids include the tetraether and diether lipids of the archaea. The alkyl ether linkage is resistant to cleavage over the wide pH range encountered by these organisms. All three classes of the archaea (extreme halophiles, methanogens, and thermoacidophiles) have membranes composed of lipids derived from one basic core structure, diphytanylglycerol ether. In addition, some archaea lipids contain one to four diphytanylglycerol ether cyclopentane rings [45]. The membranes of these organisms are very tolerant to extreme conditions. Archaea inhabit climates with temperatures above 100°C as well as niches with 36% salt content, pHs above 11 and less than 1, and pressures as high as 1100 atmospheres in the deepest part of the ocean. [46]. The extreme halophilic archaea contain only diether derivatives. This may, however, be a reflection of their typical growth temperature rather than their tolerance for salt, meaning they may not be adapted for extremely high growth temperatures which might require tetraether lipids. But as temperature increases the percent of tetraether lipids increases to the point where the thermoacidophiles contain only tetraether derivatives. Similarly, *Sarcina ventriculi* forms α,ω-fatty acids and cross-links its headgroups forming a bipolar monolayer, which is reminiscent of the tetraether lipids in archaea. *S. ventriculi* synthesizes these lipids in the presence of high temperatures, acidic and alkaline pHs, and in the presence of certain fluidizing solvents [29, 47]. The anaerobic thermophilic bacterium *Thermoanaerobacter*



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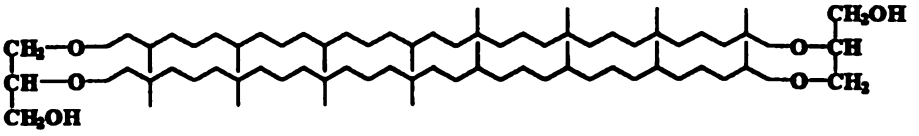

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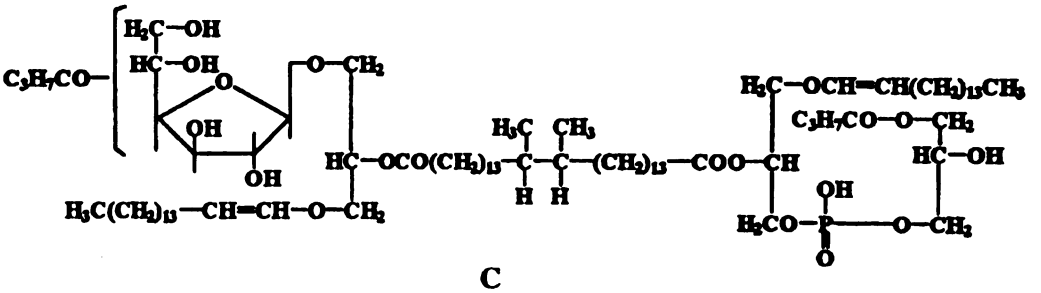
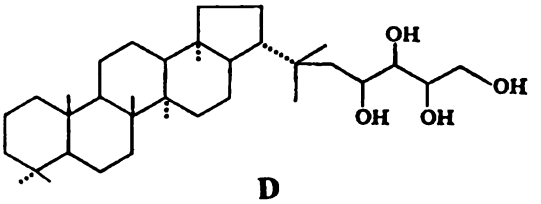


C



D

Figure 1.4: Structures of novel lipids from bacteria. (A) diether lipid and (B) tetraether lipid from Archaea; (C) a diabolic acid-containing phospholipid from *Butyrivibrio* S2; and (D) a tetrahydroxybacteriohopane lipid from *Acetobacter*.

ethanolicus 39E [48] and the hyperthermophile *Thermatoga maritima* [49] also synthesizes α,ω-dicarboxylic fatty acids in response to high temperatures. Other specialized lipids include hopanes, and ω-cyclohexyl fatty acids.

Hopanes are the bacterial equivalent of cholesterol [50]. These structures have been found in diverse bacteria including *Acetobacter* [51], cyanobacteria, obligate methylotrophs, and in many Gram-negative and Gram-positive chemoheterotrophs [52]. ω-Cyclohexyl fatty acids are the predominant fatty acids in the thermoacidophiles isolated from hot springs all over the world. Most have been initially classified as strains of *Bacillus acidocaldarius.* But ω-cyclohexyl fatty acids are also major components in some mesophilic bacteria such as *Curtobacterium pusillum* [53, 54]. Several strains of thermoacidophilic bacteria isolated from Japanese hot springs included anywhere from 74 to 93% of these fatty acids, and biosynthesis of these lipids increases with increasing glucose in the medium [53]. This is necessary because acids and solvents are made from glucose as a primary fermentation activity. To protect its membrane, the levels of these ω-cyclohexyl fatty acids must increase as glucose concentration increases and fermentation activity increases as a consequence. The region of greatest hydrocarbon chain mobility in a biomembrane is the area between the two leaflets (Figure 1.5) [55]. By incorportating an ω-cyclohexyl group in this zone, the organism can reduce the extent of chain terminus rotation and wagging. This affords a very high degree of motional restriction and hence control of dynamics. The flat (all trans-fused) hopane molecule does the same, but along the entire length of the hydrocarbon chain.

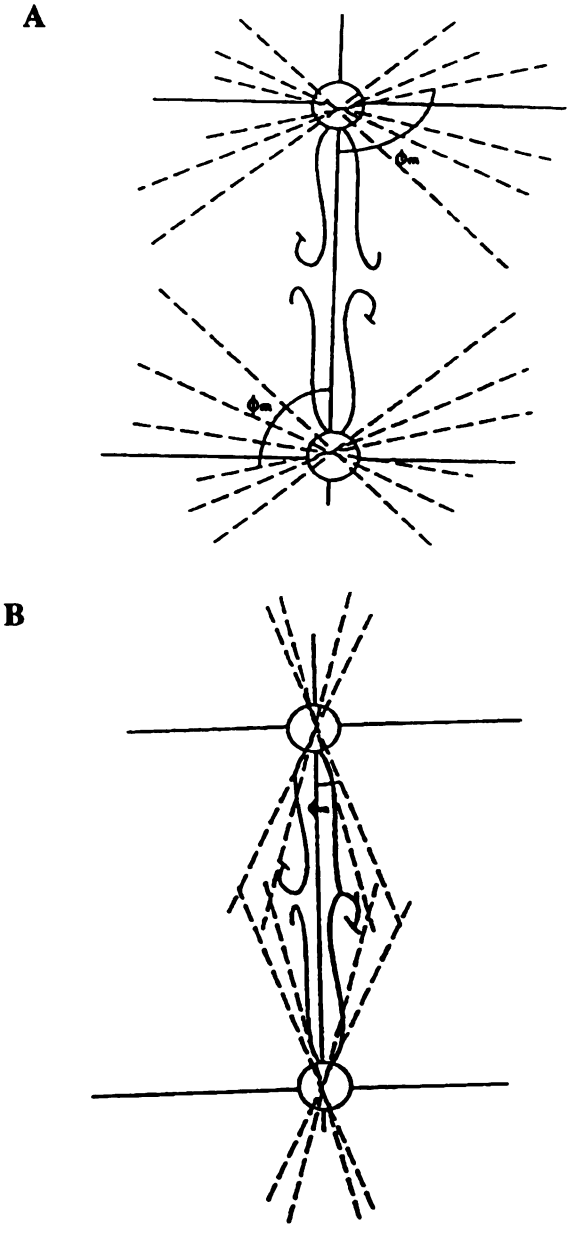


Figure 1.5: Simplified pictures of the motional spectrum of lipids. Φ_m indicates the maximum angle subtended by the two acyl chains at the headgroup. It is a measure of the extent to which the acyl chains are sprayed. (A) Monolayer form, and (B) Bilayer form.

Protein stability and function

Cells must also maintain protein stability and function. The studies to date primarily focus on protein stability at high temperatures, mainly from thermophilic organisms. While the exact mechanisms that confer high temperature tolerance are not known, some suggestions are fewer amino acids that are vulnerable to heat and the formation of "salt-bridges," which consists of ionic bonds through the interior preventing denaturation. Enzymes from thermophilic barophiles, isolated from deep sea vents, often consist of a more dense hydrophobic interior as well as containing salt bridges. Increased pressure stabilizes van der Waals interactions and is thought to destabilize salt bridges. In general enzymes isolated from bacteria adapted to high temperatures are also extremely resistant to high pressure [56]. Proteins from extreme halophilic and even some moderate halophiles have been shown to contain a high content of acidic amino acids and are also relatively deficient in nonpolar amino acids compared to nonhalophiles [43]. The tendency to increase the amount of acidic amino acids relative to basic amino acids may not be general. Increased proportions of polar amino acids will, in general, help in keeping the proteins hydrated in this highly polar environment. Increases in NaCl and KCl increases the extent of interactions of hydrophobic groups again by a "salting out effect." Proteins with less nonpolar amino acids will maintain a more flexible conformation and a higher degree of hydration with increased salinities.

In addition to protein stability, certain enzyme activities and metabolic processes must be maintained. Since membrane alterations, especially changes in acyl chain structures,

occur in response to environmental perturbations, some aspect(s) of lipid biosynthesis must be regulated by the effects of the environment on the cell. Temperature regulated changes in membrane fatty acid composition have been studied the most. Two mechanisms for making unsaturated lipids are known. These pathways are 'aerobic' or 'anaerobic' and can usually be distinguished by the position of the double bond in the acyl chain. The aerobic pathway requires molecular oxygen and results in palmitoleate (16:1Δ9*cis*) and oleate (18:1Δ9*cis*) as the primary unsaturated products. The anaerobic pathway yields palmitoleate and *cis*-vaccenate (18:1Δ11*cis*) as the major unsaturated products [57]. All anaerobic and facultative anaerobic bacteria use the anaerobic pathway of fatty acid biosynthesis. In *E. coli*, which uses the anaerobic pathway, the ratio of saturated to unsaturated acyl chains is thought to be determined by the thermal regulation of β-ketoacyl-ACP synthase. This enzyme exists in two forms in *E. coli*, type I and II, and catalyzes the two carbon elongation reaction of fatty acid biosynthesis. Type II is more efficient in elongating palmitoleate than palmitate (16:0) and is more temperature labile. Thus at low temperatures palmitoleate is elongated to *cis*-vaccenate and less palmitate is made. In this case the effect of longer chains is outweighed by the effect of increased unsaturation. In this organism the thermal regulation of fatty acid composition does not require *de novo* protein synthesis, only changes in activity of existing enzymes occurs [58].

The aerobic pathway for producing unsaturated acyl chains involves desaturation of saturated fatty acids. In this case the fatty acid synthetase yields saturated fatty acids only. The desaturases, which are membrane-bound, are believed to work on membrane

phospholipids directly. Temperature then could regulate these enzymes by inducing membrane phase changes to alter the enzymatic activity, or by induction of new protein synthesis [58]. In *Bacillus* strains the desaturase enzyme is temperature labile resulting in faster turnover rates with increased temperatures. Drops in temperature then cause an increase in the amount of desaturase present in the membrane (slower turnover rate). Lower temperatures also are known to induce desaturase activity in these organisms [59].

As noted earlier, changes in membrane acyl chain length occur with alteration of temperature. It is proposed that an elongase enzyme that is thermally regulated is responsible for this. Hence, *Micrococcus cryophilus* alters the ratio of oleic and palmitoleic acids, the only fatty acids in its membrane, in response to temperature supposedly through an elongase enzyme that interconverts C_{16} and C_{18} fatty acids by way of a C_{14} intermediate [58]. Russell and coworkers suspect an essential component of this process such as acyl carrier protein (ACP) turns over rapidly because inhibitors of protein synthesis only partially inhibit this interconversion [58]. An alternative idea is that the acyl chain length selection process is random, and that the most viable cells at a given temperature have a particular acyl chain distribution. Dramatic shifts in temperature select for the bacterial populations with the best suited acyl chain length distributions or the ones that can alter their distributions to function at the new temperature.

Other alterations of fatty acid structures are seen in response to temperature including changes in the cyclization, branching, and cross-linking of fatty acids, though the

mechanisms of thermal regulation of these syntheses has not yet been shown. The synthesis of *iso*- and *anteiso*-branched fatty acids requires *de novo* fatty acid synthesis using primers derived from amino acids. Temperature regulation of the transaminases that convert amino acids to their α-keto acids has not been demonstrated as of 1984 [58], and a subsequent literature search to the present has found no further evidence that this has been demonstrated. As will be discussed later, the thermal regulation of the synthesis of the branched fatty acids relates to the available pools of acyl-CoA primers as well as to the available thermal energy. It has been shown that the tail-to-tail cross-linking of *S. ventriculi* membrane acyl chains is temperature dependent as well. The regulation occurs presumably through activation of a constitutively expressed enzyme, since inhibitors of protein synthesis do not affect this activity [29]. Similarly, the Archaeon *Methanococcus jannashcii* has been shown to increase the proportions of tetraether lipids (C_{40}) over the level of diether lipids (C_{20}) with increasing temperature [60], indicating some aspect of the formation of tetraethers is also thermally regulated.

In addition to regulating enzymes involved in controlling fatty acid composition many organisms produce 'heat shock' proteins in response to detrimental stress factors. Though they are called heat shock proteins, they are synthesized in response to extreme changes in temperature (hot or cold), acid, ethanol, and other physical and chemical perturbations. These proteins are constitutively expressed at very low levels, but with a sudden large temperature increase (or extreme changes due to other perturbants) the levels of these proteins rapidly increase. As the cells acclimate to the new environmental condition, the heat

shock protein levels decrease slowly, usually over a couple of hours, back to basal levels. If cells, however, are slowly acclimated to a higher temperature (or other condition), the levels of heat shock proteins do not increase [61]. The major heat shock proteins (Hsp) are believed to function as molecular chaperones to aid in protein folding and prevent protein aggregation of unfolded proteins [62]. Some of these same proteins induced by heat are also induced by other stresses such as ethanol and inhibitors of oxidative phosphorylation [63]. *E. coli*, *Bacillus cereus* WSBC 10201, and *Pseudomonas fragi* all produce a new series of proteins in response to cold shock, one in particular is CspA [64-66]. Some of these cold shock proteins are thought to function in control of transcription and translation, namely to prevent initiation of protein synthesis [67]. *E. coli* and *Salmonella typhimurium* are two examples of bacteria that produce acid shock proteins [68, 69]. While the exact functions of most shock proteins are not known, they may simply reflect the shifts in metabolic pathways that must occur in response to changing environmental conditions.

Osmotic balance

Changes in concentrations of solutes in the aqueous environment surrounding organisms determines the flow of water into and out of the cell. Cell membranes are readily permeable to water but not to many solutes. As a result, these osmotic changes alter cell volume and/or pressure, and changes in cell volume lead to fluctuations in intracellular solute concentrations as well. While the cellular signals for sensing osmotic changes are not really known at this time [70], several physicochemical changes that might be used by Gram-negative bacteria have been suggested. These theories include baroreceptors for detecting

hydrostatic pressure changes in the interior bulk liquid phase of the cell, which have been viewed as unlikely [71]; turgor pressure sensors in the inner membrane which respond to compression against the peptidoglycan, and wall stretch receptors which respond to tangential stress within the peptidoglycan layer [72]; stretch receptors for detecting changes in membrane surface area [73]; and chemoreceptors for detecting internal and external solute concentration or water activity [74].

It is thought that a common adaptive response for many organisms is to alter intracellular concentrations of solutes that do not produce adverse effects on cellular macromolecules [71, 75, 76]. These solutes are referred to as osmolytes or compatible solutes. Compatible solutes include K^+ ions; amino acids such as glutamate, glutamine, proline, and glycine betaine (an N-methyl substituted amino acid); trehalose and mannosucrose, which are disaccharides; and others such as monosaccharides and glycerol. Osmolytes in high concentrations are seemingly not too toxic, but they do affect some biochemical processes. It has been proposed that increased intracellular concentrations of osmolytes has overcome temperature-sensitive mutations [76] as well as increased the upper temperature growth limit [77, 78], and conferred cryotolerance [79] on many bacteria. Results from these studies should be taken with caution, though, because the media were altered and analysis on the membrane chemistry and the levels of protein expression were not carried out. Bacteria that are thought to produce compatible solutes include *Escherichia coli* [80-82], several marine methanogens [83], *Rhizobium meliloti* [84], and many others [70]. It should be noted, however, that an increase in compatible solutes may not indicate

osmotic adaptation. The increase (or decrease) of certain metabolites may be a consequence of the new conditions to which the cell has adapted. Alterations in metabolic pathways are to be expected as environmental conditions change. So an increase in metabolites may not be the cause of adaptation to changes in osmotic pressure. For instance, if the increase in a particular osmolyte is still low compared to the external osmolality there is little significance in this event with regards to adapting to changes in osmotic pressure. For example, the glutamine pool in *E. coli* remains proportional to the external osmolality, but is 10% less than the intracellular K^+ ion concentration [80]. In halophilic archaea the glycine betaine concentration is about 100 fold less than the major compatible solutes (K^+ and Cl^- ions) [85]. So in these instances the presence of the osmolytes may serve a different function and/or be a consequence of shifts in metabolism [70]. Again, it is thought that bacteria respond to the reverse situation, hypoosmotic shock (water influx), by decreasing osmolyte concentrations via excretion, dilution by growth, catabolism, and polymerization. As will be discussed in more detail in chapter 4, osmolytes are also thought to aide in cryoprotection of microorganisms, but this is not the only means by which bacteria can survive subzero temperatures. In the next section special issues relevant to survival in subzero climates is reviewed.

Special issues in subzero temperature adaptation

A great deal of research has focused on the adaptation of organisms to extreme heat. One reason for this is the potential industrial use of enzymes and organisms that can withstand such high temperatures. However from the aspect of adaptation of organisms to

extreme environments, the majority of the earth's biosphere is subjected to subzero temperatures permanently or seasonally. Water can be supercooled to the extent that it can still be a liquid down to -40°C and below. As such, organisms deal with stresses from supercooled water as well from freezing. In supercooled water the degree of ionization is greatly decreased, affecting its ability to serve as conjugate bases or acids in reactions. Also the viscosity of the water increases and thus diffusion rates decrease [86]. In light of these and other changes that occur as water is chilled or frozen, the organisms that inhabit these climates have special circumstances which must be dealt with in order acclimate to subzero temperatures. These issues include responding to changes in salt/metabolite concentrations and oxygen availability; prevention of freezing of intracellular water; and provision of cellular maintenance energy, carbon sources and heat generation.

Salt/metabolite concentrations and oxygen availability

As water freezes it generally separates out as pure water (ice) causing an increase in salt and metabolite concentrations in the remaining liquid phase. Bacteria in the liquid phase would need to adjust to these increases. However, bacteria that may be trapped within the ice see the other extreme: a sharp decrease in available salts both organic and inorganic. Diffusion of these salts and metabolites for use by the cells is now extremely limited.

Oxygen on this planet is generated by photosynthesis, which obviously requires light. In most subzero climates, oxygen is primarily generated by algae but only on the surface of snow. For microorganisms buried underneath these frozen habitats, oxygen becomes

limiting very quickly because it cannot diffuse well through the ice. The bacteria must then develop efficient anaerobic mechanisms for generating energy using other inorganic terminal electron acceptors that are available. These electron acceptors may be nitrate, carbon dioxide, sulphate, thiosulphate, or sulfite [87].

Prevention of freezing of intracellular water

Freezing of intracellular water causes several problems, the major one is cell rupture due to the expansion of frozen water. Another problem is an imbalance in electrolyte concentration. As ice forms it forces out the solutes creating areas with high electrolyte concentrations causing osmotic changes that result in the efflux of water. It is also possible for freezing to result in eutectic mixtures in which some of the solutes remain within the ice, preventing their availability for use by the cell. Cells must also contend with decreased diffusion of metabolites as water becomes supercooled and/or freezes.

Maintenance energy

All cells expend a certain amount of energy to maintain organization at the molecular level. This includes the spatial orientation of membranes, proteins, nucleic acids, etc. As is well known, there exists a natural tendency towards randomness and disorder (increase in entropy) in the universe. Therefore maintaining the necessary levels of organization as well as carrying out the biosynthesis of macromolecules, requires energy.

More than two decades ago calculations were made by several researchers to estimate

the amount of ATP needed for maintenance energy by bacteria. John Pirt determined a maintenance coefficient for aerobic and anaerobic continuous cultures of *Aerobacter cloacae* [88]. This is an index that tells the amount of glucose needed to maintain a gram of cells. This coefficient was defined as the slope of the line of plots of the reciprocal of the yield value versus the reciprocal of growth rate. The values for the aerobic and anaerobic cultures were 0.09 and 0.47 grams of glucose per gram dry weight bacterium, respectively. The higher coefficient for anaerobic cultures is expected due to less efficient energy yields from anaerobic metabolism. Using the coefficient for the anaerobic culture, the amount of ATP needed to maintain one gram dry weight bacterium was one-twentieth (~0.75 mmole) the ATP necessary to produce one gram dry weight of this bacterium. It should be noted, though, that this value far exceeds the free ATP pool (~10 μmoles per gram dry weight bacterium) usually available in a given microorganism [89]. About ten years later Tempest and colleagues [90], by modifying Pirt's derivation, determined another linear relationship. The slope of their plot of energy source/cells/hour versus growth rate is also defined as a maintenance coefficient, but the error in this slope is primarily in the intercept. In the Pirt plots, the error is in the slope. While these two derivations for determining maintenance energy are often used, other calculations for determining maintenance yield have been done [91].

While there is no one way to calculate maintenance energy, the concept is still very important. The idea of maintenance energy was first suggested when it was observed that low levels of energy sources were not sufficient for subculturing bacteria, indicating that

cells may need some energy just to maintain viability [92]. At low temperatures cells require much more energy to produce one gram of cells (also partly due to decreased oxygen availability), but the total amount of energy put into the system (as measured by carbon source used) is even greater than that calculated to produce one gram of cells [89]. So at low temperatures, the amount of energy needed to maintain viable cells is also much greater. This can be explained by the driving force of cellular chemical processes--heat (thermal energy). Since cell maintenance requires turnover of macromolecules, to drive the chemical processes involved in turnover requires a certain amount of heat, which makes these processes more difficult at low temperatures. Heat expenditure is also necessary to sustain membrane dynamics at low temperatures. Low temperatures cause lipids to solidify, preventing the diffusion and lateral motion of substrates and enzymes used in the many chemical reactions that take place within the membrane. In addition maintaining the electromotive force across the membrane is entropically unfavorable. So a certain amount of energy is used to preserve membrane asymmetry. Enzymes, the biological macromolecular catalysts, also require thermal energy to sustain the optimal vibrational modes at which they function. Thus overall, with the decrease in available thermal energy at colder temperatures, cells must burn vast amounts of chemical fuels just to maintain an equivalent cell mass, much less allow for cell growth.

Carbon source and heat generation

This increased need for heat requires cells to be more efficient in generating heat and in using energy resources. Typically cells use three classes of substances for deriving

chemical energy: carbohydrates, amino acids, and fatty acids. Carbohydrates are the most oxidized of these compounds and hence generate the least amount of heat per gram than amino acids and fatty acids. A substantial loss in the net generation of energy from burning carbohydrates comes from the priming of them for metabolic degradation. This process requires a large investment of energy through the generation of phosphorylated species. Amino acids are an oxidation state lower than carbohydrates and fatty acids are the among the most reduced substrates for microorganisms. Both would provide more energy than carbohydrates and would be used with greater efficiency at subzero temperatures, as is indicated by their respective heats of combustion. The heat of combustion for glucose is 669.94 kg-cal/g. While for leucine (855.6 kg-cal/g) and two fatty acids, palmitate (2,384.76 kg-cal/g) and oleate (2,657.4 kg-cal/g), the heats of combustion are higher, especially for the fatty acids [93]. Further evidence for the preferred use of fatty acids as energy at subzero temperatures is the observation of lipid droplets in many Arctic and other psycrophilic organisms (Danielle Prévost, Agriculture and Agri-Food, Canada; personal communication). These lipid droplets consist of triacylglycerides, which are the storage form of fatty acids.

The universality of adaptive responses to various environmental perturbations

From the evidence documented above one can see that numerous environmental perturbations cause the same types of molecular mechanisms of adaptation. Therefore these stresses may induce the same sets of sensory mechanisms in a given organism. The peripheral membrane of procaryotes is the ideal candidate for sensing all of these variations within an environment. In surrounding the cell it has constant contact with its surroundings,

it transmits the available thermal energy to the cell interior, and its organization and adaptability allow for rapid alterations in its structure to compensate for any physical and chemical perturbations it senses. The primary sensory mechanism could be simply perturbations in the membrane dynamics sensed by disturbances of the gel and liquid-crystalline phases. As discussed above both physical and chemical stresses (including temperature, pressure, pH, salinity, and chemical substances) all result in membrane alterations which are thought to maintain a certain degree of fluidity. Cold temperatures, increased pressure, increased salinity (depending on the type and concentration of cation), and certain solvents all rigidify the membrane by inducing closer packing of the lipid acyl chains and/or by stabilizing electrostatic lipid headgroup interactions. Thus all these perturbations result in membranes with greater unsaturated acyl chains and shorter chain lengths, which disrupts this induced tighter packing. Other factors that fluidize the membrane (high temperature, pH and salinity extremes, and some solvents) by disrupting acyl chain packing and/or destabilizing electrostatic interactions of the lipid headgroups, result in membranes with longer, more saturated acyl chains which increases the acyl chain interactions causing a reduction in the acyl chain motion.

The membrane, by virtue of its component lipid acyl chains, is tuned to the available thermal energy and transmits it to the cell interior. This available energy is what determines which metabolic reactions can be carried out, and the cell's metabolic processes are adjusted accordingly. The products of the metabolic process, however, determine the available pool of acyl-CoA primers which can be used for fatty acid synthesis. So there is a circular

relationship between available thermal energy, metabolism, and membrane structure. A change in any one of these parameters by any type of perturbation affects the other two.

Psychrophilic and psychrotropic bacteria offer a unique opportunity to establish the relationship between energy, metabolism, and fatty acid structures. As discussed above, the available thermal energy is much less as temperature drops. Thus the balance between carbohydrate and amino acid metabolism is affected, which is also reflected in the membrane lipid acyl chain structures. In this work, principles of adaptation and adaptability of organisms to extreme environments are developed using an Arctic permafrost bacterium. These principles are then applied to data regarding other bacteria to test their generality.

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CHAPTER II

DEVELOPMENT OF A THEORY ON THE SIGNIFICANCE OF FATTY ACID HETEROGENEITY

ABSTRACT

The theory that the chain length distribution of the fatty acyl groups of bacteria is a Gaussian function the mean and variance of which depends on temperature was tested by examining this distribution in a permafrost bacterium that is capable of growth from temperatures close to freezing up to 30°C. The variance of the curve was a linear function of absolute temperature over most of the range with a second order deviation towards the upper end. This supports the idea that the chain length selection is a random process in which the distribution reflects the external temperature, or more correctly momentum distribution of molecules in the extracellular matrix, and is characterized by a smaller variance at lower temperatures and a higher one at higher temperatures. Fatty acid composition data from the literature for three other Gram-positive organisms were also used to support the theory. Having established that there is a consistent relationship between temperature and acyl chain distribution, then one can predict the fatty acid composition of a given organism over a temperature range. By adding a variety of chemical perturbants to Arctic strain 45-3, *Bacillus subtilis* PY79, and *Sarcina ventriculi* JK, we also show that there is a temperature equivalent for a given solvent perturbation in a particular organism. This is consistent with earlier isotope studies that indicated the mobility of the hydrocarbon chains is actually the critical dynamic factor that is influenced by temperature. The membrane acyl chain length alterations induced by chemical substances, such as solvents and antibiotics could, therefore, be explained by the structures of the perturbants and how the structures could influence the acyl chain packing. These results indicate that by knowing whether the

perturbant would fluidize or rigidify the membrane, the temperature tolerance of an organism can be increased (or decreased) by adding exogenous substances such as very long or short chain or fused-ring hydrocarbons to counteract the temperature effect. For extreme perturbations, the mean of the distribution curve shifted abruptly to form a new series of distribution curves about the new mean. For elevated temperatures, this is consistent with the increased value of kT.

INTRODUCTION

> *"Why not more quantitative data?*
>
> *Lipid data on the same strain, grown and analyzed under the same conditions in two different laboratories, or even in the same laboratory, may vary appreciably from a quantitative point of view. Thus, the most important facts to consider are the types of lipids that occur in a given organism and which of these represent the major types. Actual percentages will not be of much use."*
>
> *M. P. Lechevalier [1]*

While this quote was made in 1982, it still seems to reflect our understanding of membrane acyl chain heterogeneity today. Researchers have shown that many organisms alter their membrane acyl chain lengths, unsaturation, branching, and cyclization in response to fluctuations in temperature. The percentages are graphed or listed in many combinations, but they still seem to just end up describing an overall trend of changes with no real relationship between the magnitude of the temperature change and the percentages determined. Here we show that there is, in fact, a regular (mathematical) relationship

between the acyl chain distribution of an organism and its growth temperature.

The reason for the many types of acyl chain alterations that have been documented in response to temperature (and other perturbations) is thought to be an organism's response to maintain a necessary degree of membrane fluidity. This phenomenon is termed "homeoviscous adaption" [2]. Key questions that remain are: How do cells sense temperature? and, What is the connection between this observed membrane heterogeneity and temperature?

All cellular metabolic pathways are series of chemical reactions, and these reactions all have activation energy barriers which must be overcome. The driving force to overcome these barriers is heat (infrared quanta). Temperature dictates the available heat that can be used for driving cellular processes, including the synthesis of chemical energy (ATP). One theory is that the membrane lipid acyl chains are able to channel this energy (kT) to the cell interior via special vibrational modes called longitudinal accordian modes (LAMs) [3]. This concept is depicted in Figure 2.1. The sun emits a continuous spectrum of electromagnetic (EM) radiation, which determines the thermal energy spectrum on earth. Though the infrared part of the spectrum is of interest here because it is absorbed as heat in matter. The most abundant quanta at a given temperature (T) has the magnitude kT. This thermal energy spectrum is then transferred to the matter (water, gas, and other molecules) surrounding cells and is stored in their rotational, vibrational, and translational modes. In biological systems water plays an important role in transferring this energy through collisions with other

Figure 2.1: Diagram depicting the transfer of the thermal energy spectrum from the environment via longitudinal accordian modes of membrane acyl chains to the cell interior. The sun emits electromagnetic radiation (EM) of which the most probable quanta has the magnitude of $h\nu = kT$, where h = Planck's constant, ν = frequency of quanta, k = Boltzmann's constant, and T = temperature. This energy is stored in the rotational (rot.), vibrational (vib.), and translational (trans.) modes of the matter (such as water = blue molecules, gases = green molecules) surrounding the cells. By molecular collisions and solvent-lattice vibrations this energy is transferred to the membrane and stored in special vibrational modes of the membrane acyl chains called longitudinal accordian modes (LAMs). This energy is then transferred to the cell interior again by molecular collisions and solvent-lattice vibrations.

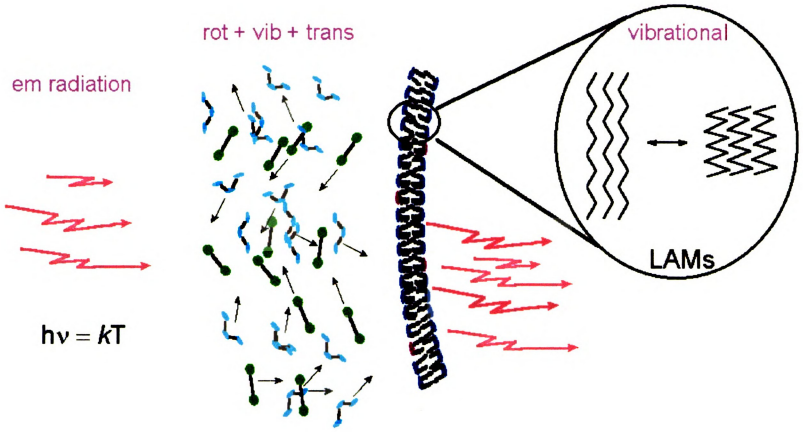


Figure 2.1

molecules and solvent-lattice vibrations. The membrane, due to its location around the cell, has the opportunity to control the thermal energy spectrum that enters the cell. This is because collisions with molecules surrounding the cell and solvent-lattice vibrations result in energy being transferred to special vibrational modes of the membrane acyl chains called longitudinal accordian modes (LAMs), which is the extension and compression of the acyl chains. The net effect is such that the energy quanta are converted into highly tailored, specific, oriented waves on passing through the membrane and into the cell. This energy spectrum is then transmitted throughout the cell interior again via molecular collisions and solvent-lattice vibrations. However, the energy that can be stored and propagated by a given chain length is a function of the chain length, with longer chains storing higher energy quanta. Therefore an acyl chain length distribution is equivalent to an energy distribution.

In a rigorous mathematical analysis of the relative energies of these LAM modes, Hollingsworth showed that both the LAM energy distribution in the membrane and its corresponding acyl chain length distribution were normally distributed, as described by the Gaussian density function:

$$f(x) = \{ 1/[\sigma(2\pi)^{1/2}] \} e^{-1/2[(\mu - x)^2/\sigma^2}$$

where $f(x)$ is the probability, σ is the standard deviation, μ is the mean, and x is the acyl chain length. He also showed that the most prominent energy stored in the most abundant acyl chain length of the membrane was equal to kT at the growth temperature of the organism.

Thus it was proposed that altering of membrane acyl chain lengths allows organisms to transmit the maximal available thermal energy spectrum [3].

It was also shown in this paper that not only is the composition of membrane fatty acid chains normally distributed when ranked by chain length, but there is a relationship between the variance (σ^2) of this distribution curve and temperature as well. Namely, as temperature decreases the distribution curve becomes taller and narrower (smaller σ^2). As temperature increases the curve becomes shorter and broader (larger σ^2) [3]. The molecular speed or momentum distribution of the molecules surrounding the cell, which is one aspect of the kinetic energy (translational energy mode) distribution of the molecules, also has a Gaussian type function described by the Maxwell-Boltzmann equation. This equilibrium distribution function $\{f_o(p)\}$ describing the momentum (p) of a dilute gas at temperature T has the form:

$$f_o(p) = Ae^{-(p-p_o)^2/2mkT}$$

where m is the mass of a gas particle, *k* is the Boltzmann constant, p_o is the mean momentum, and A is a constant involving the total number of particles (n), m, *k*, and T. Because the distribution function that describes molecular speed or momentum at a particular temperature has the same form, a relationship between temperature in the Maxwellian distribution function and σ^2 in the Gaussian density function is invited. This suggests several important questions, the main one of which is: Is there a linear relationship between σ^2 and temperature over the adaptation range of an organism? If temperature is sensed by alterations of the phase structure of membranes, then membrane-active chemical agents that

disturb the packing and fluidize or melt certain regions should have the effect of increasing temperature. On the other hand, those that cause a reduction or freezing of acyl chain motion should have the effect of reducing temperature. These effects are not limited to organic (hydrocarbon) soluble molecules. Hence antibiotics that interfere with the lipid headgroup packing thus disturbing their long range order should have the same fluidizing effects as increasing temperature. Therefore, there should be a temperature equivalence for a variety of membrane-active chemical perturbants.

Modulation of phase equilibria is another aspect of membrane structure which may enable cells to sense temperature variations. Membrane components are not uniformly dispersed, rather they are organized into phases or domains which may differ in composition, morphology, or both. The chemical make-up of the constituents (including lipid headgroups, acyl chains, and membrane proteins) dictate the types and structures of the phases. These domains exist in a very complex equilibrium between each other and with the bulk of the membrane. The phase equilibria are extremely temperature sensitive. A small temperature change can result in dramatic alterations in phase structures. Since the phase boundary is a discontinuity, the specific heat should change abruptly across it and small changes in temperature should be amplified. A change in temperature would affect gel to liquid-crystalline and other phase transitions thus altering membrane fluidity. If the new dynamical state does not adequately capture the thermal spectrum, it does so eventually. This is because an essential characteristic of adaptation is that the molecular systems that capture the available heat energy at the maximum of its distribution are the ones that are most viable.

One can, therefore, equate a change in temperature with a change in some dynamical property of the membrane and vice-versa.

In this study we use a Siberian permafrost bacterium grown at four different temperatures to determine the real dependence between σ^2 and temperature. We also demonstrate the generality of this relationship using data from the literature regarding three other Gram-positive organisms, *Bacillus subtilis, Listeria monocytogenes,* and *Bacillus amyloliquefaciens*. We also investigate the effects of several solvents and antibiotics on Arctic strain 45-3, *Bacillus subtilis* PY79, and *Sarcina ventriculi* JK to establish a temperature equivalence for the effect of chemical perturbations on membrane acyl chain distributions.

MATERIALS AND METHODS

Arctic strain 45-3 culture conditions

Arctic strain 45-3 was cultured in 100 ml cultures of trypticase soy broth at 4°C, 14°C, 24°C, and 30°C with shaking at moderate speeds.

Effects of cyclohexane and 1,4-cyclohexadiene on Arctic strain 45-3

Cultures (10 ml) of Arctic strain 45-3 were grown as described above except that 0.5 M and 1.0 M concentrations of cyclohexane (54 µl and 108 µl, respectively) and 1,4-cyclohexadiene (47 µl and 95 µl, respectively) were added to individual cultures.

Cyclohexane was obtained from Sigma, and 1,4-cyclohexadiene was obtained from Aldrich.

Effects of *cis*- and *trans*-decalins on *Bacillus subtilis* PY79

Cultures (10 ml) of *B. subtilis* PY79 were grown to mid-exponential phase (LB media: 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl; pH 7.0) at 37°C with shaking at 270 rpm. At this point 0.2 M (0.31 ml) *cis*-decalin and 0.2 M (0.32 ml) *trans*-decalin (both obtained from Sigma) were added to individual cultures, and then incubated at 37°C for another six hours. Cells were harvested by centrifugation. Fatty acid methyl ester analyses were done as described below.

Effects of antibiotics on *Bacillus subtilis* PY79

A 2.5 l culture of *B. subtilis* PY79 was grown, as described above, to mid-exponential phase, and then split into 5, 500 ml cultures. Then 5 ml of 10 mg/ml stocks of chloramphenicol (Sigma), streptomycin (Boehringer Mannheim), and tetracycline (Sigma) were added to three of the cultures. One was a 37°C control. The last was an ethanol control (5 ml added), since chloramphenicol was dissolved in ethanol. The cells were harvested by centrifugation. The total lipids were extracted two times in 4:1:4 $CHCl_3$:MeOH:H_2O by stirring for 36 hours total. The organic layers were removed and evaporated to dryness. Fatty acid methyl ester analyses were done as described below.

Fatty acid methyl ester analysis

About 1-2 mg of cells from cultures grown under the various conditions were

methanolysed in 5% HCl in methanol at 75°C for 36 hours. The suspensions were evaporated to dryness under a stream of nitrogen, and 0.5 ml H_2O, 1.5 ml hexane, and 1.5 ml chloroform were added. After vigorous shaking, the mixture was centrifuged and the organic layer was removed and concentrated to dryness. The resulting fatty acid methyl esters were analyzed by gas chromatography (GC) and GC-MS using a 30 m DB1 column. The temperature program was a ramp from 160°C to 320°C at 3°/min. The final temperature of 320°C was held for 5 min. The relative proportion of the fatty acid components were calculated from the integrated peaks. The fatty acid methyl esters were identified by GC-MS analysis using a Jeol JMS-AX505H spectrometer interfaced with a Hewlett-Packard 5890A gas chromatograph.

Variance (σ^2) calculations of acyl chain length distribution curves

Variances (σ^2) of the acyl chain length distribution curves, which are normally distributed were calculated using the Gaussian density function:

$$f(x) = \{1/[\sigma(2\pi)^{1/2}]\}e^{-1/2[(\mu-x)^2/\sigma^2}$$

where $f(x)$ is the probability, σ is the standard deviation, μ is the mean, and x is the acyl chain length. By setting the value of x equal to μ (the condition at the mean), the equation simplifies to:

$$f(x) = 1/\sigma(2\pi)^{1/2}$$

which when solved for σ becomes

$$\sigma = 1/f(x)[2\pi]^{1/2}$$

The variance is the standard deviation squared. The variances of each curve were then plotted versus temperature.

RESULTS AND DISCUSSION

The gas chromatograms for Arctic strain 45-3 at 4°C and 24°C are shown in Figure 2.2A and B, respectively. The main series of acyl chains synthesized is the odd one, with the *anteiso*-branched fatty acids predominating. In Figure 2.3, the odd series acyl chain length probability distributions (inclusive of *iso, anteiso*, alkenyl, and straight chains of the same length) are plotted for each growth temperature. These distributions had the expected Gaussian profile with the curves becoming taller and narrower with decreasing temperatures. The Gaussian character was easily verified by sampling the function at points equidistant from and on opposite sides of the mean, and verifying that it had the same value. These distributions are in agreement with those first shown by Hollingsworth [3], and support his idea that the chain length selection process is random, and the mean and variance of these distributions are determined by temperature.

To further support the generality of this relationship, fatty acid composition data for

Figure 2.2: Gas chromatographs of fatty acid methyl esters obtained from Arctic strain 45-3 cultured at (A) 4°C and (B) 24°C. The main acyl chain components were (1) $C_{14:1}$ (2) *iso*-C_{14} (3) n-C_{14} (4) $C_{15:1}$ (5) *anteiso*-C_{15} (6) *iso*-C_{16} (7) n-C_{16} and (8) *anteiso*-C_{17}.

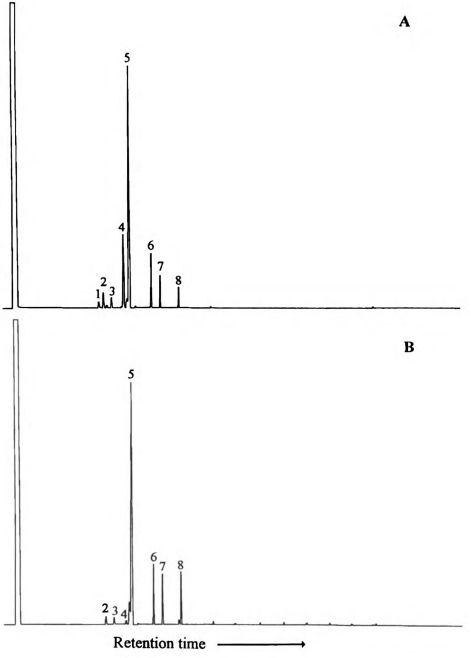


Figure 2.2

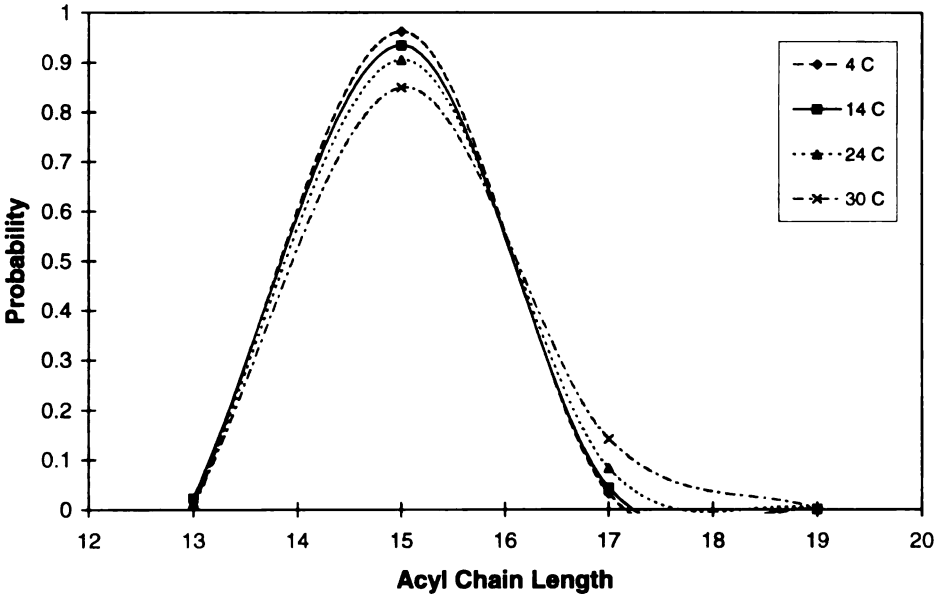


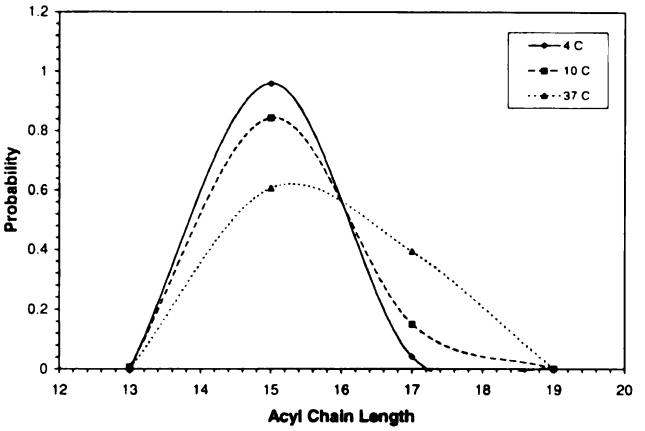
Figure 2.3: Plot of probability distributions at 4°C, 14°C, 24°C, and 30°C for Arctic strain 45-3.

three other Gram-positive organisms were taken from the literature and their probability distributions plotted for the many series of acyl chains synthesized. The branched (*anteiso and iso*) odd chain length series was plotted for *Listeria monocytogenes* (Figure 2.4A) [4], and for *Bacillus amyloliquefaciens* (Figure 2.4B) [5]. For *Bacillus subtilis* the *anteiso*, odd-chain length series was plotted (Figure 2.4C) [6]. All three of these organisms have Gaussian probability distributions in which the curves become shorter and broader with increasing temperature. The peak of the distribution curve for *B. subtilis* at 35°C lies between the curved for 20°C and 26°C, which does not follow the trend. This is the only discrepancy found so far, and since this is from the literature it is unknown whether this is true or due to experimental error. These results indicate, at least for Gram-positive bacteria, that the random acyl chain length selection process is a general rule for these organisms as shown by the Gaussian distributions, of which the mean and variance of the curves are determined by temperature.

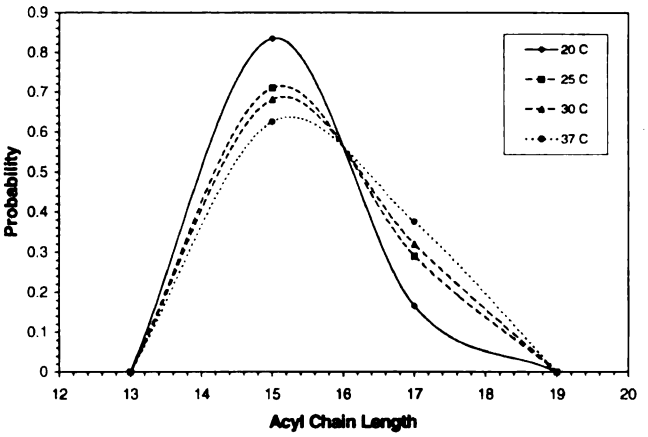
For *L. monocytogenes* and *B. amyloliquefaciens*, the means of the probability curves also shift toward the right as temperature increases (Figure 2.4A and B, respectively), showing that indeed longer chain lengths become more dominant. This suggests that bacteria have "basins of stability," in which they can alter their acyl chain length distributions over a certain range dictated by kT. At extremes of temperature (hot or cold) there are limits to which the organism can adjust around a particular acyl chain length distribution. This would be shown be the Gaussian profiles such that at high temperatures the curve would become very broad (flatten out) while at low temperatures the curve would become very tall and

Figure 2.4: Plots of probability distributions at several growth temperatures for (A) *Listeria monocytogenes* (B) *Bacillus amyloliquefaciens* and (C) *Bacillus subtilis*.

A



B



C

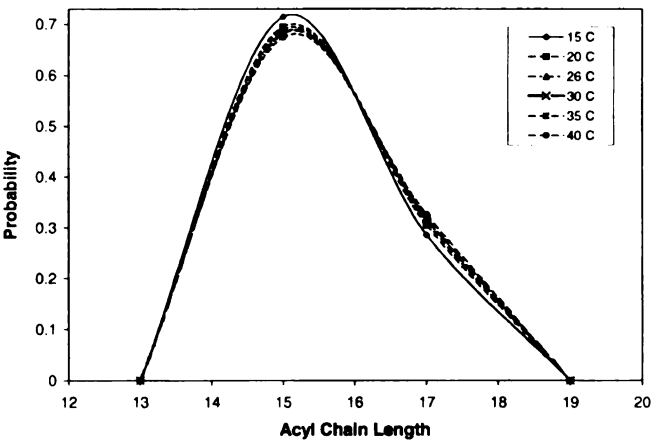


Figure 2.4

narrow. So as the temperature (kT) approaches the limits of this stability range, those bacteria which can reestablish their acyl chain length distributions about a new mean (acyl chain length) will survive. At higher temperatures this new mean would be shifted to longer acyl chain lengths, and at lower temperatures the mean would be at shorter acyl chain lengths. Consistent with this idea, the acyl chain length probability distributions for the extreme thermophilic Gram-positive bacterium, *Bacillus caldolyticus*, is shown in Figure 2.5 [7]. As predicted, as the temperature increases the means of the distribution curves shift from 15.1 to 17, indicating that at higher temperatures this bacterium is able to establish a new distribution of acyl chains in which the mean is now reset to a longer fatty acid chain.

Since there is a definite pattern of height and width changes of the Gaussian distribution curves with respect to temperature, we determined the variances (σ^2), the distribution about the mean, of these curves and plotted them versus the growth temperatures. These graphs and the equations for the curves are shown in Figure 2.6 for Arctic strain 45-3 (A), *L. monocytogenes* (B), *B. amloliquefaciens* (C), and *B. subtilis* (D). Over the broad permissible growth range for Arctic strain 45-3 (Figure 2.6A), the relationship between σ^2 and temperature is linear. The other three organisms also have regions of linearity as well, particularly *L. monocytogenes* (Figure 2.6B) and *B. amyloliquefaciens* (Figure 2.6C). However, towards the extremes of growth temperatures for the organisms, the data for the variance plots have more pronounced curves. Thus, the functions that best fit the data are all second order. In any event, these results show then that for a given organism there is a smooth relationship between the variance of the acyl chain

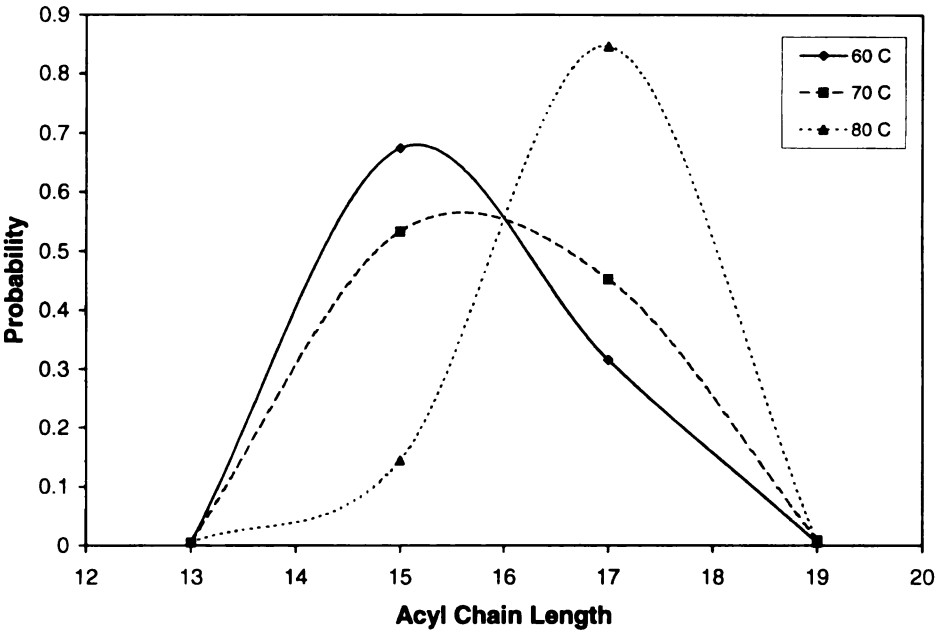
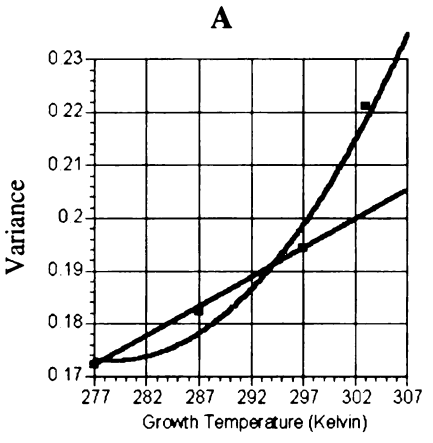
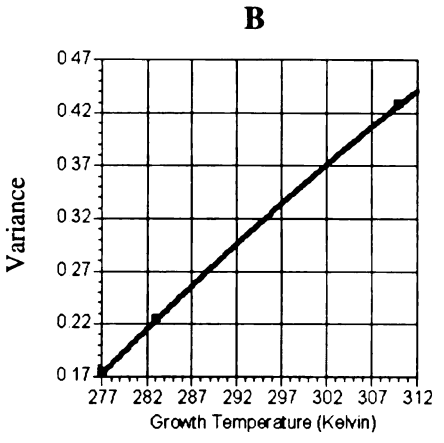


Figure 2.5: Plot of probability distributions at 60°C, 70°C, and 80°C for *Bacillus caldolyticus*. Note the shift in the mean as the temperature increases.

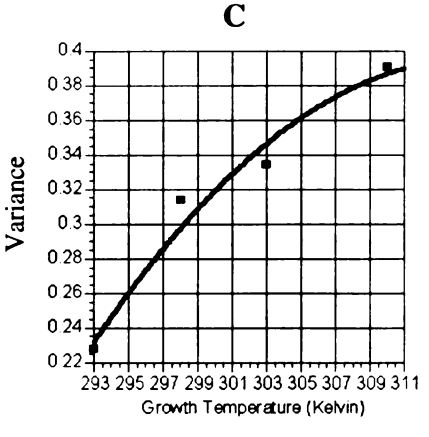
Figure 2.6: Plots of the variances of the acyl chain distribution curves versus growth temperature (in degrees Kelvin) and the equations for the curves for (A) Arctic strain 45-3, (B) *L. monocytogenes*, (C) *B. amyloliquefaciens*, and (D) *B. subtilis*. Note that the 35°C data point for *B. subtilis* was left out of the graph for curve fitting purposes due to possible errors in the data for that growth temperature (see text).



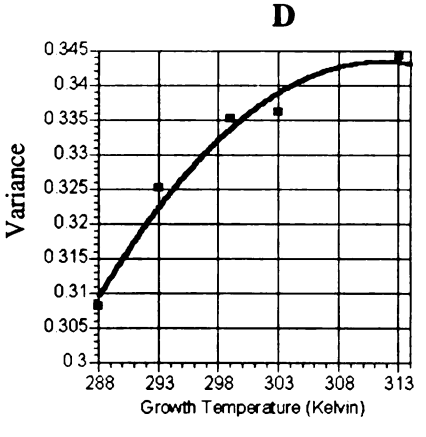
$y = (7.70 \times 10^{5})x^2 + (4.29 \times 10^{2})x + 6.15$



$y = (-2.86 \times 10^{-5})x^2 + (2.45 \times 10^{-2})x - 4.42$



$y = (-3.38 \times 10^{-4})x^2 + (2.12 \times 10^{-1})x - 33.1$



$y = (-6.11 \times 10^{-5})x^2 + (3.81 \times 10^{-2})x - 5.59$

Figure 2.6

length distribution and the growth temperature. Therefore knowing the temperature, one can predict the fatty acid composition over a temperature range for a particular bacterium.

The effects of two solvents, cyclohexane and 1,4-cyclohexadiene, on the membrane acyl chain distribution of Arctic strain 45-3 was also determined. These two solvents were chosen because they interact with the hydrocarbon chains and because L. Bérubé in our lab demonstrated that cyclohexane had a rigidifying effect on *S. ventriculi* membranes while 1,4-cyclohexadiene fluidized them. These solvent effects were confirmed by further analysis using T_1 NMR experiments (explained in detail in Chapter 5), in which these solvents mixed with DPPC (DL-α-phosphatidlycholine, dipalmitoyl) vesicles indicated the terminal methyl and methylene groups of the acyl chains had greater motional freedom in the presence of 1,4-cyclohexadiene, but were more restricted in the presence of cyclohexane.

However, in the case of Artic strain 45-3 both cyclohexane and 1,4-cyclohexadiene acted as fluidizers. This is best shown in the GC chromatograms (Figure 2.7 A, B, and C) in which there is an increase in the amounts of longer acyl chains (namely *anteiso*-C_{17} and *n*-C_{16}) in the presence of both cyclohexane and 1,4-cyclohexadiene as compared to the 24 °C control. This increase in saturated and longer chains fatty acids shows the solvents act like high temperatures and fluidize the membrane. The difference in effects on the Arctic strain 45-3 and *S. ventriculi* bacteria can be explained by the structures of both the solvents and the main membrane acyl chain structures. As shown in Figure 1.5, the greatest degree of acyl chain motion is at the terminus of the chain in the middle of the bilayer. The T_1 NMR

Figure 2.7: Gas chromatograms of fatty acid methyl esters obtained from Arctic strain 45-3 cultured in the presence of two solvents. (A) 24°C control, (B) 0.1 M cyclohexane, and (C) 0.1M 1,4-cyclohexadiene. The main acyl chain components were (1) $C_{14:1}$ (2) *iso*-C_{14} (3) n-C_{14} (4) $C_{15:1}$ (5) *anteiso*-C_{15} (6) *iso*-C_{16} (7) n-C_{16} and (8) *anteiso*-C_{17}.

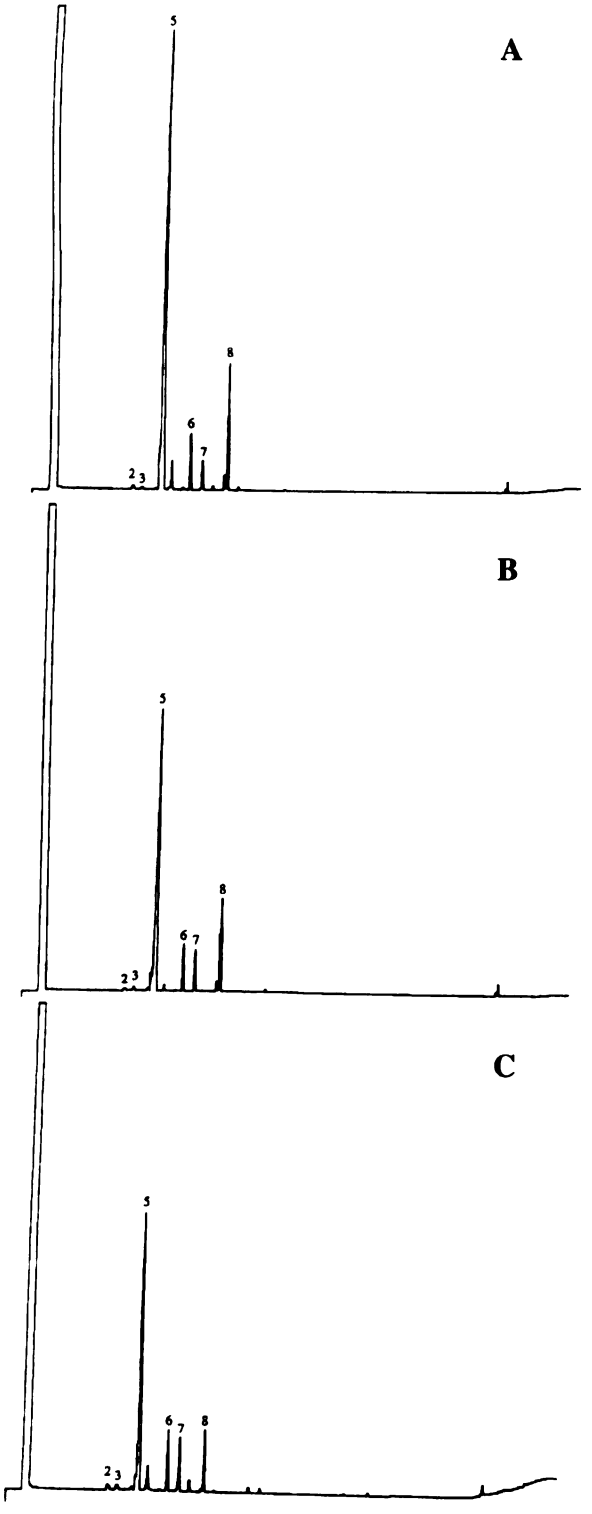
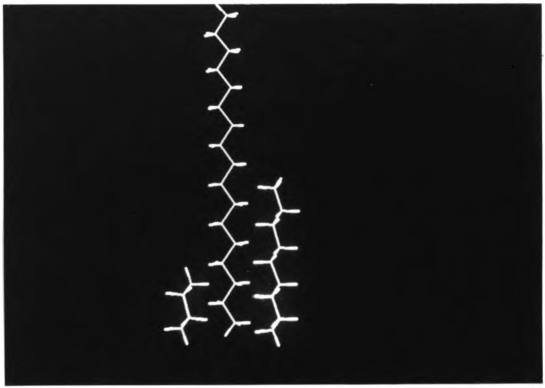


Figure 2.7

experiments discussed above, also show that the primary effect of these solvents takes place at these methyl termini of the fatty acids. In Figure 2.8 the packing interactions of cyclohexane and an ω-cyclohexyl acyl chain with a straight hydrocarbon chain are shown. The chair conformations of both these cyclic hydrocarbons enable them to pack closely in a nesting fashion with the methyl terminus of the straight hydrocarbon chain, effectively reducing the motional freedom of the chain resulting in a rigidifying effect. 1,4-Cyclohexadiene, which is a more planar molecule is not capable of this nesting arrangement with the straight acyl chains and as a result fluidizes the membrane. This is what is observed with *S. ventriculi*, which has primarily straight acyl chains. In the Arctic bacterium, however, the main membrane fatty acids are *anteiso*- and *iso*-branched, and as such neither cyclohexane nor 1,4-cyclohexadiene pack well with the branched acyl chain termini, and both therefore would increase the random motion at the methyl termini. 1,4-Cyclohexadiene was particularly toxic to the Arctic bacterium, such that the cells showed limited growth after its addition. This is probably due to the more planar conformation of 1,4-cyclohexadiene which disturbs the packing of the chain termini more, or to its greater diffusibility within the membrane because of the absence of stable packing arrangements along the hydrocarbon chain.

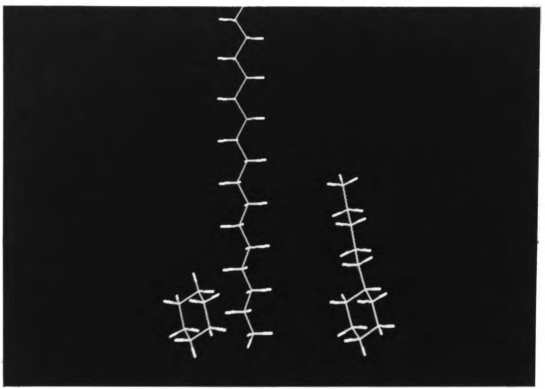
Further substance perturbation studies were done using *cis*- and *trans*-decalin and three antibiotics (Figure 2.9) on *B. subtilis* PY79. The GC chromatogram for *B. subtilis* PY79 at 37°C is shown in Figure 2.10. The primary membrane acyl chains are the branched, odd chain length series. In Figure 2.11 the effects of *cis*- and *trans*-decalins on *B. subtilis*

Figure 2.8: Computer generated pictures showing the packing interactions between a straight chain hydrocarbon and cyclohexane and an ω-cyclohexyl acyl chain from two different views (A) and (B). Note the *trans* conformations of the rings allows close packing in a nesting fashion with the methyl terminus of the hydrocarbon chain.



A

Figure 2.8



B

Figure 2.8 (cont.)

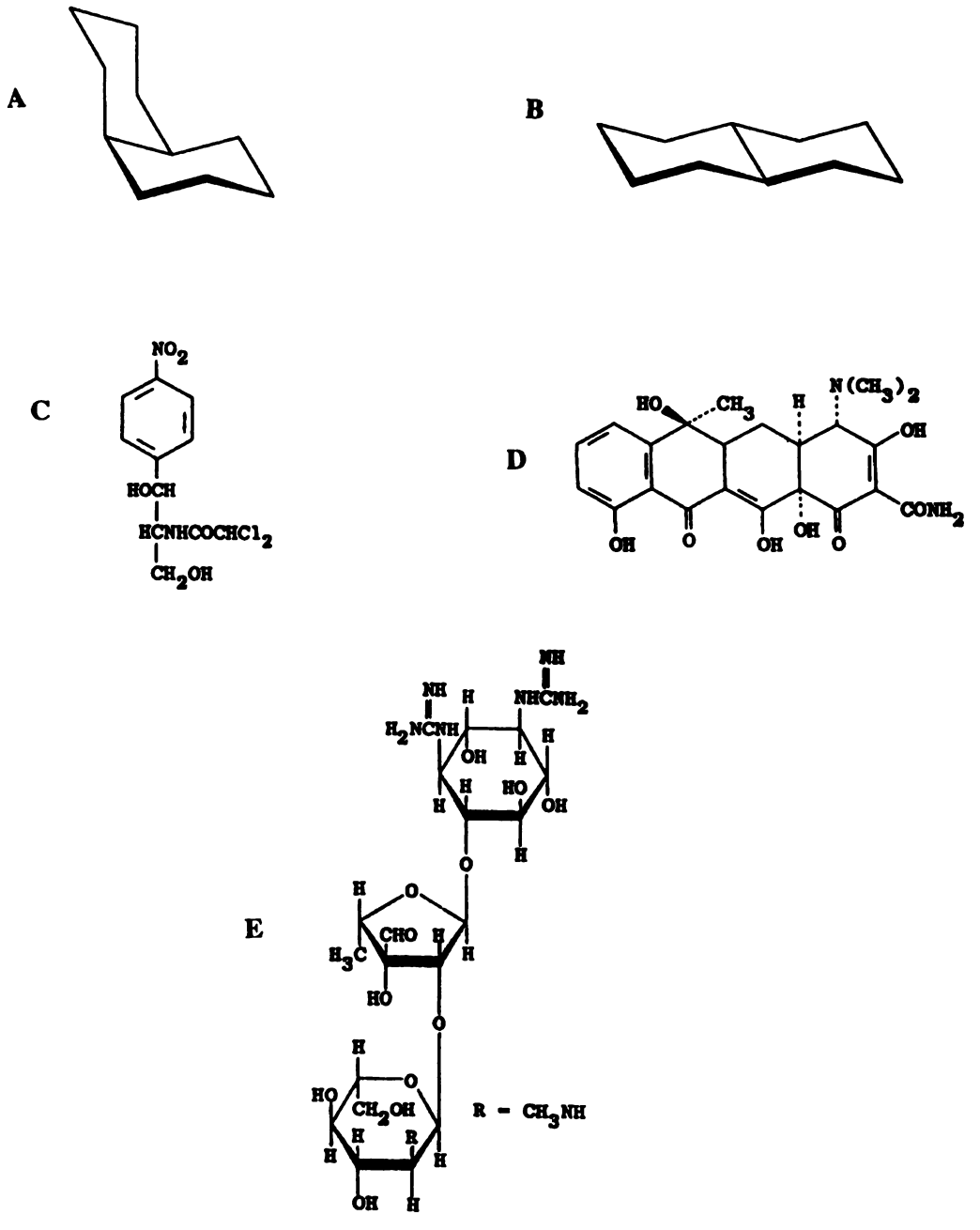


Figure 2.9: Structures of the chemical perturbants used studies with *Bacillus subtilis* PY79. (A) *cis*-decalin, (B) *trans*-decalin, (C) chloramphenicol, (D) tetracycline, and (E) streptomycin.

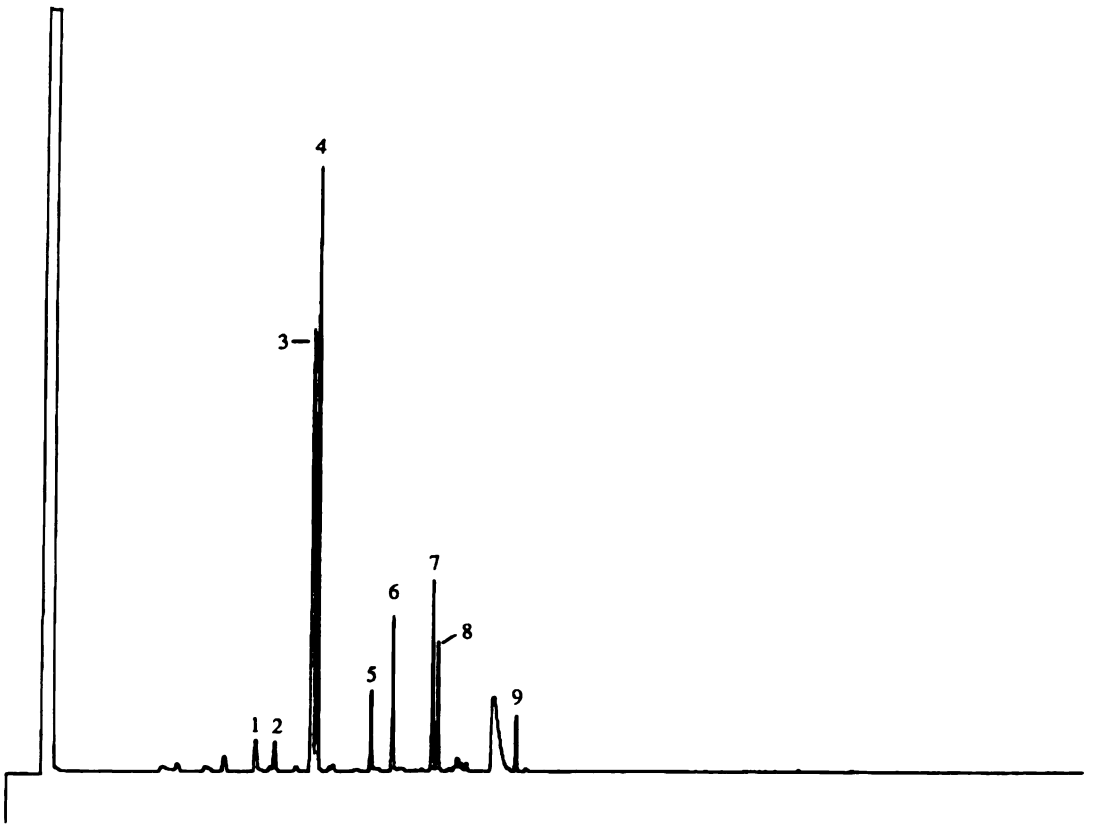


Figure 2.10: Gas chromatogram of fatty acid methyl esters of *Bacillus subtilis* PY79 from a 37°C culture. The main acyl chain components were (1) *iso*-C_{14} (2) n-C_{14} (3) *iso*-C_{15} (4) *anteiso*-C_{15} (5) *iso*-C_{16} (6) n-C_{16} (7) *iso*-C_{17} (8) *anteiso*-C_{17} (9) n-C_{18}.

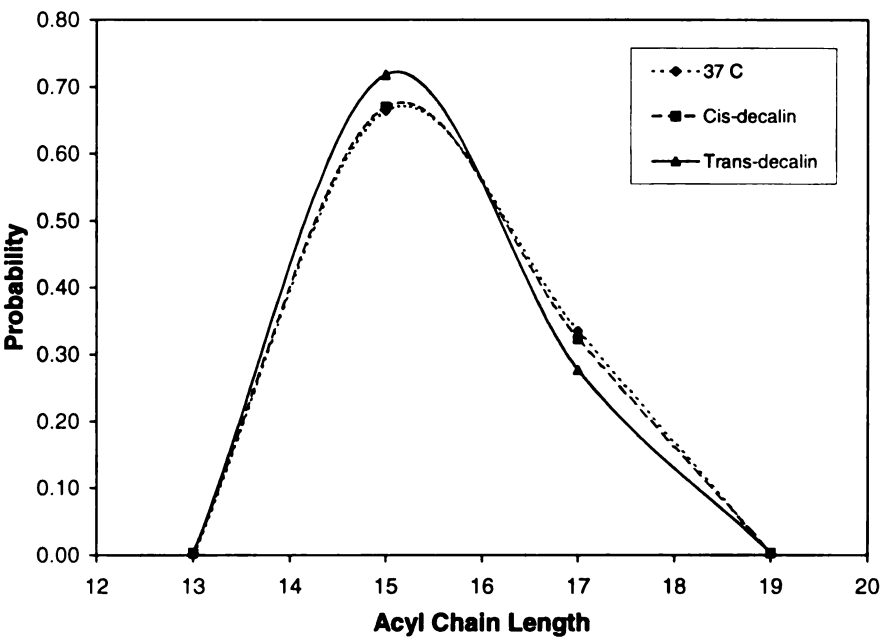


Figure 2.11: The effects of *cis*- and *trans*-decalins on the plot of the probability distributions of *Bacillus subtilis* PY79.

acyl chains distributions is shown, in which *cis*-decalin has no perturbing effect on the membrane while *trans*-decalin acts as a rigidifier. Again this can be explained by interaction of the perturbants with the type of membrane acyl chains in this organism. *cis*-Decalin has an L-shaped structure and as such the fluidizing effect of one ring is offset by the nesting capability of the second ring, and thus would not disrupt the membrane packing anymore than the branched acyl chains already cause. The two rings of *trans*-decalin, on the other hand, are both chair conformers and can nest between the hydrocarbon chains along their length causing an overall decrease in acyl chain motion. The effects of the antibiotics are also related to their structures. Chloramphenicol and tetracycline are largely hydrophobic and interact with the membrane acyl chains. Both of these antibiotics have planar ring systems and would not pack well with the membrane acyl chains. As expected these two antibiotics fluidize the *B. subtilis* membranes (Figure 2.12). In fact the large ring system of tetracycline fluidizes the membrane to such an extent that the mean of the curve is shifted towards longer chains lengths (similar to that seen by extremely high temperatures). Streptomycin, though, is a polar molecule consisting of sugar derivatives. As a result it most likely interacts with membrane headgroups such that the amidino groups, which are positively charged, interact with the negative phospholipid charges and stabilize the membrane. This headgroup stabilization would result in closer packing of the acyl chains, rigidifying the membrane. Again this predicted effect is demonstrated with *B. subtilis,* as seen by the taller, more narrow distribution curve for streptomycin as compared to the 37°C control (Figure 2.12).

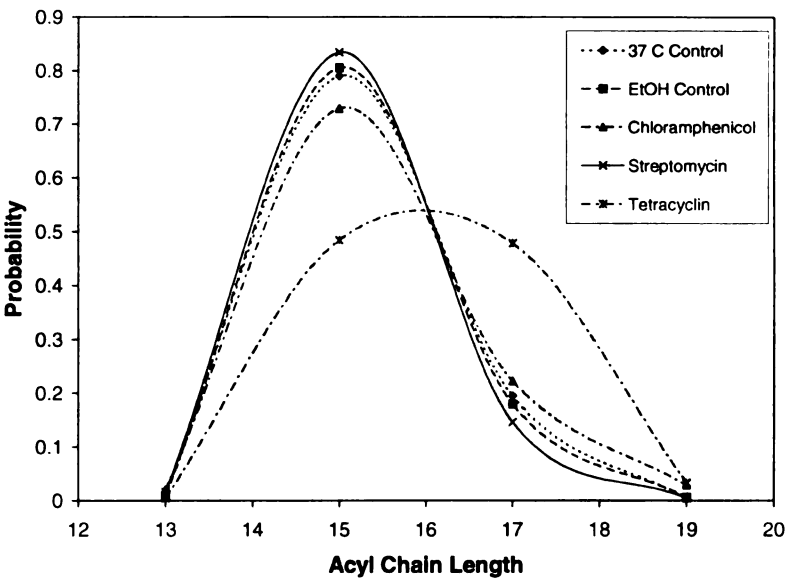


Figure 2.12: Effects of three antibiotics on the plot of the probability distributions of *Bacillus subtilis* PY79.

These results indicate that the effect of chemical substances on acyl chain length distributions can be explained based on their structures and the membrane acyl chain components of a given organism. As a result there is a temperature equivalent shift in membrane acyl chain length compositions based on whether the perturbant acts as a fluidizer or rigidifier of the particular membrane. The ability of organisms to adapt to environmental perturbations by alteration of their acyl chain distributions would be reflected in their growth rate under these new conditions. These results indicate that there might be a relationship between temperature, solvent concentration, and growth rate. In Figure 2.13 hypothetical growth rate curves for conditions of high and low temperatures and solvent concentrations. Such a three dimensional graph of these factors for a given bacterium would allow one to determine the solvent concentration needed to offset the temperature shift to allow the organism to grow outside its temperature range. A temperature offset could also be determined to allow a bacterium to grow in the presence of higher solvent concentrations.

In living systems something must fuel the molecular motion of the cellular processes, since these processes are not self-sustaining. This driving force is the heat (kinetic energy), determined by temperature, available to the organism. Therefore, there has to be a match between the thermal energy spectrum and the cellular chemical properties, and this match is characteristic of a given organism. Organisms capture the available kinetic energy by collisions with molecules whose momentum distribution is a Gaussian type function, the mean and variance of which is determined by temperature according to the Maxwell-Boltzmann distribution law. The collisions between the surrounding solvent and gas

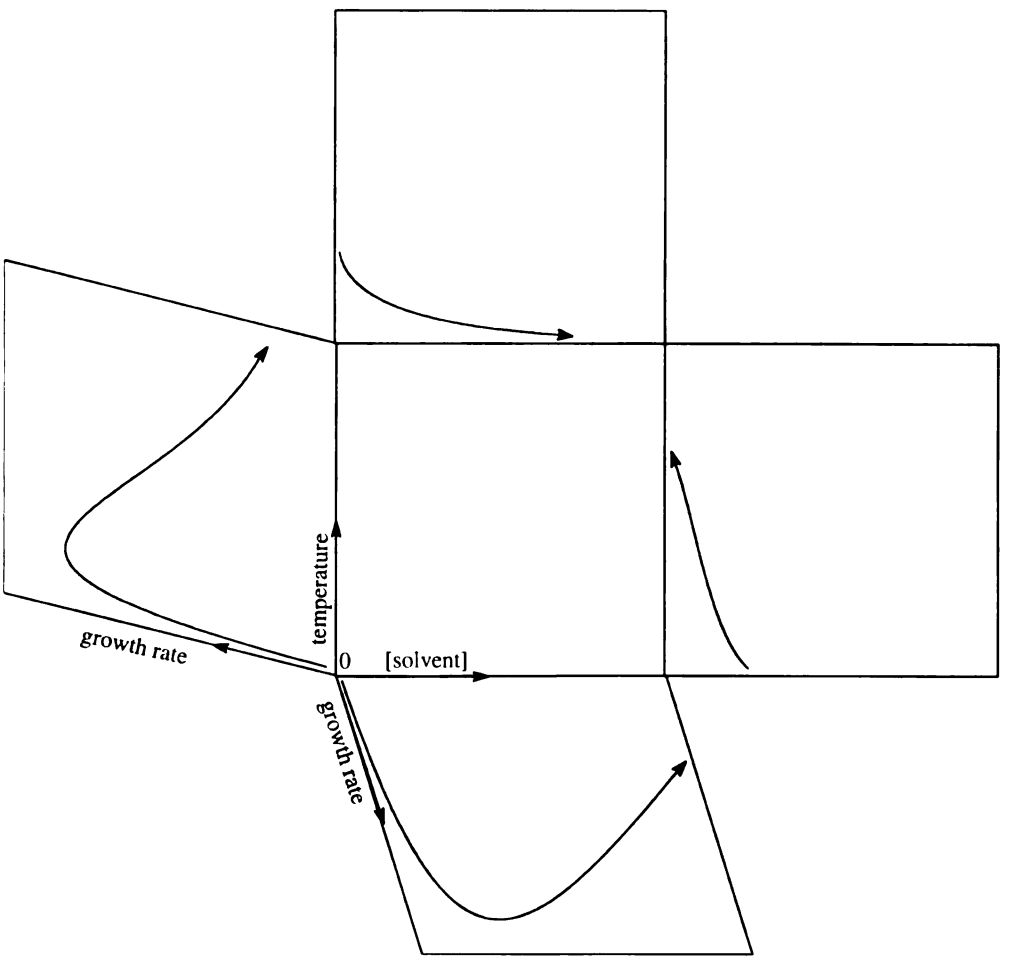


Figure 2.13: A three dimensional graph showing hypothetical growth rate curves for conditions of high and low temperatures and solvent (fluidizing type) concentrations.

molecules and the molecules in the boundary structures of the cells form the basis of the heat exchange process. To effectively capture this kinetic energy distribution, the surface of the cell must have an array of molecules or some molecular system that can absorb the energy given up in these exchanges into some vibrational mode because the cellular chemistry is driven by these vibrations. So then the best adapted organism should have some energy harvesting system that traps this energy that is passed on in these collisions in vibrational modes with the same mean energy as in the collisions and over a distribution that is narrowly defined around the mean energy. Since the energy distribution of the collisions to start with is Gaussian as well, it makes sense that the vibrational modes are also Gaussian, with the most abundant acyl chain length in the membrane trapping the most abundant energy quanta stored in the matter surrounding the cell.

The energy trapped by the membrane and propagated to the cell interior is used to drive the cellular chemical processes. Not only does this energy determine the rate of diffusion of molecules (and thus the collision rate), it also dictates various motions of proteins that determines the activity of these proteins, and as a result the types of cellular processes that can be carried out. Two types of large-scale motions observed in proteins are hinge bending motions and single strand motions, both of which may produce large relative displacements of atoms within proteins and are usually involved the biological activity of the protein. As will be discussed the frequencies, by relation to the time scale of the motions, of these motions are also of the magnitude kT.

In 1995, Hollingsworth showed that the frequency of the most abundant membrane acyl chain length corresponded to 215.4 cm^{-1} (in the spectroscopic unit of wavenumbers) or 6 $psec^{-1}$, which corresponded to kT at the growth temperature of the organism [3]. This energy (as well as the rest of the energy distribution stored in the acyl chains) is then transferred to the cell interior, mostly to the water molecules. Recently terahertz (Thz) laser vibration-rotation-tunneling spectroscopy has been used to characterize the frequencies of the water solvent cage. The first calculations were for trimers of water molecules, and they found that the characteristic frequency (in wavenumbers) for one aspect of water vibrations was 40-50 cm^{-1} [8]. For water tetramers this frequency increased to 2.04 Thz (67.9 cm^{-1}) [9]. This frequency continued to increase with the size of the water cage to 2.4 Thz (81.2 cm-1) for pentamers [10] and 2.491 Thz (83.03 cm-1) for hexamers [11]. So one would expect that as this water cage approached infinity, this water vibrational frequency would get larger and eventually settle to a frequency probably in the range of 100-200 cm^{-1} (since the rate of increase in frequency between the pentamer and hexamer is smaller), which would correspond to the characteristic energies transmitted by the membrane acyl chains through their LAM modes. This is also only one aspect of water cage vibrations, and there are certainly many others that would contribute to the total energy that is transmitted by the solvent molecules. This energy stored in the solvent molecules in the cell interior is now redistributed among the other low frequency modes of proteins and other molecules (such as DNA and RNA) for use in carrying out many of the cell processes.

Many enzymes and binding proteins consist of several globular domains connected

by relatively flexible polypeptide chains [12-14]. The binding sites for substrates and ligands are typically located between these domains, and thus the time scale and relative motion of the domains would play an important role in the binding and activity of the enzymes (proteins). These large scale motions would involve collective motions with the concerted displacement of many atoms. Normal mode calculations done on the small protein bovine pancreatic trypsin inhibitor (BPTI) showed that some of these large scale motions were low frequency modes on the order of 3.6-6 ps^{-1} (100-200 cm^{-1}), which typically involved motion of several neighboring residues. More global motions have frequencies less than 3.6 ps^{-1} (100 cm^{-1}) [15]. Adiabatic mapping calculations for hinge bending motions, one type of global motion, were first done for lysozyme, in which its active site is located in a cleft between two globular domains [16]. These calculations, and further improved calculations that take into account solvent damping effects, indicate that the hinge bending motion is greater than 10 ps (less than 0.1 ps^{-1}). In fact with solvent damping effects, the hinge motion is expected to be Brownian in nature with a typical fluctuation opening and closing the cleft by 0.1 nm and will persist for about 20 ps [17]. In regards to other types of proteins, fluorescence depolarization techniques were used to determine the time scale of hinge bending motions of immunoglobulins is in the range of 10-50 ns [18, 19]. Also, indirect evidence provided by X-ray crystal structures indicate that tRNA molecules may even have hinge bending motions [20]. Initial investigations of this movement for $tRNA^{Phe}$ have been done [21, 22], and molecular dynamics simulations show a periodic motion of the bending between the two arms of the $tRNA^{Phe}$ molecule which is 24 ps [23]. It is expected that solvent damping effects would increase this time scale to the characteristic nanosecond range

[12]. It has also been shown that several proteins have biologically important strand folding and unfolding motions, which often occur in peptide segments near binding or active sites. Upon binding of the substrate or ligand these segments assume ordered conformations in which they cover or otherwise interact with the bound molecule [12-14]. For example, the active site of trypsin, which consists of several polypeptide loops, is highly ordered; but in its inactive precursor, trypsinogen, these loops are disordered. The apparent characteristic time scale of this loop motion is about 11 ns [24], which is in agreement with studies done on the growth kinetics of alpha helices [25]. The time scale of these global motions of proteins and other macromolecules are limited by the viscosity of the solvent, which is water in biological systems. Thus solvent damping impedes these global motions, and hence their rates of motion are diffusion limited and are in a very low frequency range (less than 100 cm^{-1}).

Based on all of this evidence, though some of it is indirect from various types of simulations, one can perceive that the energy transmitted by the membrane acyl chains is disseminated among the many vibrational modes of the solvent cage (and other molecules) in the cell interior. These numerous solvent-lattice vibrational modes store and propagate many frequencies of energy, in the low frequency range, to the proteins and other large molecules in the cell, which are then stored in these molecules in their various vibrational modes, thus allowing them to carry out the myriad cellular processes.

In this chapter, focus is on the acyl chain length heterogeneity of membranes and their

function in transmitting the available thermal energy spectrum. However, membrane lipid acyl chain heterogeneity also includes differences in unsaturation, branching, and the presence of carbocyclic rings. It has been shown that functionalization of an acyl chain by single alkenyl, methyl, and cyclopropyl groups does not cause a significant difference of the acyl chain LAM frequencies [26]. These functionalizations, though, do affect the packing arrangement of the membrane acyl chains. Thus these alterations in acyl chain structure most likely affect other aspects of membrane motional dynamics as is predicted by the homeoviscous adaption theory. As a result these changes may play important roles in maintaining other aspects of membrane function, such as their selective permeability functions or control of certain enzymatic processes by altering the membrane domain compositions.

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CHAPTER III

THE INTERRELATION BETWEEN TEMPERATURE-INDUCED SHIFTS IN CARBON METABOLISM AND MEMBRANE LIPID CHEMISTRY

ABSTRACT

The available thermal energy, determined by temperature, drives the chemical processes carried out in living organisms. The ability to utilize a particular substrate to derive chemical energy (typically ATP) is dependent upon the transition state (activation) energies of all of the steps in the metabolic pathway for that substrate. If the available thermal energy is not enough to overcome the activation energy for one or more steps in the metabolic process, the pathway requires significant energy investment or priming, and/or the energy yield is too low, then the organism might not be able to utilize the given substrate effectively. This is true for the more highly oxidized substrates like carbohydrates. As a result, the balance of metabolism might conceivably shift to utilization of more reduced substrates, like amino acids and fatty acids. The utilization of which might require lower activation energies and certainly requires less of a chemical energy investment and can yield much more chemical energy per gram. The notion that more energy is required to support cellular upkeep at lower temperatures is well established and embodied in the concept of maintenance energy. Based on this premise we investigated the ability of Arctic strain 45-3, which thrives in extremely cold environments, to utilize glucose at 4°C and 24°C. One special advantage of such a strain is that it allows a substantial spread of temperature. Most organisms that are available in culture do not grow well below 15°C. Our results, using NMR spectroscopy and radioactively labeled glucose, indicate that this strain utilizes glucose much more slowly at 4°C than at 24°C. We also show that enzymatic control of metabolic processes, such as carbohydrate and amino acid metabolism, must be done by regulation of constitutively expressed enzymes because the total cellular protein profiles at the two

temperatures are essentially identical. The fact that this strain can use glucose more readily at 24°C is in agreement with our observation that more straight membrane acyl chains are produced at 24°C than at 4°C. These results support the idea that the available thermal energy regulates the balance of chemical processes carried out in the cell, and this balance determines the metabolic intermediate profile which is reflected in the membrane acyl chain structure.

INTRODUCTION

As temperature fluctuates the amount of heat (infrared quanta) available for use by cells changes in turn. This energy (kT), which is propagated into the cell interior by the longitudinal accordian modes (LAMs) of the membrane acyl chains, provides the necessary energy for various metabolic pathways [1]. In Figure 3.1 hypothetical energy profiles for two different metabolic pathways are shown. At high temperatures (T_1) there is most likely enough energy to carry out either metabolic pathway. At lower temperatures (T_2), though, the available energy may not be enough to get over the activation barriers of the pathway indicated by profile A, so the cell has no choice but to use the metabolic pathway indicated by profile B. In general the more oxidized substrates, such as carbohydrates, require more chemical energy input in their metabolism. More reduced substrates such as amino acids and fatty acids require less energy input.

The structure of the cell membrane reflects the type of metabolism carried out by the

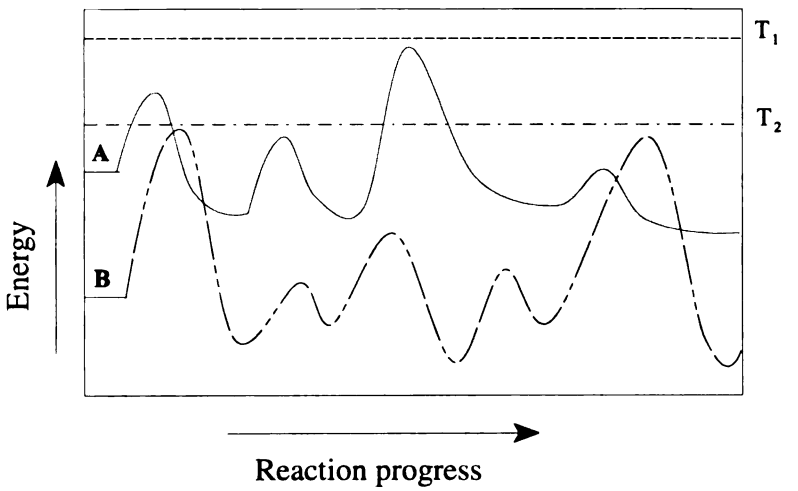

Figure 3.1: Hypothetical energy profiles for two different metabolic pathways are shown, a higher energy requiring profile in (A) and a lower energy requiring profile in (B). Note that at high temperatures (T_1) there is most likely enough energy to carry out either metabolic pathway. At lower temperatures (T_2), though, the available energy may only be enough to use the metabolic pathway indicated by profile B.

cell. Evidence of this is suggested by the literature documenting the greater occurrence of branching in membrane fatty acids at low temperatures [2, 3]. The structures of these fatty acids are determined by the acyl-CoA primers. For the branched fatty acids the primers result from metabolism of the amino acids, leucine, valine, and isoleucine [4]. The tendency to synthesize branched fatty acids at lower temperatures therefore could be interpreted to mean that amino acid metabolism is more favorable than carbohydrate metabolism at low temperatures.

Changes in metabolism can be accompanied by changes in either the activity or the levels of synthesis in the enzymes involved. The controls of metabolic pathways could be accomplished by *de novo* protein synthesis, or induction or suppression of constitutively expressed enzymes. Changes in metabolism with temperature can also occur in the absence of both these two control mechanisms. This is because at higher temperatures, there may be enough activation energy to make some pathways active that are not active at low temperatures. Here we studied the ability of an Arctic permafrost bacterium to utilize glucose over its growth temperature range as well as monitor its protein profiles at two temperatures. This bacterium, Arctic strain 45-3, utilizes glucose much more slowly when grown at 4°C than at 24°C. The protein profiles for this bacterial strain at 4°C and 24°C are essentially the same, indicating that the control of different metabolic pathways likely occurs through thermal or constitutive enzyme control.

MATERIALS AND METHODS

Culture conditions of Arctic strain 45-3

Siberian strain 45-3 was cultured in trypticase soy broth (TSB) at 4°C and 24°C with shaking at moderate speeds.

Analysis of trypticase soy broth before and after bacterial growth

Samples of TSB media before and after Arctic strain 45-3 was cultured at 4°C and 24°C (both cultures were grown to about the same optical density) were taken and concentrated to dryness. The samples were then resuspended in 0.6 ml of D_2O. ^1H NMR spectroscopy was done on each sample. Two dimensional DQF-COSY (double quantum filtered-correlated spectroscopy) and TOCSY (total correlated spectroscopy) spectra were also done for the unused TSB media sample.

Rate of utilization of carbon sources by Arctic strain 45-3

Five milliliter aliquots of TSB media were made, and 0.2 μCi of ^{14}C-glucose, ^{14}C-leucine, and ^{14}C-valine (each) were added to the media. Then the media was inoculated with Arctic strain 45-3, and cultures were grown at 4°C and 24°C. Samples (100 μl) were taken before the cells were added and then every 4 hours for at least 36 hours (longer for the 4°C cells). The cells were centrifuged out and then 20 μl of the media were spotted on TLC plates. The metabolites on the TLC plates were separated using a solvent system of 7:3:1 1-propanol:ammonium hydroxide:H_2O. The plates were then exposed to a phosphoimager.

The intensities of spots were determined using the integration routines available in the ImageQuant™ package.

Total protein profiles of Arctic strain 45-3 grown at 4°C and 24°C

Cultures of Arctic strain 45-3 at 4°C and 24°C were grown as described above. Total cell lysates were prepared from 4°C and 24°C cultures by adding two volumes of 10 mM Tris-HCl, pH 8.0 and 3 mg/ml lysozyme and incubating for one hour on ice. One volume of 2X SDS-PAGE sample buffer solution [0.125 M Tris-HCL (pH 6.8), 0.04% sodium dodecylsulphate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.0025% bromophenol blue] was then added. The samples were mixed well, boiled for 5 minutes, and centrifuged briefly to remove any precipitate. The lysates from 4°C and 24°C cultures were separated using Laemmli's discontinuous buffer system [5] on 12% polyacrylamide slab gels containing 0.32% bisacrylamide, 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel contained 4% polyacrylamide, 0.1% bisacrylamide, 0.125 M Tris-HCl (pH 6.8), and 0.1% SDS. Electrophoresis was carried out at 80 V for about 3 hours. Gels were stained in a solution of 0.1% Coomassie blue in 40% methanol/10% acetic acid, and destained in a solution of 40% methanol/10% acetic acid.

RESULTS AND DISCUSSION

Arctic strain 45-3 has predominantly branched chain fatty acids indicating the use of amino acid derived acyl-CoA primers for fatty acid synthesis. While amino acid metabolism

predominates in generating these primers throughout the growth temperature range, the amount of C_{16} straight chain fatty acids increases by almost a factor of six in going from 4°C to 30°C (Table 3.1), which is consistent with an increased utilization of carbohydrates. Also at 24°C and 30°C, C_{18} straight chains (not shown) are detected. Based on the reasons that the

Table 3.1: Percent of fatty acid methyl ester derivatives in the total methanolysate of the cells grown at 4°C, 14°C, 24°C, and 30°C in order of elution. The numbered components correspond to the numbered GC peaks in Figure 4.12.

Peak No.	Structure	% in 4°C Cells	% in 14°C Cells	% in 24°C Cells	% in 30°C Cells
1	$C_{14:1}$	1.30	1.41	0.00	0.00
2	*iso*-C_{14}	2.65	3.18	2.24	1.08
3	n-C_{14}	1.55	3.02	2.78	3.74
4	$C_{15:1}$	17.58	9.09	0.87	0.28
5	*anteiso*-C_{15}	62.67	64.53	68.82	60.87
6	*iso*-C_{16}	8.72	10.22	10.38	6.31
7	n-C_{16}	3.11	5.43	8.61	17.69
8	*anteiso*-C_{17}	2.42	3.10	6.31	10.02

pathway requires significant energy investment or priming or the energy yield is insufficient when the available thermal energy is too low, it appeared possible that carbohydrate metabolism might be less effective at lower temperatures than other forms of metabolism. We investigated the ability of Arctic strain 45-3, which survives in extremely cold climates, to utilize glucose and amino acids at 4°C and 24°C. ^{1}H NMR of TSB media before (Figure

3.2A) and after growth of Arctic strain 45-3 at 4°C (Figure 3.2B) and 24°C (Figure 3.2C) are shown. The signals for the α- and β-anomeric protons of glucose at 5.18 and 4.58 ppm, respectively, have almost completely disappeared from the media of the 24°C culture, while the relative decrease in these signal intensities for the 4°C culture is much smaller.

To further investigate this bacterium's ability to metabolize glucose, ^{14}C-labeled glucose, leucine, and valine were added to the TSB media for 4°C and 24°C cultures. Figure 3.3 shows the TLC separation of ^{14}C-glucose, ^{14}C-valine, and ^{14}C-leucine for one time point and its corresponding analog profile indicating the relative intensities of the three spots. Figure 3.4 shows the extent of utilization of ^{14}C-glucose by the bacterium at 4°C and 24°C. The time points at 24 hours and 67 hours for the 24°C and 4°C, respectively, are when the two cultures are at about the same optical density. The results from this radioactive label experiment confirm the NMR results, which indicate that Arctic strain 45-3 utilizes glucose at a greatly reduced rate at 4°C, while at 24°C the glucose becomes limiting very quickly. Since the trypticase soy broth medium is largely peptides and amino acids (17 g/l pancreatic digest of casein; 3 g/l papaic digest of soybean meal; and 2.5 g/l glucose), the percent reduction of ^{14}C-valine and ^{14}C-leucine was small. These results, however, support our hypothesis that the ability to use more oxidized substrates at low temperatures is impeded. Thus the bacteria shift the balance of their metabolic pathways to use more reduced substrates, amino acids for instance.

To determine whether this shift in metabolism is reflected in variations of this strain's

Figure 3.2: ^{1}H NMR of TSB media before and after growth of Arctic strain 45-3. (A) Unused TSB media, (B) TSB media from 4°C culture, and (C) TSB media from 24°C culture. Most of the glucose signals are in the 3.2-3.6 ppm range, while some of the free amino acid and polypeptide signals are in the 1.0-1.5 ppm range. However, note the almost complete disappearance of the (1) α-anomeric [5.18 ppm] and (2) β-anomeric [4.58 ppm] glucose signals in the 24°C culture, as compared to the unused media and the 4°C culture.

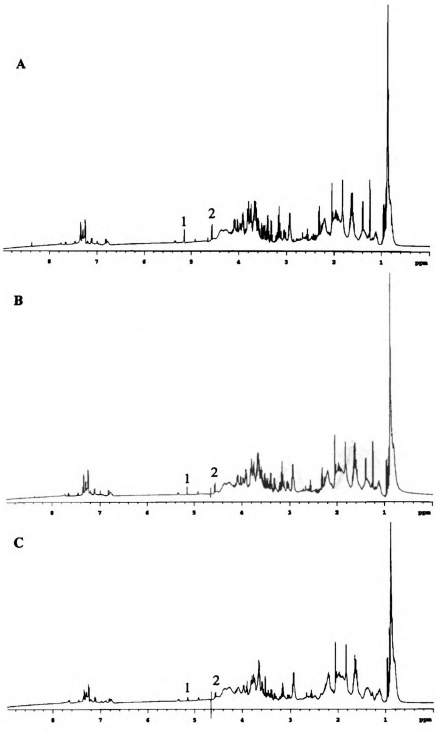

Figure 3.2

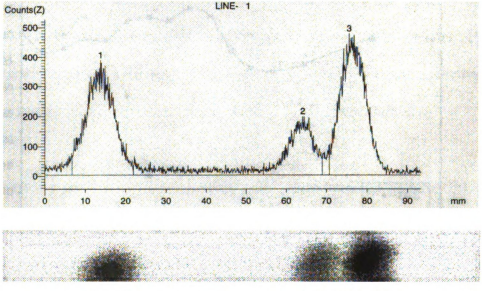

Figure 3.3: TLC separation of ^{14}C-glucose, ^{14}C-valine, and ^{14}C-leucine for one time point and its corresponding analog profile indicating the relative intensities of the three spots.

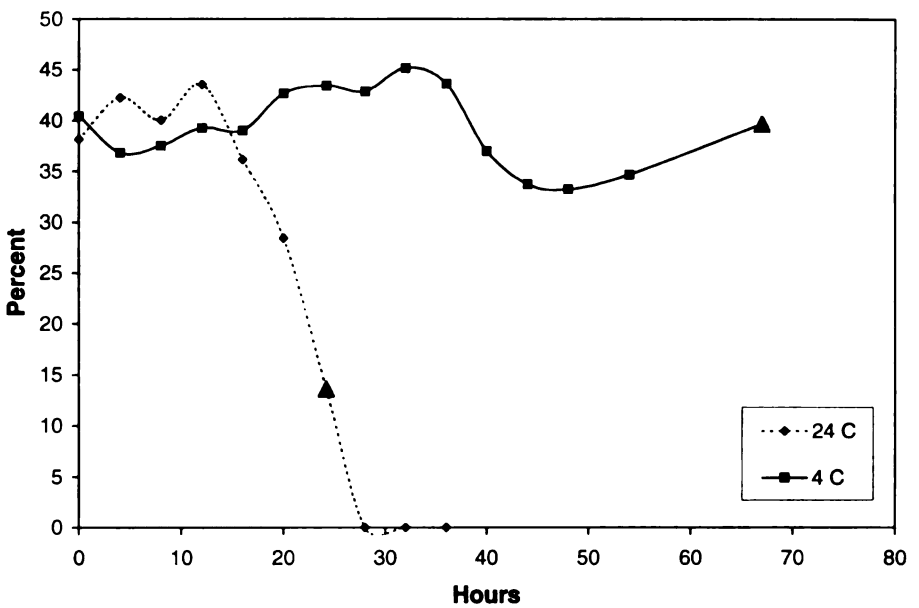

Figure 3.4: The extent of utilization of 14C-glucose by Arctic strain 45-3 at 4°C and 24°C. The time points at 24 hours and 67 hours for the 24°C and 4°C cultures, respectively, are when the optical densities for the two cultures are about equivalent.

enzyme production, total protein profiles from 4°C and 24°C cultures were determined. These profiles are shown in Figure 3.5. The profiles for the two temperatures are essentially the same, showing no appearance or disappearance of different proteins at the two temperatures. The differences seen in some band intensities is not consistent upon repeated culturing of the organism at the two temperatures. In many gels of the total cellular proteins from subsequent cultures of this organism at the two temperatures, no significant differences in band intensities was seen. This indicates that regulation of the metabolic pathways in this organism must occur largely by induction or suppression of constitutive enzyme activity, or by thermal regulation in which higher temperatures may provide enough activation energy to make some pathways active that are not active at lower temperatures.

As has been discussed previously, there is a relationship between the types of metabolism carried out by an organism and its membrane acyl chain structure. This relationship is represented in Figure 3.6. The balance between carbohydrate and amino acid metabolism, for instance, can be regulated by *de novo* enzyme synthesis, or by activation or suppression of constitutively expressed enzymes, or by thermal regulation as described above. The balance between these two types of metabolism determines the available acyl-CoA primers for fatty acid synthesis. An increase in amino acid metabolism, in particular the levels of leucine, isoleucine, and valine, would increase the pool of branched acyl-CoA primers. In contrast, an increase in carbohydrate metabolism would increase the pool of acetyl-CoA leading to straight acyl chains.

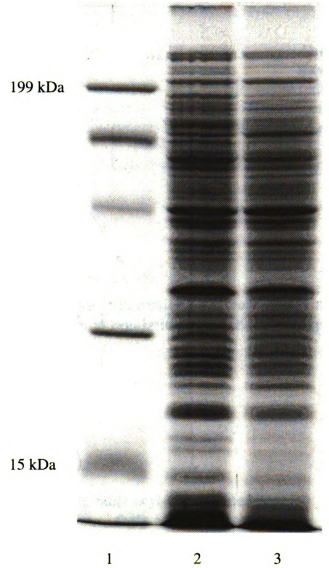

Figure 3.5: Total protein profiles for Arctic strain 45-3. Lane (1) low molecular weight standards, (2) 4°C, and (3) 24°C.

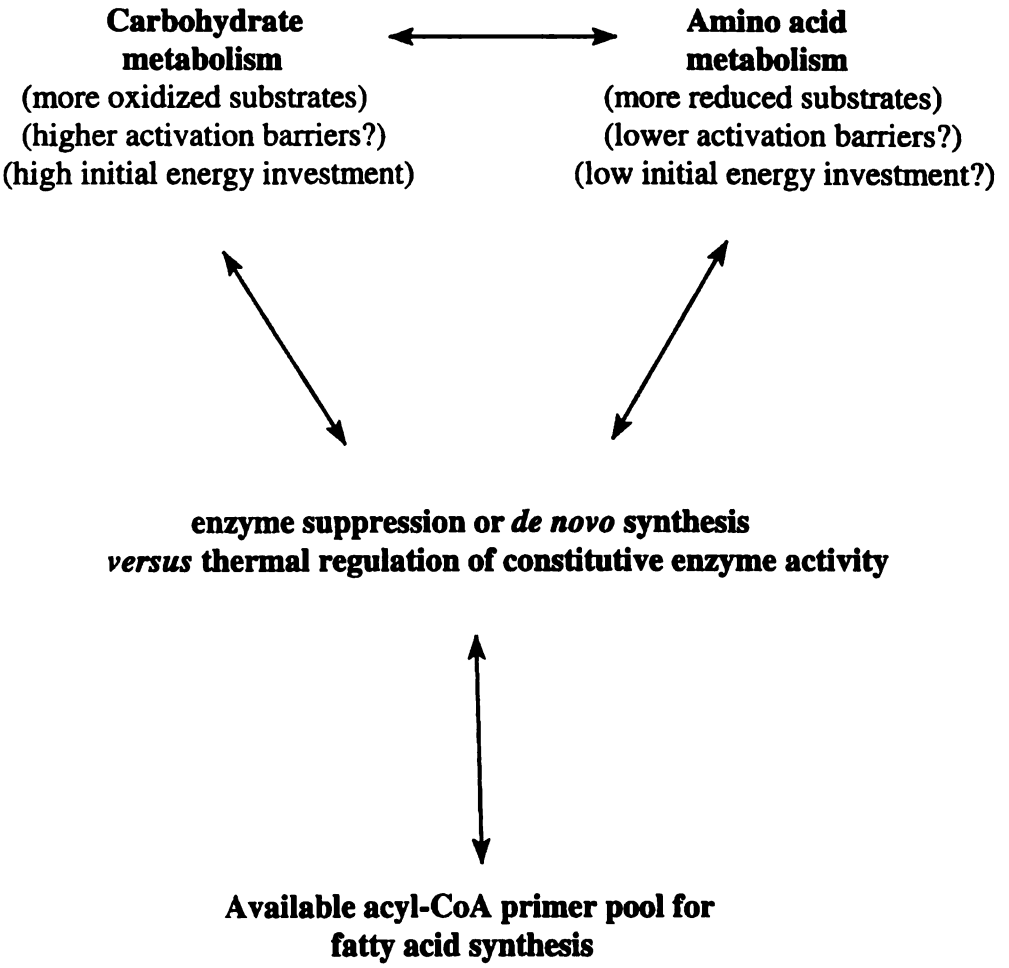

Figure 3.6: Schematic representation of the relationship of the balance between two types of cellular metabolism, the potential thermal or enzymatic control of the metabolic processes, and the pools of available acyl-CoA primers for fatty acid biosynthesis.

The balance between these two metabolic pathways is controlled by the available thermal energy. Since carbohydrates are more oxidized substrates, they generate less energy per gram (669.94 kg-cal/g for glucose) than amino acids (855.6 kg-cal/g for leucine) [6]. Carbohydrates also require more energy to prime (in phosphorylation steps) and is oxygen dependent. In low temperature climates not only is the available thermal energy reduced, but the oxygen availability is also much less (due to decreased diffusion of oxygen through cold or frozen soils). So as temperature drops the highly oxygen dependent and energy costly metabolic pathways, i. e. carbohydrate metabolism, shut down in favor of utilization of the more reduced substrates, amino acids and fatty acids. This is also consistent with the fact that maintenance energy is higher at lower temperatures [7-9]. Therefore organisms that use energy sources that yield more energy per gram would be more successful at low temperatures as well. Therefore the balance of metabolic processes, which is determined by the available thermal energy, is reflected by the structure of the membrane acyl chains. At higher temperatures more straight chains are produced, while at lower temperatures more branched chains are made.

Since the available thermal energy determines the balance of cellular metabolic processes, the outcome of competing cellular reactions is determined by kinetics (the activation energy barriers of the steps in the pathways) and not thermodynamics (the difference in free energies of the starting materials versus the products). In other words, if two chemical processes in the cell are competing, then the short-term outcome of the competition is determined by the activation energy barriers the two processes face. The

ability to overcome these barriers is determined by the available thermal energy spectrum that has been transferred to the cell interior. One reaction may have a lower activation energy barrier, but the resultant product has a higher free energy content. So these products are kinetic products, and they accumulate the fastest because of the lower activation barriers to their formation and are not determined by the final free energies of these products. If the cellular systems were closed systems and were in equilibrium, these higher energy kinetic products, if there is some reversibility in the processes, would revert to the lower energy thermodynamic products. However, cellular metabolic pathways are an intricate web of reactions, and the kinetic products are often entrained in subsequent reactions such that the entire process is determined by kinetics and not by thermodynamics (free energy differences). Also in living systems, energy is constantly being fed in as heat, light, and/or nutrients, and energy is constantly being given off in completely unpredictable patterns in swimming, crawling, or walking. Thus an equilibrium state of cellular processes, as would be required for thermodynamic control of chemical processes, is never reached. Consequently, the nature of living systems is determined by kinetics, not thermodynamics.

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CHAPTER IV

THE ADAPTATION OF ARCTIC BACTERIA TO LOW PRE-FREEZING TEMPERATURES INVOLVES MEMBRANE ALTERATIONS BUT NOT INCREASED PRODUCTION OF OSMOLYTES

ABSTRACT

An Arctic bacterial strain isolated from the Siberian permafrost grown at 4°C produced undetectable levels of osmolytes such as glycerol, sugars, other sugar alcohols and glycine betaine in the cytoplasm. The relative proportions in the total carbohydrate and amino acid metabolic profiles between cells cultured at 4°C and 24°C using NMR spectroscopy showed no significant changes. There was a net reduction by a factor of 4.6 in the total amount of amino acids at 4°C compared to 24°C. In the cells cultured at 4°C, major changes in membrane chemistry and cell surface chemistry were observed compared to those grown at 24°C. There was a change in composition and a dramatic reduction in the amount of polysaccharide capsule formed when cells were grown at 4°C compared to 24°C. Because of this, cells from the lower temperature culture were hardly wettable. This would negate the effect of freezing water on the membrane surface. Cells grown at the lower temperature were smaller in volume by a factor of ~14. These results indicate that the major mechanism for low temperature adaptation in this organism is alteration of the surface and membrane structure.

INTRODUCTION

The processes bacteria use to allow them to survive and function at close to freezing temperatures and below is a mystery that has fueled much interest and spawned several theories. It is generally believed that there are two major physical or physico-chemical threats that the bacterium needs to respond to in these temperature regimes. The first is ice formation

within the cell which might lead to cell lysis because of the volume increase on expansion of water as ice is formed. The second is the increased salinity outside the cell as ice formation leads to the separating out of pure water (as ice) and a corresponding increase in salt concentration in the liquid state. It is thought that a general mechanism to counteract both of these phenomena would be an increase of solute concentration inside of the bacterial cell. A reasonable response then would be for the organism to increase the intracellular amounts of certain metabolites especially glycine betaine, glycerol, mannitol and sorbitol both as cryo-protectants and as osmolytes (1-3). There are certain to be other mechanisms at work to protect against freezing since as temperature decreases the solubility of most organic molecules and salts decreases. The decrease is rather dramatic in the case of phosphates and carbonates. This solubility trend is in the opposite direction to that required for freeze protection by increasing osmolyte concentration. There are other aspects of bacterial cell structure that can contribute to this adaptation. These include membrane structure, capsular polysaccharide structure and amount and cell size. For instance, the membrane can be protected from freezing water by increasing its hydrophobicity. This can be accomplished by changing the structure and reducing the amount of the hydrophilic capsule. A change in structure might lead to a microenvironment that reduces the tendency of water associated with it to freeze. Making the actual surface more hydrophobic by removing charged groups from the lipids will reduce the interaction between the actual membrane surface and external water.

Psychrophilic bacteria (organisms that are adapted to these near-freezing and below

conditions) are very good systems in which to examine the mechanisms of bacterial adaptation to these extremes. In this study, we used a strain of bacteria that was obtained from the permafrost sediment core of the age 60-100 thousand years deposited in the Siberian tundra zone on Kolyma-Indigirka Lowland (152-162°E and 68-72°N), see Figure 4.1. The permafrost sample was received by rotary drilling without any solution or chemical reagents. The temperature of the extracted core was no higher than -7°C. The methods of sampling, storage, transportation and contamination control were described previously (4). Using these methods, it was shown that the micro flora in the samples were there *in situ* and were not the result of contamination. The organism studied was isolated from a sample at a core depth of 4.0 meters and was named 45-3. The bacterium was a gram positive and according to its morphological properties and 16S rRNA sequence (Jim Tiedje, Center for Microbial Ecology, Michigan State University, Michigan; personal communication) was an *Arthrobacter* sp. The bacterial morphology existed as rods and V-forms in young cultures and coccus type in old cultures. The strain has a temperature range for growth of 4 to 30°C and an optimal growth temperature of 23°C.

MATERIALS AND METHODS

Culture conditions

Siberian strain 45-3 was cultured in trypticase soy broth at 4°C and 24°C with shaking at moderate speeds.

Figure 4.1: Siberian permafrost bacterial sampling region in the tundra zone of north-eastern Russia, Kolyma-Indigirka Lowland (152-162°E and 68-72°N). Strain 45-3 was isolated from a core sample from site D.

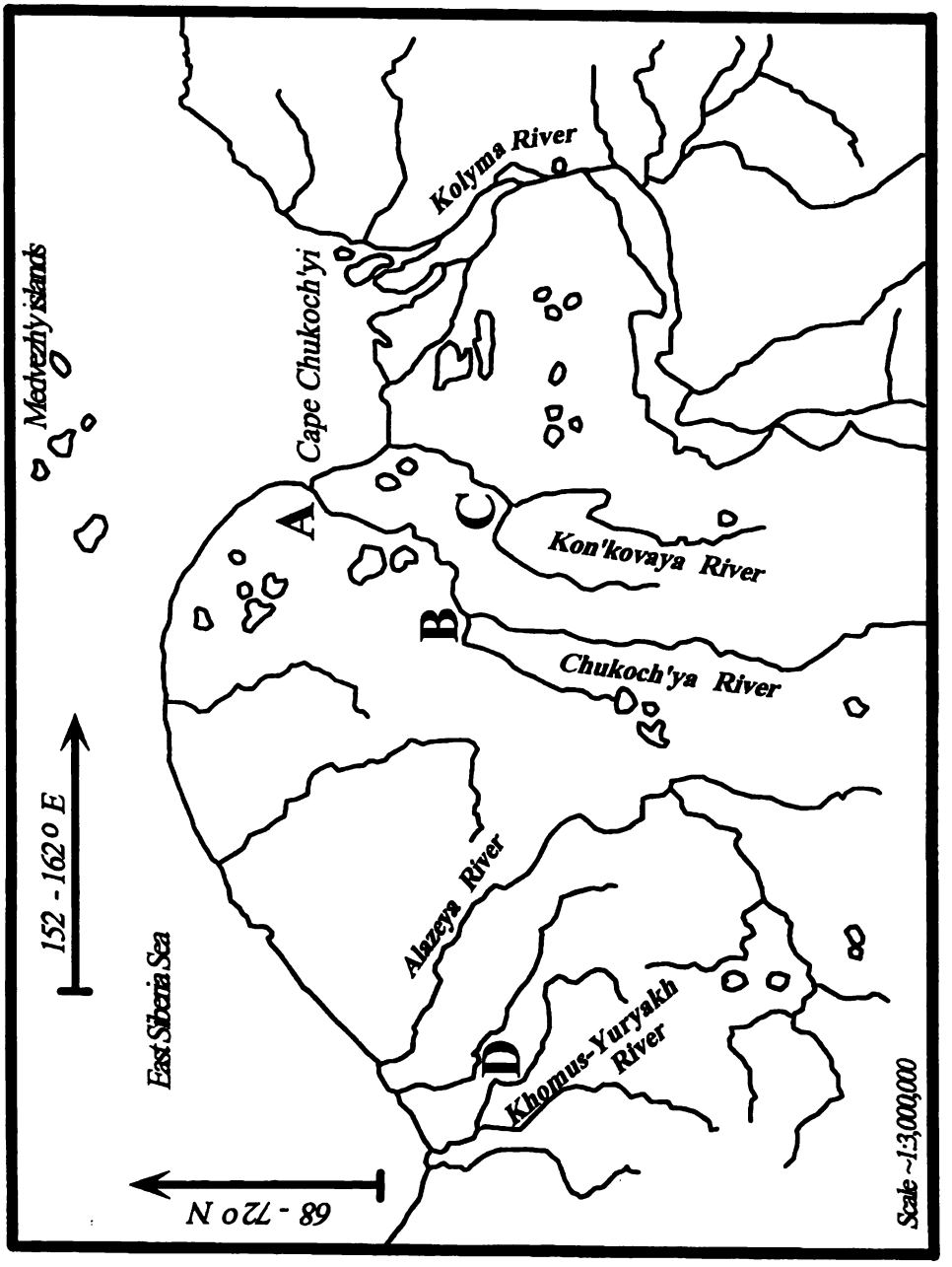
Kolyma River
Medvezhy islands
Cape Chukoch'yi
A
B
C
D
Kon'kovaya River
Chukoch'ya River
Alazeya River
Khomus-Yuryakh River
152 - 162° E
East Siberia Sea
68 - 72° N
Scale ~1:3,000,000

Figure 4.1

Hydrophobicity analysis

Cells from 4°C and 24°C cultures were diluted to the same optical density. Glass coverslips coated with chlorotrimethylsilane were dipped into the cultures for several minutes and then rinsed thoroughly in a stream of fresh media. The coverslips were placed on slides and the cells were then stained with several drops of 0.1% acridine orange for five minutes. The coverslips were again washed thoroughly using fresh media. The cells were viewed using rhodamine *iso*-thiocyanate (RITC) optics with excitation and primary barrier filters at 529 and 550 nm respectively.

Total lipid extraction

Cells from the 4°C and 24°C cultures were stirred with 60 ml H_2O, 130 ml $CHCl_3$, and 15 ml MeOH at 30°C for 12 hours. The layers were separated by centrifugation. The aqueous layers were extracted once more with 30 ml $CHCl_3$ for 15 min., and the organic layers were then combined. The aqueous layers were again centrifuged and the clear solutions were kept. The organic layers were analyzed by NMR spectroscopy and by thin layer chromatography using 10:4:2:2:1 chloroform/acetone/methanol/acetic acid/water. TLC analyses were performed on silica gel plates. The spots were visualized by spraying either with orcinol or 10% phosphomolybdic acid in ethanol and heating at 120°C to visualize the organic components.

Polysaccharide isolation

The aqueous layers from the total lipid extracts were concentrated to 4 ml and 12 ml of ethanol was added. The polysaccharide which precipitated was recovered with a glass rod

and dissolved in 10 ml with water, and 10 mg $MgCl_2$, and 10 units each of RNase A and DNase were added. The solutions were kept at room temperature for 2 hours. They were then dialyzed for 4-5 hours, changing the water twice during that time, lyophilized and the resulting solids were weighed. These polymers were analyzed by NMR spectroscopy and by gas chromatography-mass spectrometry (GC-MS) after converting them to alditol acetate derivatives [5]. The remaining aqueous alcoholic solution was centrifuged to remove any precipitated solids. The supernatants which contained free amino acids, carbohydrates, and the like were removed and analyzed by NMR spectroscopy and a sample subjected to amino acid analysis on a Waters Millipore system which included a WISP 710B autosampler, a Waters 510 pump, and a Waters 440 absorbance detector.

Fatty acid methyl ester analysis

Total cells from cultures grown at 4°C and 24°C were methanolysed in 2% HCl in methanol at 75°C for 30 hours. The suspensions were blown to dryness, and 1 ml H_2O and 2 ml hexane were added. After vigorous shaking, the mixture was centrifuged and the hexane layer was removed and concentrated to dryness. The resulting fatty acid methyl esters were analyzed by gas chromatography (GC) and GC-MS using a 30 m DB1 column. The temperature program was a ramp from 160°C to 320°C at 3°/min. The final temperature of 320°C was held for 20 min.

Lipid headgroup analysis

Approximately 5 mg of total lipids (isolated as described above) from cells cultured at 4°C and 24°C were dried, and then 0.5 ml of 2 M trifluroacetic acid was added. The

lipids were hydrolyzed at 120°C for 1.5 hrs. The lipid headgroups were obtained by adding 0.5 ml of H_2O and extracting twice with 2 volumes of $CHCl_3$. The chloroform layers were removed and combined. The aqueous layers, containing the lipid headgroups, were dried and analyzed by NMR spectroscopy.

Protein analysis by SDS-PAGE

Total cell lysates were prepared from 4°C and 24°C cultures by adding two volumes of 10 mM Tris-HCl, pH 8.0 and 3 mg/ml lysozyme and incubating for one hour on ice. One volume of 2X SDS-PAGE sample buffer solution [0.125 M Tris-HCL (pH 6.8), 0.04% sodium dodecylsulphate (SDS), 10% glycerol, 5% β-mercaptoethanol, 0.0025% bromophenol blue] was then added. The samples were mixed well, boiled for 5 minutes, and centrifuged briefly to remove any precipitate. The lysates from 4°C and 24°C cultures were separated using Laemmli's discontinuous buffer system [6] on 12% polyacrylamide slab gels containing 0.32% bisacrylamide, 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel contained 4% polyacrylamide, 0.1% bisacrylamide, 0.125 M Tris-HCl (pH 6.8), and 0.1% SDS. Electrophoresis was carried out at 80 V for about 3 hours. Gels were stained in a solution of 0.1% Coomassie blue in 40% methanol/10% acetic acid, and destained in a solution of 40% methanol/10% acetic acid.

Electron microscopy

Arctic strain 45-3 cells from 4°C and 24°C cultures were negatively stained with 1.5% potassium phosphotungstate and viewed by transmission electron microscopy.

RESULTS

The morphologies of the cells cultured at the two temperatures were very different. The cells that were cultured at 4°C were waxy and oily. These cells would not form a suspension even after prolonged agitation. In contrast, the cells cultured at 24°C were fluffy and readily suspendable. This indicated that there was a significant layer of polysaccharide material on their surface and that the 4°C cells did not have much of a polysaccharide capsule. This was confirmed by the weight of capsular polysaccharide material obtained from the cells. Even though the packed volume of the 4°C cells was over twice that of the 24°C cells, the yield of polysaccharide in the latter cells was 2.4 times greater than from the 4°C cells. The cells grown at 4°C were also much more susceptible to osmotic shock in a hypotonic medium compared to those grown at 24°C. Hence these cells were completely lysed on storing at 10°C in distilled water in less than 4 hours whereas the cells cultured at 24°C were still intact after 2 days under the same conditions.

Transmission electron microscopy revealed that there was a dramatic difference in cell sizes for bacteria grown at the two temperatures. Those grown at the higher temperature had twice the length and more than twice the diameter of the cells grown at the lower temperature. Since bacteria were rods at both temperatures, this represented a difference in volume of a factor of ~14 (Figure 4.2). Electron microscopy also indicated that the 4°C cells had only a microcapsule and had a very non-polar membrane surface since these took up the ionic stain only with difficulty. In agreement with these results, the hydrophobicity experiment showed that more cells from the 4°C culture (Figure 4.3A) attached to the

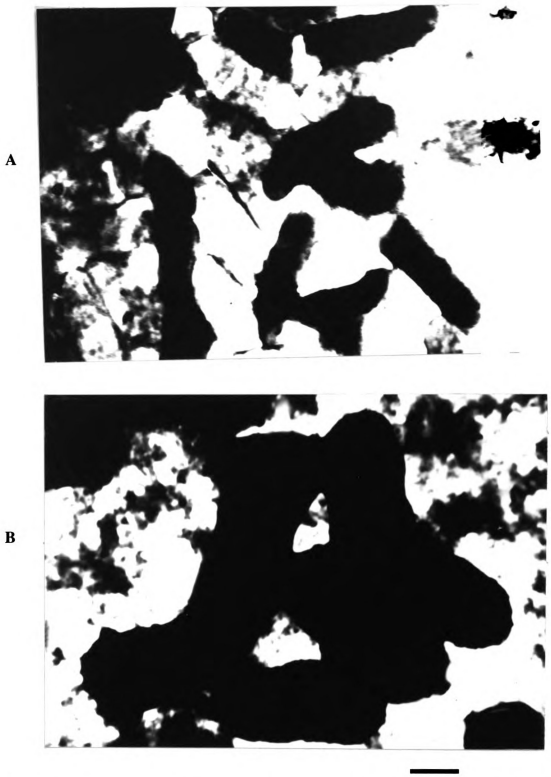

Figure 4.2: Electron micrographs (at the same magnification) of Arctic strain 45-3. Bar = 0.66 µm. (A) Cells cultured at 4°C. (B) Cells cultured at 24°C. Note that the 24°C are about two times the width and length of the cells grown at 4°C.

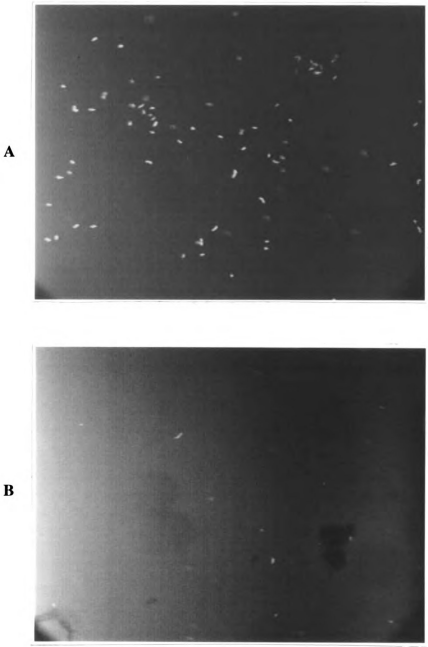

Figure 4.3: Fluorescent micrographs using RITC optics (at the same magnification) of Artic strain 45-3 stained with 0.1% acradine orange attached to silanized coverslips. (A) Cells cultured at 4°C. (B) Cells cultured at 24°C.

silanized coverslips. than from the 24°C culture (Figure 4.3B), which indicated the 24°C cells have a more polar surface and were unable to attach to the coverslips.

Proton NMR spectroscopy was used to provide a comprehensive determination of the relative amounts of the various metabolic intermediates elaborated by strain 45-3 at 4°C and 24°C. Analyses were carried out on the supernatant of the ethanol precipitation of the aqueous layer which would contain free amino acids, sugars, sugar alcohols, and nucleotides. Proton NMR spectra of these fractions from the 4°C (Figure 4.4A) and 24°C cells (Figure 4.4B) indicated that the major components were amino acids. Two dimensional DQF-COSY (Figure 4.5) and TOCSY (data not shown) experiments showed that these were glutamate, valine, glycine, methionine, threonine, and a compound tentatively identified as 3-aminobutyric acid. These results were in agreement with amino acid analyses. From the NMR spectra, the relative proportions of the amino acids in each culture did not change significantly between cells cultured at the two temperatures, which was again confirmed by the amino acid analyses in which the relative amounts varied by about 1-4%. Signals for common osmolytes such as free sugars and sugar alcohols such as glycerol, sorbitol and mannitol, which have proton shifts in the 3.0 - 4.5 ppm region were noticeably absent or at most present in only trace amounts (Figure 4.6). Signals for the methyl group of glycine betaine (~3.2 ppm), another common osmolyte were also absent.

Gas chromatography mass spectrometry analysis of the capsular polysaccharides obtained from the bacteria grown at 4°C and 24°C indicated that they contained fucose, ribose, mannose, galactose, glucose, and glucosamine (Figures 4.7A and B). There were

Figure 4.4: ^{1}H NMR spectra of the cytoplasmic metabolites. (A) Cells cultured at 4°C. (B) Cells cultured at 24°C. The peaks are labeled with the one letter amino acid codes. The major components were glutamate (E), valine (V), glycine (G), methionine (M), threonine (T), and a compound tentatively identified as 3-aminobutyric acid (see also figure 5.5). Note that there are no significant differences in the relative proportions of amino acid components.

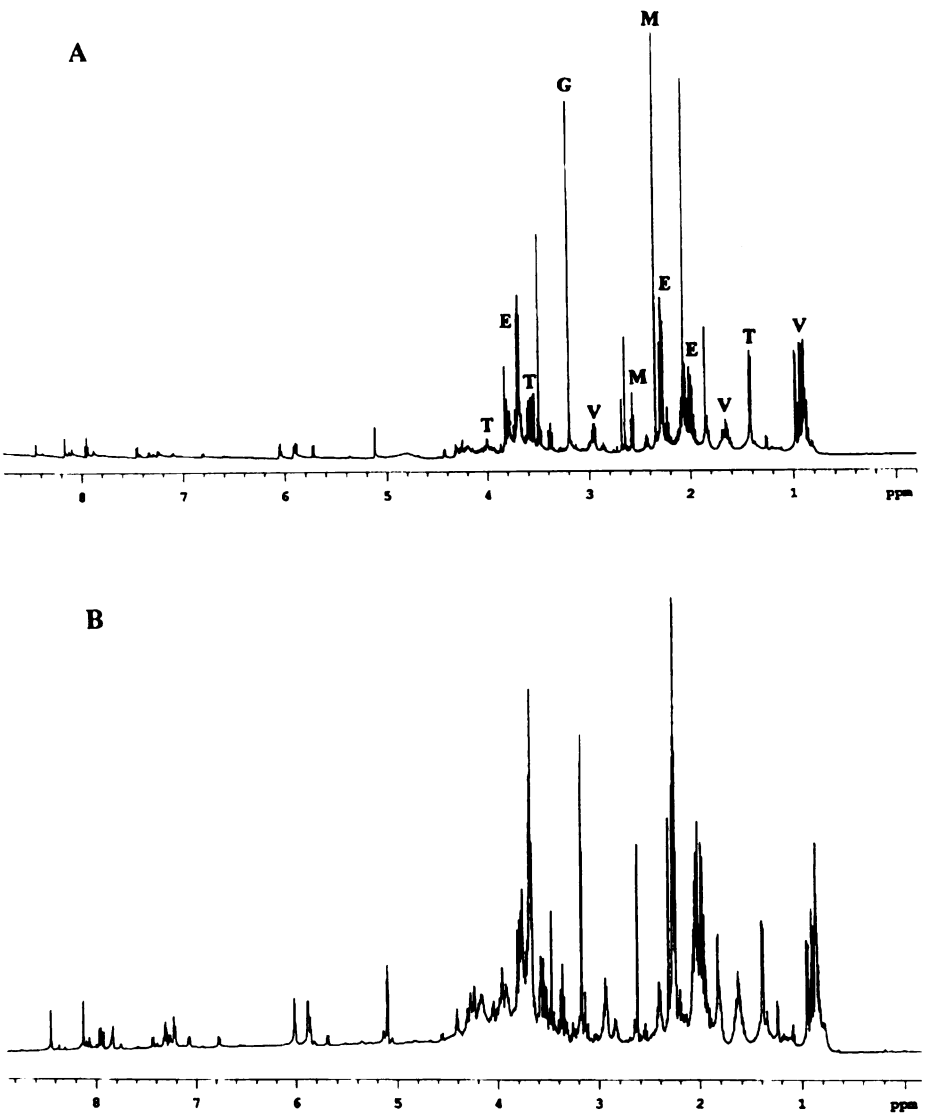

Figure 4.4

Figure 4.5: Two dimensional DQF-COSY spectrum of the cytoplasmic metabolites from cells cultured at 4°C. The cross peaks are labeled with the one letter amino acid codes.

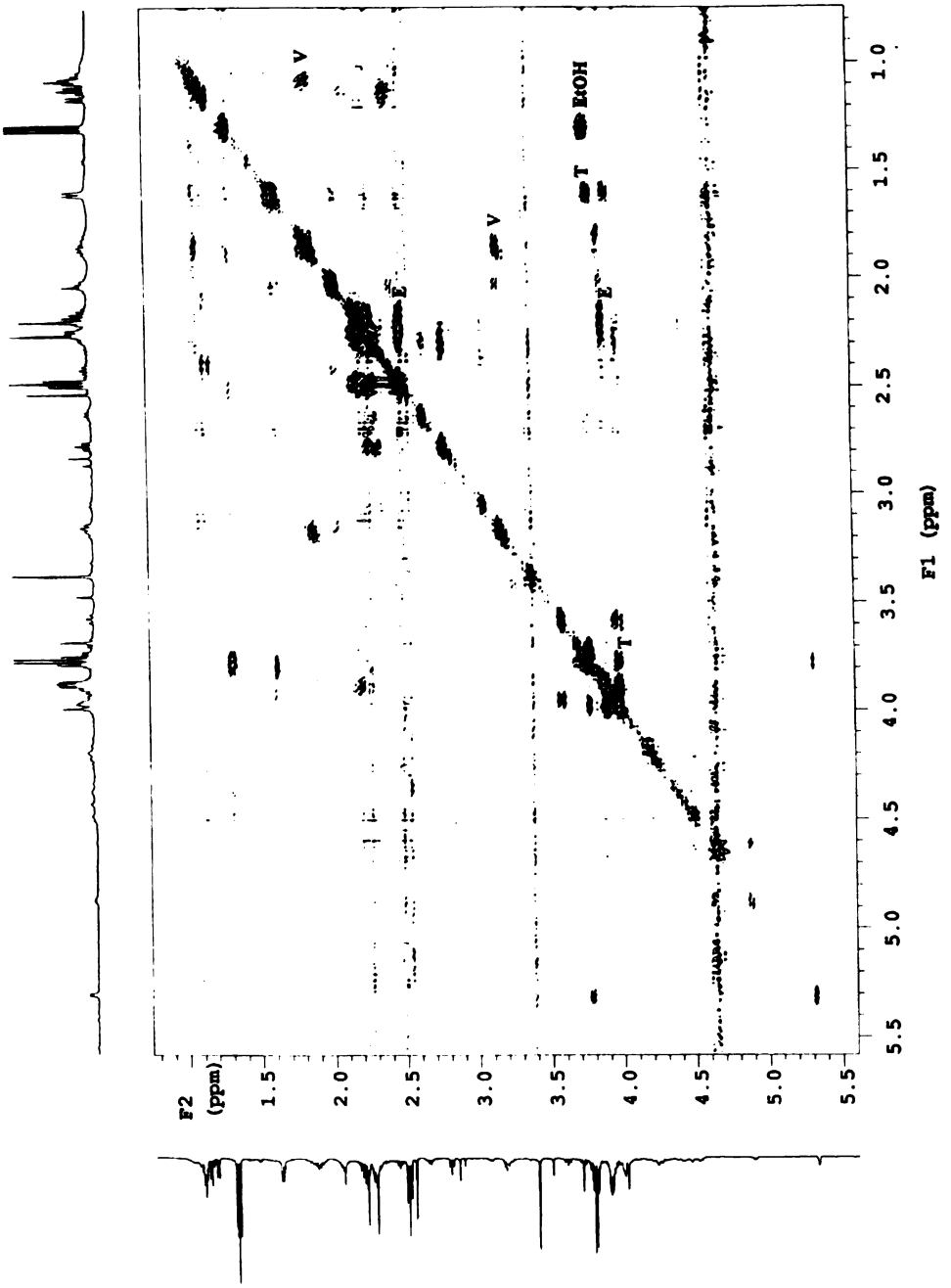
F2 (ppm)
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
F1 (ppm)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
V
V
V
E
E
T
T
EtOH

Figure 4.5

Figure 4.6: ^{1}H NMR spectra of some common osmolytes. (A) glucose (B) glycerol (C) mannitol (D) sorbitol.

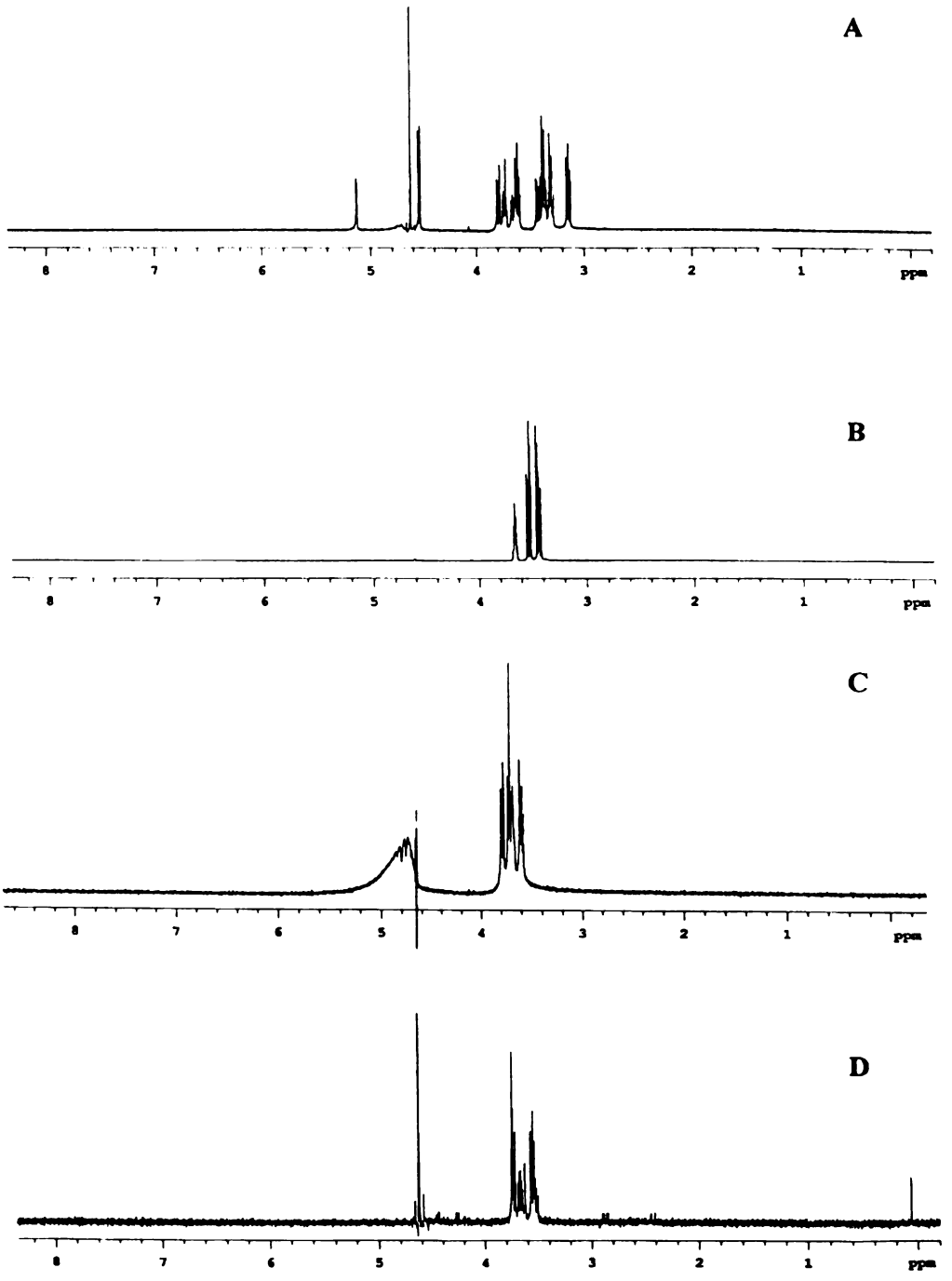

Figure 4.6

Figure 4.7: Gas chromatograph profiles of the capsular polysaccharides from the bacteria cultured at (A) 4°C and (B) 24°C. The peaks indicated are (1) fucose, (2) ribose, (3) mannose, (4) galactose, (5) glucose, and (6) glucosamine. (Note: The peak seen at 58 min. was due to a transient shift in the baseline.)

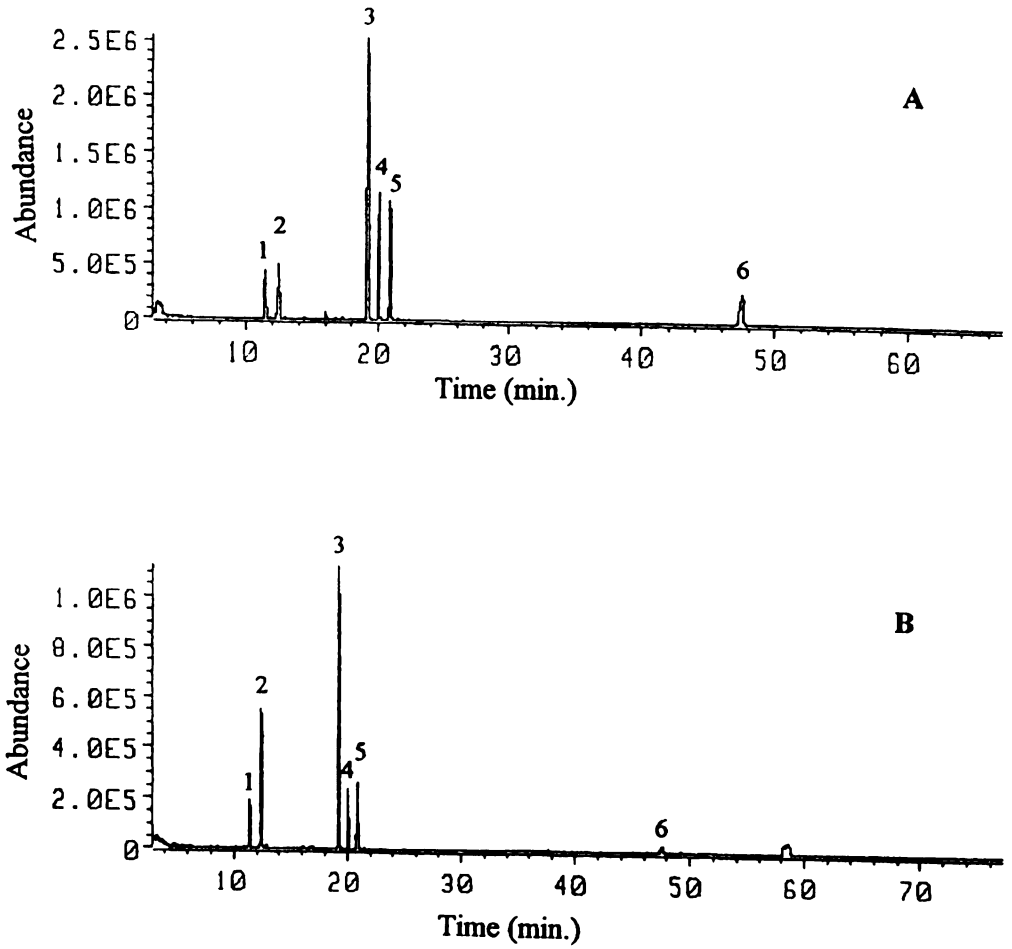
A
Abundance
2.5E6
2.0E6
1.5E6
1.0E6
5.0E5
0
1
2
3
4
5
6
10
20
30
40
50
60
Time (min.)
B
Abundance
1.0E6
8.0E5
6.0E5
4.0E5
2.0E5
0
1
2
3
4
5
6
10
20
30
40
50
60
70
Time (min.)

Figure 4.7

quantitative differences in the relative proportions between the cells from the two different cultures. Mannose was the major component in both cases. Ribose was the next major component in the case of the cells grown at higher temperature whereas galactose and glucose were the next major components in the cells grown at lower temperature. Another major difference was that the polymer from the 4°C cells contained a greater amount of glucosamine.

Thin layer chromatography of the total lipids from the 4°C and 24°C cells showed three major components namely a fast moving component which was tentatively identified as triacylglycerol, diacylglycerol and free fatty acids. The latter two had retention times consistent with those observed for standards run under the same conditions. The levels of triacylglycerol were much higher in the cells grown at 4°C and appeared as a methanol insoluble oil that was soluble in chloroform or hexane. In the case of the 4°C cells, triacylglycerols accounted for more than half the total lipids. Diacylglycerol was the next predominant component, while only traces of phosphoglycerol were present. Other minor components were observed in the lipid preparations from the 24°C cells but the quantities were too small to allow characterization. Triacylglycerol probably serves as an energy source and not as a membrane component. Its presence, though, precluded a meaningful estimate of the total amount of membrane lipids obtained from the 4°C cells. Triacylglycerol is only sparingly soluble in methanol, however, and this was used as a way of separating it from the total lipids for NMR analyses. The recovery of lipids (excluding triacylglycerol) from the 4°C cells was ~6 mg per gram (wet weight) and about half this amount for the 24°C cells.

The ^{1}H NMR spectra of the total lipids from the 4°C and 24°C cells are shown in Figure 4.8A and 4.8B, respectively. The two dimensional DQF-COSY and TOCSY experiments (not shown) indicated that the signals between 5.30 and 5.43 ppm were due to vinyl protons because of correlations with those at ~2.05 ppm. These were much more abundant in the lipids from the 4°C culture than from the 24°C culture. Connectivities between signals assignable to diacylglycerol and what later proved to be triacylglycerol were also evident. In the spectrum of the 24°C lipids, extra signals due to the glycerol group of phosphatidyl glycerol at 3.71 and 4.12 ppm were also observed.

In agreement with the TLC analyses and the ^{1}H NMR spectra of the total lipids, the ^{1}H NMR spectra of the isolated lipid headgroups of the 4°C cells (after precipitating triacylglycerol) showed definitively that the only lipid headgroup was glycerol (Figure 4.9A). The two doublet of doublets at 3.47 ppm and 3.57 ppm were assigned to the C1 and C3 protons of glycerol. The multiplet at 3.7 ppm was assigned to the proton on carbon 2 of glycerol. The lipid headgroup components of the 24°C cells were glycerol and phosphoglycerol (Figure 4.9B). The chemical shifts of the glycerol signals were the same as mentioned above. Two doublet of doublets at 3.65 ppm and 3.76 ppm were assigned to the C1 and C3 protons of phosphoglycerol. The multiplet at 3.84 ppm was assigned to the proton on carbon 2 of phosphoglycerol. From these data the proportion of phosphoglycerol was determined to be 40% by integration of the peaks. This method is much more reliable than charring the TLC plates and integrating densitometer traces.

Figure 4.8: ^{1}H NMR spectra of the total lipids extracted from the bacteria grown at (A) 4°C and (B) 24°C. Note the much higher relative proportion of vinyl protons relative to the head group protons in the 4°C cells. TAG = triacylglycerol, DAG = diacylglycerol, PG = phosphatidylglycerol.

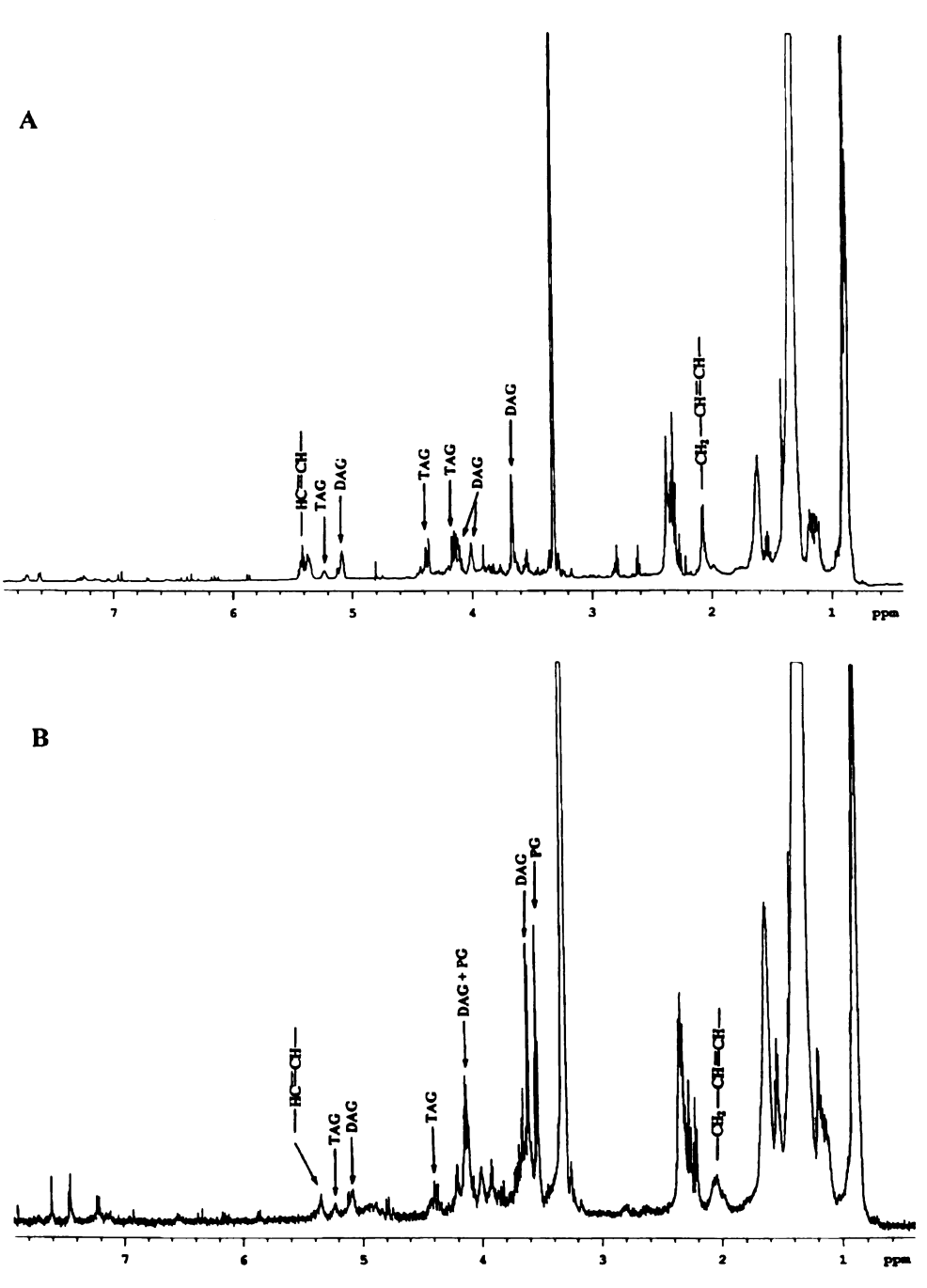
A
HC=CH
TAG
DAG
TAG
TAG
DAG
DAG
CH2—CH=CH
7
6
5
4
3
2
1
ppm
B
HC=CH
TAG
DAG
TAG
DAG + PG
DAG
PG
CH2—CH=CH
7
6
5
4
3
2
1
ppm

Figure 4.8

Figure 4.9: ^{1}H NMR spectra of the lipid headgroups from the bacteria grown at (A) 4°C and (B) 24°C. The only lipid headgroup in the 4°C cells was glycerol. The two doublet of doublets at 3.47 ppm and 3.57 ppm were assigned to the C1 and C3 protons of glycerol. The multiplet at 3.7 ppm was assigned to the proton on carbon 2 of glycerol. The lipid headgroups of the 24°C cells were glycerol and phosphoglycerol. The chemical shifts of glycerol were the same as mentioned above. The two doublet of doublets at 3.65 ppm and 3.76 ppm were assigned to the C1 and C3 protons of phosphoglycerol. The multiplet at 3.84 ppm was assigned to the proton on carbon 2 of phosphoglycerol.

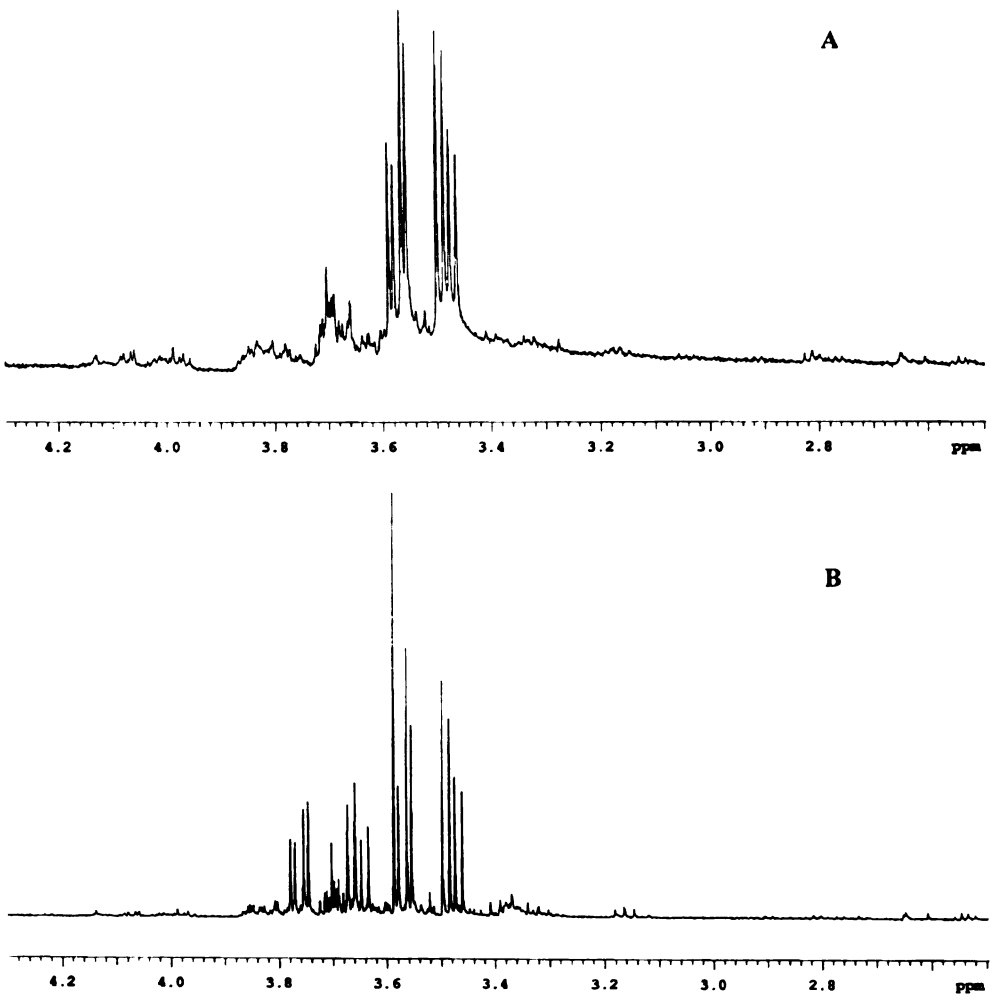

Figure 4.9

The total lipid sample from the 4°C cultured cells contained a component that was insoluble in pure methanol. With the aid of ^{1}H NMR spectroscopy using homonuclear decoupling (Figure 4.10) this component (in $CDCl_3$) was identified as triacylglycerol. Three sets of signals in the downfield region could be assigned to a glycerol moiety using the coupling information from the double resonance experiments. A multiplet at 5.25 ppm was assigned to the H2 of glycerol. The C1 and C3 protons appeared at 4.1 ppm and 4.3 ppm. The identity of this peak was confirmed by mass spectrometry using electrospray ionization (Figure 4.11). The peak at m/z 605 is due to the protonated form of a triacylglycerol species containing two C12 and one C10 fatty acid with a total of 3 double bonds between them. Other homologous species separated by 14 or 28 mass units i.e. 1 or 2 methylene groups (e.g. m/z 591 and 619) were also present.

The gas chromatograms of the fatty acid methyl esters of the membrane lipids from the 4 and 24°C cells are shown in Figures 4.12A and 4.12B. GC-MS analysis of these methyl esters indicated the major lipid in both cell types was a *anteiso*-C_{15} lipid (peak 5). However, the 4°C grown cells contained a large amount of a $C_{15:1}$ lipid (peak 4), and in general had shorter chain lengths and a greater degree of unsaturated and branched chains (Table 4.1). The cells cultured at 24°C contained a much smaller amount of the $C_{15:1}$ lipid (peak 4), while the relative proportions of *iso*-C_{16} , *n*-C_{16} and *anteiso*-C_{17} (peaks 6, 7, and 8 respectively) increased over the 4°C cells.

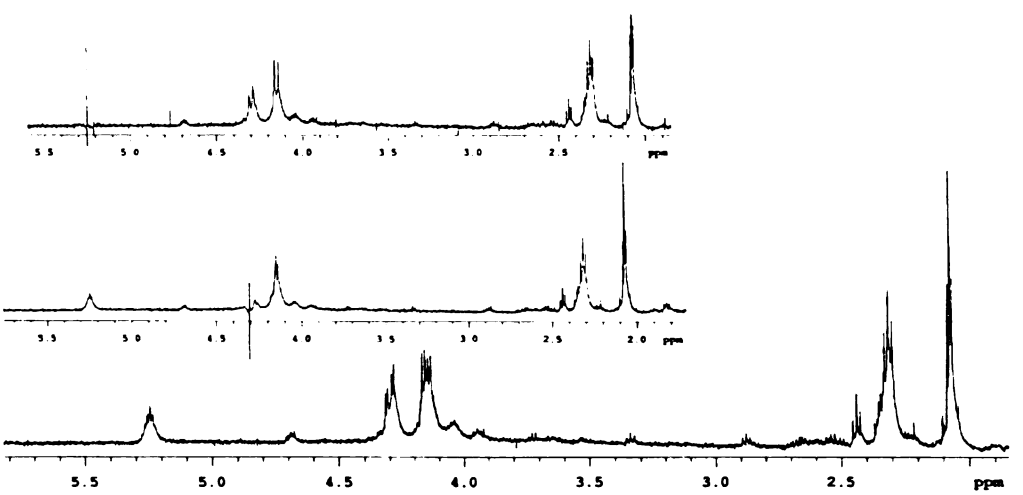

Figure 4.10: ^{1}H NMR spectrum of the methanol insoluble component from cells cultured at 4°C. The two insets show double resonance experiments carried out at 4.3 ppm and 5.25 ppm, which indicate the component was triacylglycerol. The multiplet at 5.25 ppm was assigned to the H2 of glycerol. The C1 and C3 protons appeared at 4.1 ppm and 4.3 ppm.

Figure 4.11: Positive ion electrospray mass spectrum of the triacylglycerol from cells grown at 4°C. The peak at m/z 605 is due to the protonated form of a triacylglycerol species containing two C_{12} and one C_{10} fatty acid with a total of 3 double bonds between them. Other homologous species separated by 14 or 28 mass units (e.g. m/z 591 and 619) were also present.


Scan ES+
9.31e6
100
%
0
605.6
606.5
619.6
620.6
673.6
687.5
735.6
791.8
591.5
564.6
423.3
437.3
441.4
451.4
456.4
484.4
338.3
339.3
340.3
358.3
392.4
297.2
299.2
283.4
243.2
222.2
211.2
122.2

Figure 4.11

Figure 4.12: Gas chromatographs of fatty acid methyl esters obtained from bacteria cultured at (A) 4°C and (B) 24°C. The main acyl chain components were (1) $C_{14:1}$ (2) *iso*-C_{14} (3) n-C_{14} (4) $C_{15:1}$ (5) *anteiso*-C_{15} (6) *iso*-C_{16} (7) n-C_{16} and (8) *anteiso*-C_{17}.

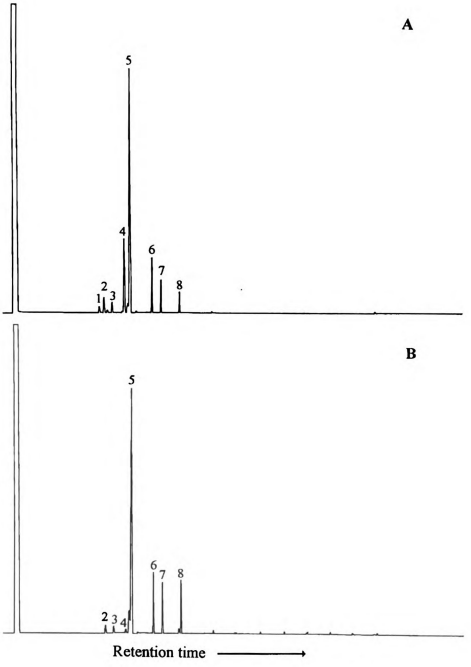

Figure 4.12

Table 4.1: Percent of fatty acid methyl ester derivatives in the total methanolysate of the cells grown at 4°C and 24°C in order of elution. The numbered components correspond to the numbered GC peaks in Figure 4.12.

Peak No.	Structure	% in 4°C Cells	% in 24°C Cells
1	$C_{14:1}$	1.75	0
2	*iso*-C_{14}	3.55	1.89
3	*n*-C_{14}	2.38	1.78
4	$C_{15:1}$	17.82	0.87
5	*anteiso*-C_{15}	58.95	67.04
6	*iso*-C_{16}	7.9	10.39
7	*n*-C_{16}	4.7	8.72
8	*anteiso*-C_{17}	2.96	9.31

Total lysates from cells cultured at 4°C and 24°C were run on 12% SDS-PAGE gels (Figure 4.13). There were no significant changes in the protein profiles, either in new proteins produced or suppressed, from the two cell cultures. Also there were no significant changes in the amounts of the various proteins produced at both temperatures, as was confirmed by running subsequent gels of total cellular proteins from other cultures grown at these two temperatures.

DISCUSSION

As the growth temperature of bacteria is reduced two possible scenarios can occur as the freezing point is reached. In the first scenario, pure water should separate out as ice leading to a sudden increase in the extracellular salt concentration. In the second scenario, if freezing does not occur, the ionic strength of the medium should actually decrease since the dielectric constant increases. In addition, some salts might actually fall out of solution because solubility is generally lower in cold water. This would also result in a decrease in ionic strength. The ionic strength of the external medium therefore decreases progressively as the freezing point is reached from above and then it suddenly increases at freezing. There is no way that this can be anticipated by the bacteria and so it is not surprising that if compatible solutes are synthesized to cope with the sudden increase in ionic strength on freezing, none are synthesized even a few degrees above freezing. Hence, in this study, the relative proportions of the components in the amino acid pools did not vary significantly between cells cultured at 4°C and 24°C and the total mass of metabolites per unit volume of packed cells decreased by a factor of 4.6 in going from 24 to 4°C. It is important to note

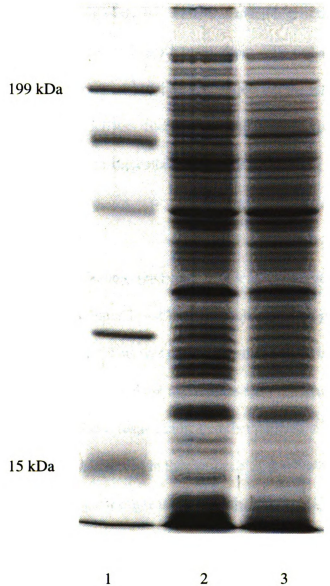

Figure 4.13: SDS-PAGE gel of total proteins of lysates from cells cultured at 4°C and 24°C. Lane (1) Low molecular weight standards (2) 4°C lysate (3) 24°C lysate.

here that the 4°C cells were much smaller than the 24°C cells and more of the former are therefore present per unit volume of wet cells. This is also borne out by the fact that the total lipids recovered per unit gram of the 4°C cells was much higher than the quantity recovered from the 24°C. In other words, there are many more cells per unit volume or wet weight present in the 4°C pellets compared to the 24°C pellets but the total quantity (as determined by NMR spectroscopy) is much less. Electron microscopy definitively showed that there was no breakage (thus leakage) of the cells at 4°C thus accounting for the lower amounts of metabolites.

In addition to these findings (and consistent with them) there was no evidence for the presence of such common compatible solutes as glycine betaine, glycerol, glucose, sorbitol, and mannitol in the 4°C cells. This was definitively determined by amino acid analysis and by NMR spectroscopy. Signals for these metabolites were completely absent from the NMR spectra of the 4°C cells. These results indicated that, at least in this case, the induction of catalytic systems (enzymes) involved in the synthesis of metabolites is not triggered by low temperature, when within a few degrees of freezing. This was also clear from SDS-PAGE where there were no new protein bands and no increase in the relative amounts of bands found in 24°C cultures was observed for the 4°cells.

The large decrease in the intracellular metabolite and general ion concentration of the organism as the temperature neared freezing had a predictable impact on the chemistry of the bacterial membrane. The low solute concentration means that high concentrations of ions are not available to act as counter-ions for the membrane lipids. As a result, the synthesis of

charged lipid components was almost totally suppressed. Only diacylglycerols and triacylglycerols (which were likely contaminants of the membrane preparation) were found in the membrane of the 4°C cells but the 24°C cells contained both phosphatidyl glycerol and diacylglycerol. Smaller amounts of triacylglycerol were also found. The degree of unsaturation was higher and the average length shorter in the fatty acid chains of 4°C cells compared to 24°C as was expected if the average motional dynamics (fluidity) of the membrane lipids was to be conserved. Because no charged lipids are present in the membranes of the 4°C cells, there is no need for counter ions to be localized there to stabilize the lipid headgroups. So these counter ions would now be in the actual free circulating ion concentration in the bulk solution. Thus the cell now has a significantly higher free circulating ion concentration without a real increase (by metabolite synthesis) in ion concentration. This is another beneficial strategy for adaptation.

Another major change between the bacteria cultured at the two temperatures occurred in the amount and composition of the capsular polysaccharides. The mass of the capsular polysaccharide coating in the 24°C cells was reduced by a factor of 4.8 per unit packed cell volume in the 4°C cells. A shift in the relative proportions of the carbohydrates making up this capsule was also observed. This decrease in capsular polysaccharide is a very reasonable observation. As the temperature decreases, the motional dynamics and diffusivity of molecules slow as well. The presence of a thick capsular polysaccharide coating would impede water flux through the cell and prevent nutrients from entering or metabolic waste from leaving. This reduction in capsule size is another critical adaptative mechanism that is probably as important as osmotic balance.

From the above discussion, it can be concluded that the conditions of high salinity in the extracellular medium that might accompany freezing cannot be anticipated by the cellular mechanisms that respond to osmolality even fractions of a degree before freezing. In fact, the indications of an incipient increase in salinity on freezing are quite to the contrary. The imbalance in osmolality that accompanies the freezing event is therefore a sudden and catastrophic one. The proposed shift in metabolite production of the organism in response to this freezing event must therefore occur after the fact. Most of the adaptative events prior to freezing revolve around maintaining the functioning of the cell membrane and coping with the low ionic strength and diffusivity of the medium. Perhaps these conditions can help counter a sudden increase in external electrolyte concentration since as we have stated earlier, the absence of charged groups on the membrane lipids increases the availability of ions to act as osmolytes. Such an adaptative mechanism to negate the effects of freezing of the extracellular matrix is more in keeping with the ion and metabolite concentrations of the cell just prior to freezing. The ordering of water at the membrane surface should also increase as temperature drops providing further freeze protection. The membrane, therefore, plays a central role in the physiology and physico-chemistry of cold adaptation. According to SDS-PAGE, there was no dramatic increase in new protein synthesis on going to 4°C from 24°C and so anti-freeze proteins, which would need to be produced in large quantities, cannot be involved.

One limitation of the cryoprotection by colligative property model is that it treats the environment inside of the cell and in its immediate vicinity as if the water there behaved as "bulk" water and its properties could be controlled strictly by colligative effects. It is possible

that the tendency for water to freeze can be reduced by interaction with the membrane surface. In fact, the ordering of water at regular hydrophilic surfaces is a well recognized phenomenon (7). The fact that salt in aqueous solutions is excluded from an ordered surface is utilized in desalination systems. In such systems, water is passed through membranes and only pure water flows through because the water close to the pores in the membrane is ordered and excludes salts. This is a very general effect. If it were an adaptative mechanism to low temperature in bacteria, there would be no need for an increase in osmolyte concentration at low temperatures. In fact, such an increase might be deleterious and a decrease might be required instead. This allows us to propose a model for freeze resistance which is consistent with our data. We propose that a good strategy would be for the cell to increase the relative amount of (membrane) bound water by reducing the cell size and change the membrane surface chemistry so that ions are not required on its surface. The water thus arranged will not freeze and the small amount of bound water in the center of the cell will contain all of the electrolytes and will not freeze because of colligative effects (Figure 4.14).

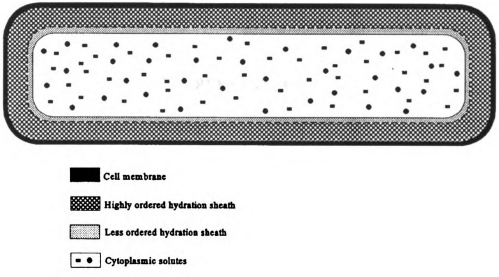

Figure 4.14: Model showing the solvation of the region between two bilayer membranes. Note the ordered water (checkered pattern) structure close to the membrane surfaces. In this region ice formation cannot take place. Note that the solutes (solid particles) are restricted to the disordered region where their effective concentrations are now so high that ice formation does not take place in this region either. The width of the ordered solvation layers should increase as temperature decreases.

ACKNOWLEDGMENTS

We thank Dr. Vera Soina for isolating the Siberian permafrost strain 45-3, and Dr. Yin Tang (from our laboratory) for doing the transmission electron microscopy.

This work was supported in part by the Center for Microbial Ecology, a National Science Foundation Science and Technology Center (NSF grant# BIR 912-0006) and by the National Science Foundation (Grant IBN 9507189 to RIH).

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CHAPTER V

CHARACTERIZATION OF A MECHANISM OF ADAPTATION OF A pH AND SOLVENT RESISTANT BACTERIUM *Sarcina ventriculi*

ABSTRACT

The formation of carbon-carbon bonds remains one of the most challenging and important endeavors of synthetic organic chemistry. There are no chemical or biological systems in which this has been realized at centers that are not activated by some functionality such as a carbonyl group. Here we report on such a reaction that occurs at isolated, completely unactivated carbon centers. Free foreign fatty acids were joined tail-to-tail at their ω-1 positions to fatty acids in the membrane lipids of *Sarcina ventriculi* simply by adding a membrane preparation to the fatty acid suspension in the presence of a low molecular weight ketone. In similar experiments, n-heptane and hexadecylpyridinium chloride were linked at the 2-position to the ω-1 position of *S. ventriculi* membrane lipids by simply adding the hydrocarbons to growing cultures of the organism. The coupling mechanism is temperature dependent, is triggered both by high and low pH values and is inhibited by thiols but not by 1,4 cyclohexadiene although, interestingly, it is suppressed by the addition of cyclohexane. The process is suppressed if cells are cultured in deuterium oxide indicating that the trigger is a kinetic factor related to motional dynamics. These results and results from NMR spectroscopy indicate that the process is regulated by membrane dynamics and occurs via a radical mechanism in which a hydrogen atom is extracted from each alkyl chain and the two alkyl radicals join to form a new carbon carbon bond. Experiments using the radical trap 2-nitroso-2-methyl propane (to which the process is insensitive) and the fact that coupling is stereospecific indicated that the radicals are caged species that never escape. Radicals that escape would be susceptible to combination or trapping by external agents and would

undergo rapid planarization and loss of chirality. The stereospecific formation of carbon-carbon bonds between unactivated carbon centers by this system has very high potential for use in synthetic organic chemistry.

INTRODUCTION

Because of its very unfavorable energetics, the formation of carbon-carbon bonds between unactivated carbon centers by the removal of hydrogen atoms from the two bonding sites and direct connection of the two centers is a process for which there are no known biological nor many laboratory precedents. In the case of alkyl chains, carrying this out with any regio- or stereospecificity is a daunting task because energetics would be expected to be the same for any methylene group along the chain. The stability of the radical formed by removal of a hydrogen atom from the unique terminal methyl groups will be less than that of one formed from a methylene group and so the specific coupling of the chain termini through the methyl groups is actually less likely. Despite the high degree of difficulty of this coupling process it might, apparently, be a very common event in the membranes of some bacteria. One such organism is the anaerobic gram positive bacterium *Sarcina ventriculi*. In a series of earlier studies [1-3] we demonstrated that this organism adapted to changes in environmental conditions such as temperature and pH by forming new lipids species containing α,ω-very long chain dicarboxylic acids of up to 36 carbons in length. Over 80% of the total membrane acyl chains can be these very long bifunctional molecules. Both the relative proportions of these unusual fatty acid species and their structures suggested they

might be formed by tail to tail coupling of existing regular chain fatty acids probably via the radical species formed by simple removal of a hydrogen atom from the ω-1 positions of two opposing acyl chains (Figure 5.1). A vicinal dimethyl function is therefore introduced at the site of linkage. Even if the two fatty acid species are identical, the isolated fatty acids (or dimethyl ester) must have the R, R or S, S configuration at the two new stereocenters and will be optically active. This has been observed [2].

There are several necessary elements which have to be understood if this reaction could ever be exploited. Firstly, coupling two alkyl chains in this fashion with the liberation of two molecules of hydrogen is an energetically uphill task. Fortunately, even bacteria have to conform to the laws of thermodynamics so there is a chance that this activity could be understood and harnessed. To drive such a process, the chain joining would have to be coupled to a reduction event and so this process would require an oxido-reducing system. One would not necessarily need to know what the intermediate redox system is, but it is important to know the nature of the final molecular species accepting the two hydrogen atoms. By adding this terminal hydrogen acceptor, the intermediate species that accepts the two hydrogens from the coupling can be regenerated thus allowing the process to be a catalytic one.

Another element of the mechanism which has to be defined is the trigger or controlling factor regulating the activity. It seems clear that the coupling process is activated by any stimulus or condition causing an increase in fluidity or motion in the membrane lipid

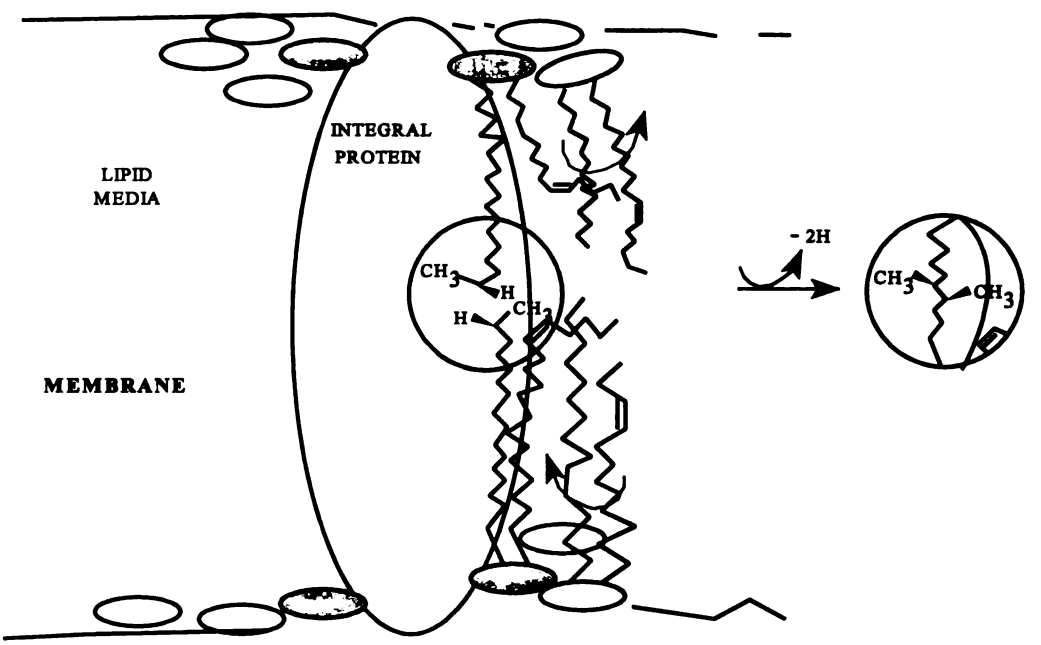


Figure 5.1: Conceptual model for the coupling of alkyl chains of the lipid components by an integral membrane catalytic system. Note that if the two hydrogen atoms are removed from the same side, the final product will have the methyl groups on the same side.

ensemble. Chemical studies show that the amount of chain coupling in this bacterial species grown at different temperatures increases with the growth temperature from essentially zero at low temperature (~ 30°C) to greater than 80% at temperatures of 45°C. Studies using dynamic NMR spectroscopy [4] show quite clearly that even when this high degree of coupling occurs in response to depression of pH, the fluidity and dynamics of the alkyl chains do not change when measured at the temperature and pH at which the bacteria are grown. The coupling activity is therefore activated and also back-regulated by molecular dynamics always conserving the motional freedom of the membrane lipids within a tight range. This dynamic regulation is an important feature of the membranes of living systems and has been termed "homeoviscous adaptation" [5].

Since this coupling reaction seems to be a general response to a perturbation of membrane dynamics, a broad spectrum of perturbations should (and do) trigger it. One parameter that should affect the expression of this catalytic activity is pH. This catalytic activity should be buried in the inner core of the membrane but it should, however, be sensitive to pH since the state of ionization of the membrane lipid head groups also helps determine the closeness of their packing [6]. The headgroup packing will, in turn, influence the order and dynamic state of the lipid alkyl chains. It should therefore be possible to use pH (at either extreme) to activate and control the reaction.

The possibility of forming these transmembrane fatty acids by the joining of existing chains suggests some very interesting and exciting possibilities. The most obvious one is

that if the enzyme system could be isolated, or harnessed directly using membrane preparations or whole cells, it could be utilized in synthetic organic chemistry for the stereospecific formation of carbon-carbon bonds between unactivated centers under very mild conditions. If the coupling process is independent of the nature of the group at the distal ends of the hydrocarbon chains, then an entire family of α,ω-bifunctional molecules can be made. This would have important implications for the polymer industry. The coupling activity could be used to stabilize liposomes by joining together alkyl chains at the interface between the layers of lipids or it could be used for anchoring monolayers to hydrocarbon substrates such as those formed by treating noble metal layers with alkane thiols. Here we describe how this coupling activity can be controlled and regulated and demonstrate that the potential uses cited above are well within the realms of possibility.

MATERIALS AND METHODS

General methods

Cells were grown under strict anaerobic conditions in liquid media as described earlier [1]. All cell transfers were carried out in a glove box under an oxygen free atmosphere provided by argon which was passed over heated copper to remove traces of oxygen. Cells were harvested by centrifugation at 8000 rpm in a Sorvall centrifuge equipped with a GSA rotor. For experiments that required pH control of growth conditions, bacteria were grown in a chemostat equipped with a pH probe. The chemostat was also fitted with an inlet for adding sodium hydroxide or hydrochloric acid solution via a peristaltic pump

under pH feedback. Cells were cultured at 37°C at pH 3, 7 or 9.7. The pH conditions were maintained by adding 5 M sodium hydroxide for growth at pH 7 or 9.7 or hydrochloric acid for growth at pH 3. For growth of cells in 99% deuterium oxide, cells were preconditioned by culturing them in increasing concentrations of deuterium oxide until they were adapted to growth at 99%. The growth temperature was 37°C. In these experiments, the pD or pH of the growth medium was allowed to drop to ~4, conditions that normally trigger the formation of very long α,ω- bifunctional fatty acids. It should be noted that the measured pD is typically about 0.4 units lower than the corresponding pH value [7].

Cell-free coupling involving free, exogenous fatty acids

For the cell-free coupling of free fatty acids to membrane lipids, cells (200 ml) were cultured anaerobically in the absence of exogenous fatty acid at 37°C and a pH of 7. They were then harvested in an inert atmosphere (argon) by centrifugation and resuspended in 2 ml of oxygen free growth medium. They were lysed in a French pressure cell under anaerobic conditions (by setting up the pressure cell in a glove box, and collecting the lysate in an anaerobic vial) and hexadecanoic acid (10 mg) and ethyl ketone (10 ml) were then added. The mixture, under anaerobic conditions, was incubated at 45°C for 3 hours and then the fatty acids were released from the lipids by methanolysis with 5% HCl in methanol at 75°C for 24 hours. The mixture was concentrated to dryness under a stream of nitrogen and the fatty acid methyl esters were extracted by partitioning between chloroform and water. The chloroform extract was subjected to GC-MS analysis using a Jeol JMS-AX505H spectrometer interfaced with a Hewlett-Packard 5890A gas chromatograph.

Coupling of heptane chains to membrane lipid fatty acid termini

N-heptane (0.29 ml) was added to a growing 10 ml culture of *Sarcina ventriculi* cells incubated at 37°C in roller bottles to effect mixing. After 2 hours, the cells were harvested by centrifugation and the methyl ester derivatives of the membrane lipid fatty acids were prepared and analyzed by GC-MS.

Coupling of hexadecylpyridinium chloride to membrane lipid fatty acid termini

Hexadecylpyridinium chloride (50 mg in 800 µl sterile water) was added to each of four exponentially growing cultures (500 ml) of *Sarcina ventriculi* at 37°C. After 24 hours, the cells were harvested by centrifugation. Membrane fatty acid methyl esters (FAMEs) were made and isolated by refluxing for 2 days in 4:1 5% HCl in MeOH:1,2-dichloroethane at 74°C, then extracting twice by phase separtation in 50 ml H_2O:100 ml $CHCl_3$:100 ml hexane. The organic layers were removed and evaporated to dryness. ^1H NMR was done on the total FAMEs. A family of methyl esters that were visible by UV detection was isolated by preparative thin layer chromatography (TLC) and characterized by various spectroscopic methods.

Effects of chemical agents on transmembrane fatty acid synthesis

Cells (10 ml) were cultured at 37°C as described above except that *tert*-butyl thiol (0.23 ml), n-butane thiol (0.21 ml), cyclohexane (0.22 ml), 1,4-cyclohexadiene (0.19 ml) or 2-nitroso-2-methyl propane (34.8 mg) was added. In the case of 2-nitroso-2-methyl propane, the solid was predissolved in 1.5 ml of ethanol to a concentration of 23.2 mg/ml. The

reagents were added to the cultures in early log phase to the final concentrations indicated, then the temperature was shifted to 50°C, and the cells were harvested in early stationary phase (about 2 hours later). The pH was not regulated.

NMR spectroscopy analysis of the effect of cyclohexane and 1,4-cyclohexadiene on membrane lipid alkyl chain dynamics

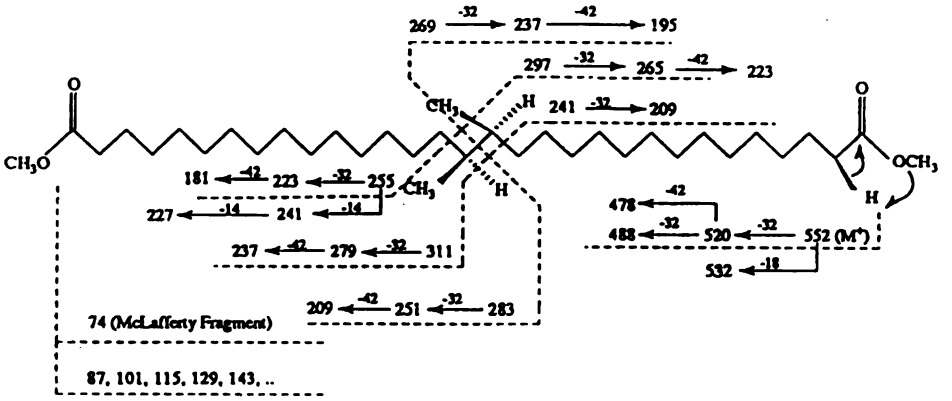
Five, 20 mg samples of dipalmitoyl phosphatidylcholine (DPPC) were lyophilized for 12 hours. Each sample was resuspended in 0.6 ml of a 10 mM phosphate buffer (pH 7.0) containing the following components at the final concentrations indicated: NaCl, 0.1 mM; KCl, 2.5 mM; $MgCl_2$, 0.5 mM; and $CaCl_2$, 1.0 mM. The DPPC samples were then frozen in dry ice/acetone bath, heated to 55°C for 10 min., and vortexed. These three annealing steps were repeated five times. Cyclohexane (5 μl) was added to the first sample and 10 μl to the second. The same amounts of 1,4-cyclohexadiene were added to the third and fourth. The fifth was kept as a control. The samples were vortexed and from each 50 μl was withdrawn and added to 0.6 ml of 5:1 D_2O:D_4-MeOH, mixed and transferred to NMR tubes. ^1H NMR measurements were performed on a Varian VXR-300S spectrometer operating at 300 MHz. The temperature was held at 60°C, above the phase transition for DPPC.

RESULTS AND DISCUSSION

Since the very long bifunctional fatty acid species in *Sarcina ventriculi* are formed by random, indiscriminate (but enzymatic) tail-to-tail joining of existing fatty acids, it should

be possible to demonstrate that foreign, exogenously-added fatty acids could be taken up intact into membrane vesicles of *Sarcina* and could be incorporated (intact) into the transmembrane lipid species. Addition of heptadecanoic acid (or any fatty acid which is not found in membranes of *Sarcina*) to a growing culture of that organism at 37°C, followed by cell lysis and a temperature shift to 45°C resulted in the formation of chimeric fatty acid species with structures one half of which was formed from the foreign fatty acid and the other from a native species (Figure 5.2). In this experiment, methyl ethylketone was added as a final hydrogen acceptor (with the formation of 2-butanol, catalysed by native oxidases) to ensure that all of the potential cofactors were always oxidized and able to accept the two hydrogen atoms generated in the coupling process. The reaction did not take place to any measurable extent if the ketone was left out (data not shown) indicating that the hydrogens were funneled (presumably via a species such as ferredoxin) to a flavin cofactor and finally to the ketone. The addition of n-heptane (which was added to a growing culture in mid-log phase) to the ω-1 position of the membrane fatty acyl chains was also realized. This was evident from the GC-MS analyses in which a peak for a component with a mass spectrum (Figure 5.3) corresponding to a 23 carbon fatty acid was observed, indicating the n-heptane was added to a 16 carbon membrane lipid. The predominant alkyl chains observed in *Sarcina* are 16:0, 18:0, and 18:1 at 37°C. However, carbon chains longer than 23 (as in addition to the 18 carbon long chains) were not observed in the n-heptane experiment, possibly because they adversely affect the membrane motional dynamics. Longer carbon chains would probably extend too far into the opposing membrane leaflet or would have to fold back on themselves, in either case increasing the fluidity of the membrane. To further

Figure 5.2: The electron impact mass spectrum of one of the chimeric bifunctional fatty acid dimethyl esters formed by tail-to-tail coupling between an exogenously added fatty acid (heptadecanoic acid) and a hexadecanoic acid residue from an *S. ventriculi* lipid component. The fragmentation pattern is indicated above the spectrum. Detailed discussions of the fragmentation modes for these molecules have been presented earlier [1, 2].


269
-32
237
-42
195
297
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265
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223
241
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209
CH3
H
O
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OCH3
181
-42
223
-32
255
CH3
H
227
-14
241
-14
237
-42
279
-32
311
478
-42
488
-32
520
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532
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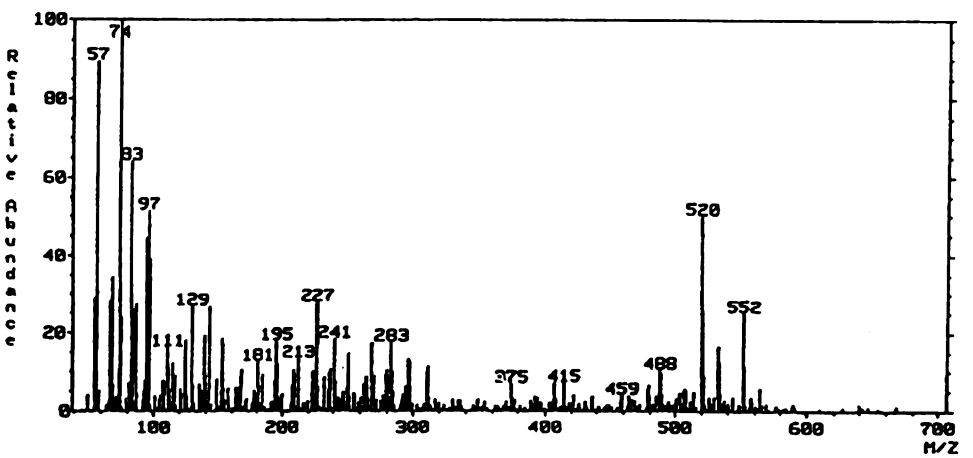
283
74 (McLafferty Fragment)
87, 101, 115, 129, 143, ..


Relative Abundance
M/Z
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80
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40
20
0
100
200
300
400
500
600
700
74
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Figure 5.2

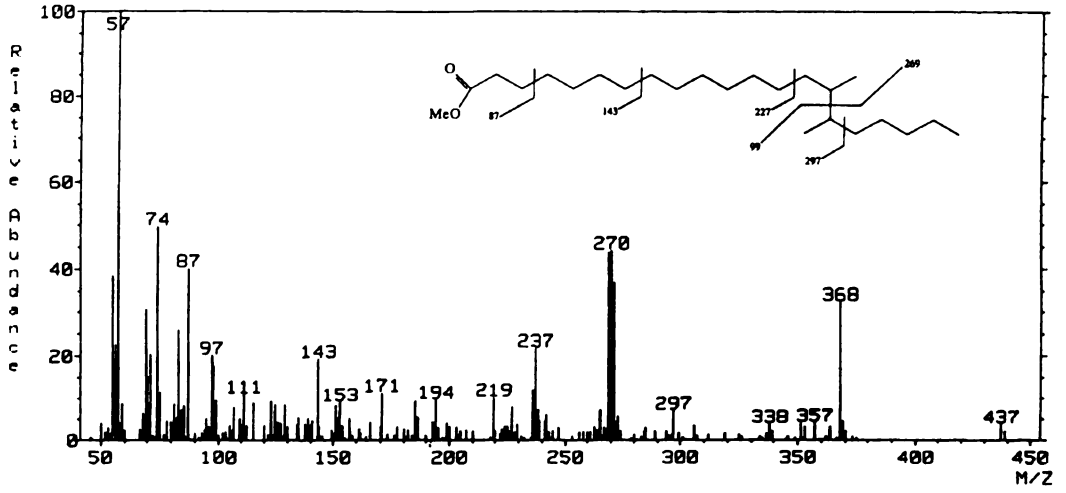


Figure 5.3: The electron impact mass spectrum of a 23 carbon fatty acid methyl ester resulting from coupling of n-heptane to a hexadecanoic acid residue from a *S. ventriculi* lipid component at the ω-1 position. The molecular weight is 368. The inset shows some of the major fragmentation patterns.

show that tail-to-tail coupling of exogenous lipids occurs without regard to its headgroup, the addition of hexadecylpyridinium chloride was also demonstrated. The ^{1}H NMR spectrum of the total fatty acid methyl esters (Figure 5.4A), which included free hexadecylpyridinium chloride, regular chain fatty acids and transmembrane fatty acids, indicated that some of the hexadecylpyridinium compound had been converted to a reduced pyridine system since there were new signals in the region of 6.9 - 7.2 ppm. The total fatty acid methyl esters were separated by thin layer chromatography. A family of transmembrane fatty acids (visible by UV detection), now separated from the free hexadecylpyridinium chloride and regular chain fatty acid methyl esters, gave a mixture in which the ^{1}H NMR spectrum indicated that the hexadecylpyridinium was coupled to native membrane lipids (indicated by the complex splitting of the methyl signals at about 0.9 ppm), but the aromatic ring had been transformed to a 1-alkyl-4-pyridone (Figure 5.4B). This was indicated by the disappearance of signals for the pyridine nucleus and the appearance of a narrow doublet at 7.72 ppm (2-position protons) and a multiplet at 7.54 ppm (3-position protons). The signals between 6.9 and 7.2 ppm were hardly visible in the spectra of the total extract prior to TLC indicating that they probably arose by oxidation of a dihydropyridine during handling. This is not surprising since the chimeric fatty acids were formed under reducing and anaerobic conditions. The infrared spectrum contained strong resonances for the C-H stretch at 2956 cm^{-1}, 2928 cm^{-1}, and 2859 cm^{-1}. The peak for the ester carbonyl stretch was observed at 1725 cm^{-1} (m), while the C=C stretch was observed at 1600 cm^{-1} (w). Electrospray mass spectrometry also supported the presence of chimeric fatty acid methyl esters containing the 4-pyridone nucleus. To test the theory that the bacteria reduced the pyridine ring, with subsequent

Figure 5.4: ^{1}H NMR spectra of (A) total fatty acid methyl esters from *S. ventriculi* cultured with hexadecylpyridinium chloride, and (B) isolated fatty acid methyl esters containing hexadecylpyridinim coupled to native *S. ventriculi* membrane lipids. The signals at 8.1 ppm, 8.45 ppm, and 9.25 ppm in (A) are due to the pyridine nucleus of free hexadecylpyridinium chloride. Note the new signals in (A) in the region of 6.9-7.2 ppm which indicated that some of the hexadecylpyridinium had been converted to a reduced pyridine system. In (B) note the essential disappearance of signals for the reduced pyridine nucleus between 6.9 and 7.2 ppm, and the appearance of the narrow doublet at 7.72 ppm (2-position protons) and a multiplet at 7.54 ppm (3-position protons) indicate that during handling the aromatic ring was oxidized to a 1-alkyl-4-pyridone.

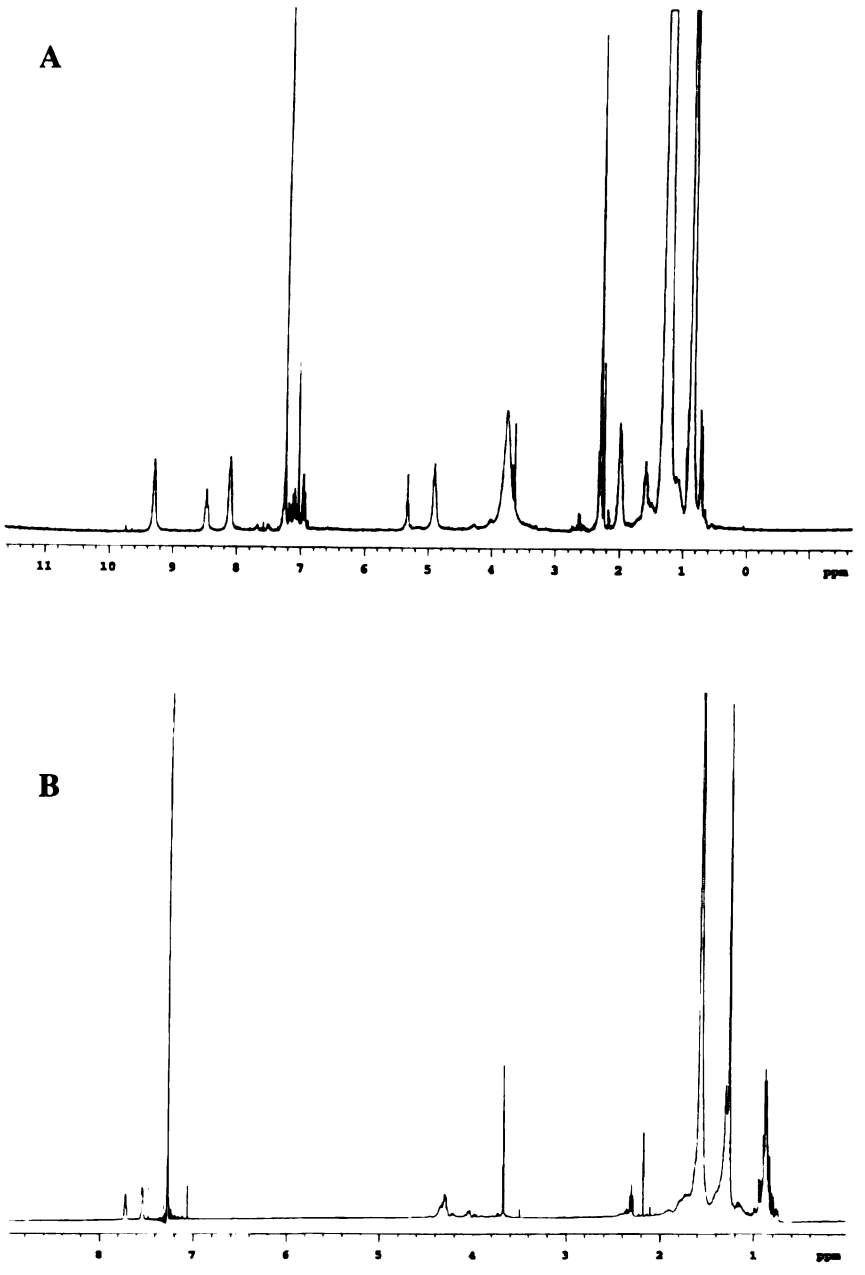


Figure 5.4

oxidation to form the pyridone (probably during handling) (Figure 5.5), we reduced hexadecylpyridinium chloride using sodium borohydride to form a dihydropyridine, and then attempted to oxidize it to a pyridone using ceric ammonium nitrate (Figure 5.5). The reduction step was very successful but oxidation yielded a variety of products and ^{1}H NMR spectroscopy indicated that only trace amounts of product corresponded to the desired material.

In a prior study, it was demonstrated that *Sarcina ventriculi* carries out tail-to-tail coupling of its membrane lipids in response to agents lowering the pH [1]. It might be argued that the coupling response might have some ionic component which is acid catalyzed. An explanation that appears to be more consistent with the profile of this response, however, is simply that lowering of pH should suppress the ionization of the phosphate groups in the membrane lipids thus reducing their electrostatic cross-linking with calcium ions thus decreasing membrane stability. Such an argument would require that we also see coupling at high pH, since then the phosphate groups would be completely ionized. The resulting electrostatic repulsion should also destabilize the membrane thus triggering the process. Observing coupling at high pH would also rule out any acid catalysis. The gas chromatography profile of fatty acid methyl esters from *Sarcina ventriculi* cells cultured at pH 9.7 is shown in Figure 5.6. A family of peaks with very long retention times is seen in the pH 9.7 chromatograms. The inset shows a mass spectrum of peak 3, which corresponds to a $C_{34:1}\alpha,\omega$-dicarboxylic acid dimethyl ester formed at high pH. This was the case for all of the other late eluting peaks in the profile. They were all readily identifiable as very long

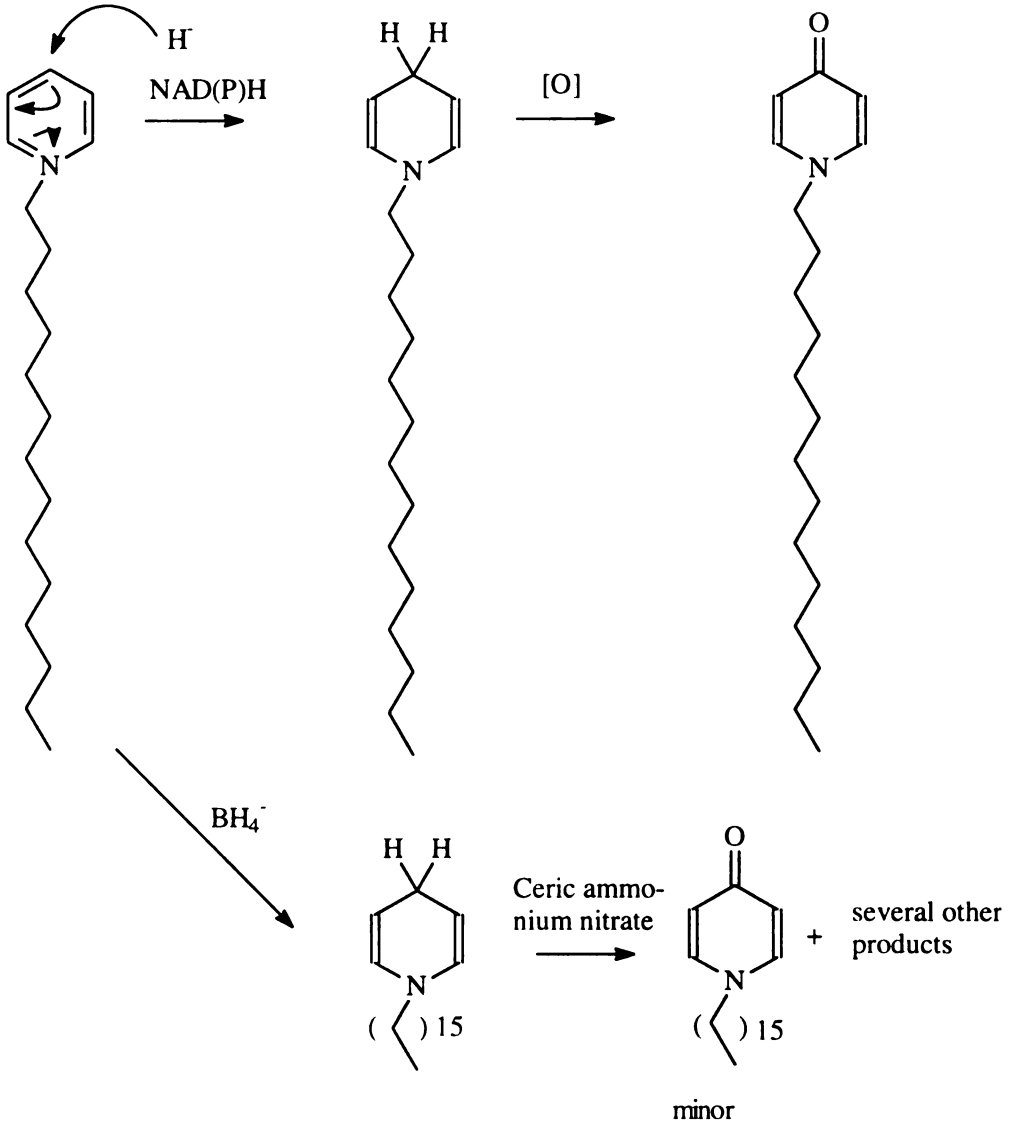


Figure 5.5: Bacterial (proposed) and synthetic mechanisms of the reduction and subsequent oxidation of the pyridine ring of hexadecylpyridinium chloride.

Figure 5.6: Gas chromatogram of fatty acid methyl esters derivatized from total lipid extracts of *S. ventriculi* in culture at pH 9.7. The major components are (A) $C_{17:1}$ fatty aldehyde, (B) $C_{16:0}$ carboxylic acid methyl ester, (C) $C_{18:1}$ carboxylic acid methyl ester, (D) $C_{18:0}$ carboxylic acid methyl ester, (1) $C_{32:1}$ ω-formylmethyl ester, (2) $C_{32:0}$ α,ω-dicarboxylic acid dimethyl ester, (3) $C_{34:1}$ α,ω-dicarboxylic acid dimethyl ester, (4) $C_{36:2}$ α,ω-dicarboxylic dimethyl ester. The inset shows an electron impact mass spectrum of peak 3, which is formed by coupling a $C_{18:1}$ and a $C_{16:0}$ lipids on opposing membrane leaflets.

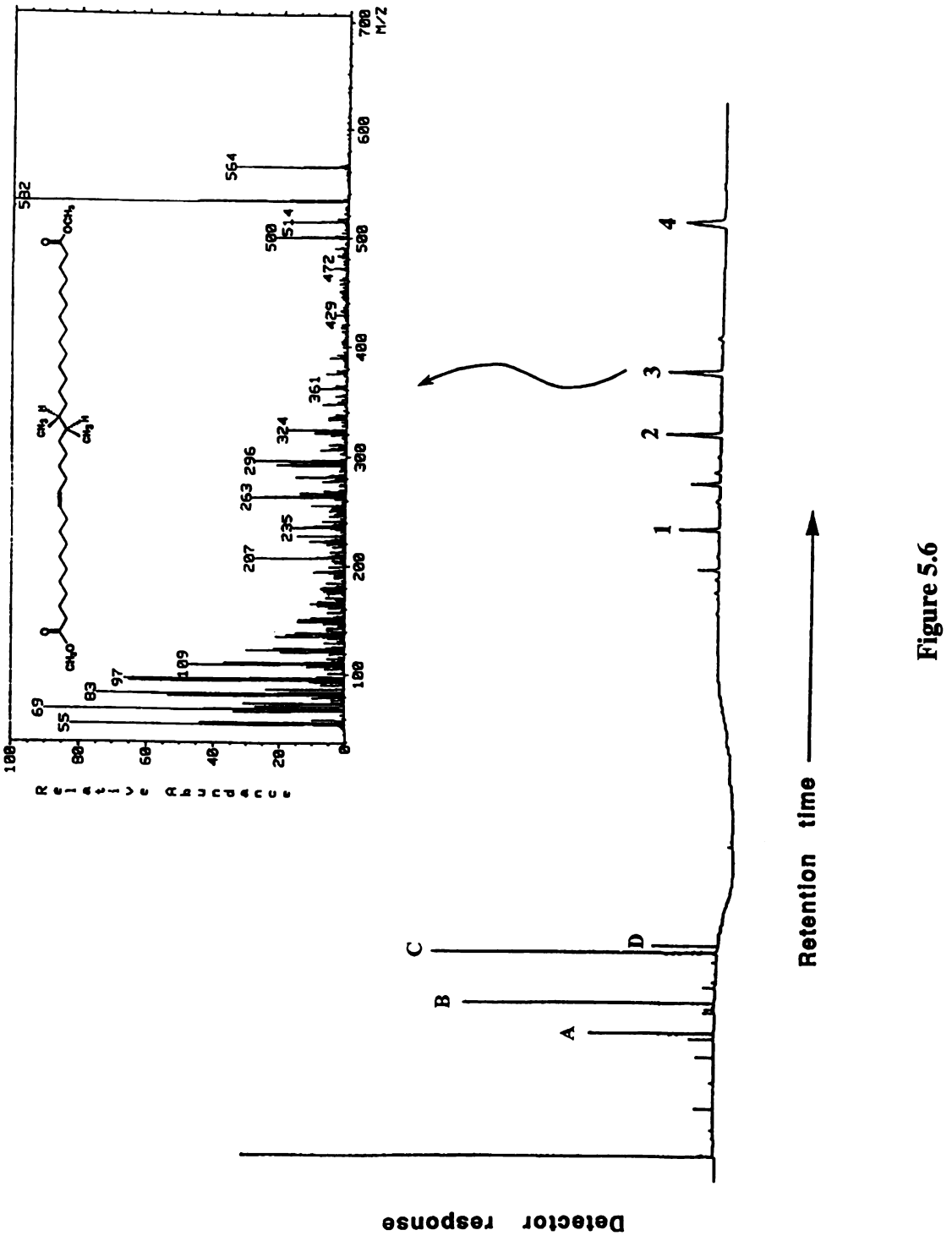
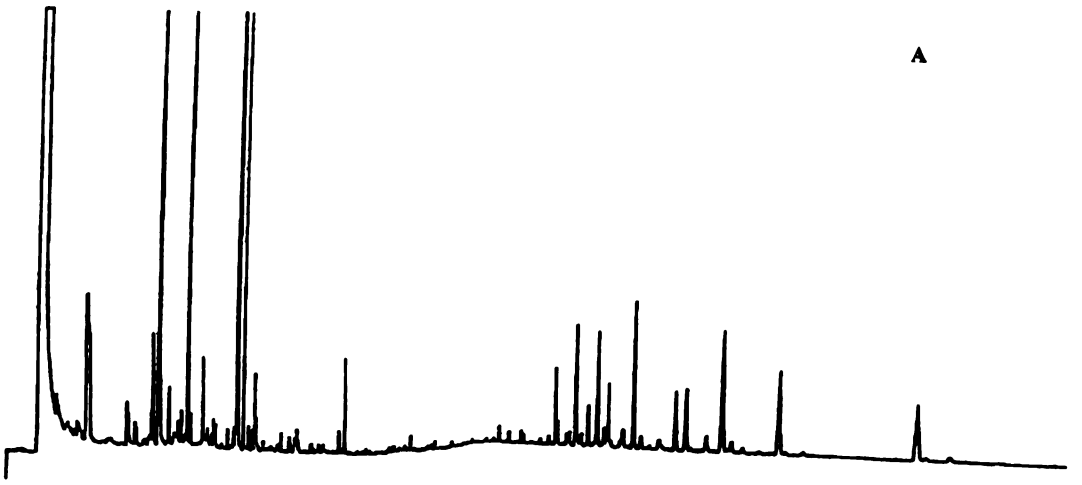

Detector response
Retention time
A
B
C
D
1
2
3
4
Relative Abundance
M/Z
582
564
514
500
472
429
361
324
296
263
235
207
109
97
83
69
55

Figure 5.6

chain bifunctional species observed when these bacteria are cultured at low pH, high temperature or in the presence of organic solvents again demonstrating the universality of the response.

To further test the hypothesis that tail-to-tail coupling is triggered by changes in the physical state of the membrane, *Sarcina ventriculi* was cultured in D_2O. The stronger hydrogen bonds in D_2O should slow down the lipid molecular motions, which would be equivalent to a reduction in temperature. The proportion of transmembrane lipids should differ between cells cultured in H_2O versus those cultured in D_2O. In these experiments, the pH was allowed to drop freely as a result of acetic acid production by the bacteria [8], usually reaching a minimal pH of 4.0. A reduction in pH is known to promote the formation of long chain lipids leading to a high proportion of transmembrane lipids. If the cells are cultured in D_2O, however, the reduced motional dynamics in the hydrogen bonding network in the lipid headgroup and in the various solvent cages should stabilize the membrane thus off-setting the fluidizing effects of the low pH. This is exactly what was observed (Figure 5.7). A high proportion of transmembrane fatty acids is seen in the cells grown in H_2O. In contrast, a very small proportion is observed in the cells cultured in D_2O. These results are consistent with the idea that substituting D_2O for H_2O is equivalent to a reduction in temperature causing an increase in membrane viscosity. The cells respond by maintaining a low level of long chain lipids. A similar kinetic effect of D_2O on lipid composition has recently been reported [9]. In this study *Bacillus subtilis* was grown in D_2O and the proportion of lipids with alkyl chain length of 16 carbons increased relative to those of 18

Figure 5.7: Gas chromatographs of fatty acid methyl esters from *S. ventriculi* cells cultured in (A) H_2O and (B) D_2O. Note the high proportions of late eluting peaks in A compared to B.



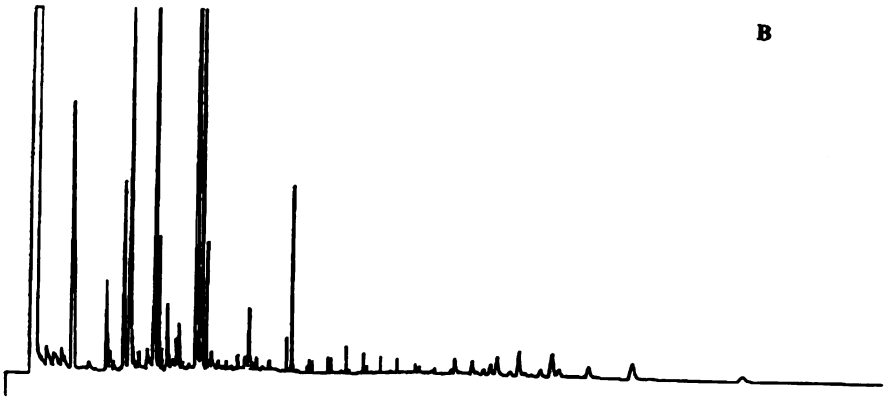


Figure 5.7

or 20 carbons.

The proposed mechanism for tail-to-tail coupling of membrane lipids is that hydrogens are removed from the ω-1 positions of two opposing alkyl chains forming radicals which quickly combine (Figure 5.1). Since these bifunctional fatty acid species are chiral, they should not be readily accessible to radical traps or scavengers. Radicals planarize rapidly, much faster than the timescale for diffusion from the cage in which they are formed [10-12]. This notwithstanding, it was of interest to assess the effects of various radical traps and scavengers on this system. Such experiments were important from another perspective. It was clear from previous experiments that oxygen had an adverse effect on the coupling activity. This would indicate either a radical process or the presence of an oxygen labile species such an iron-sulfur complex. Reagents which would allow us to test these possibilities were added to *Sarcina* cultures growing at 37°C, then the temperature was raised to 50°C to trigger the coupling reaction. The use of a radical trap is a very direct way of testing whether any accessible radical species are generated in the coupling reaction. This was done using 2-nitroso-2-methylpropane [13]. In these experiments, transmembrane cross-linking was not inhibited but, in fact, increased to levels (19%) much higher than observed in the 50°C control (Figure 5.8). Thiols too react very rapidly with radicals and would also be good probes to see if, during the coupling process, any radicals which might be generated are accessible. Two thiols, *t*-butyl thiol and n-butane thiol, were used and contrary to the nitroso methylpropane results, both inhibited coupling (Figure 5.8). Iron-sulfur species are very sensitive to thiols [14-17] and this inhibition could be indicative of the presence of such

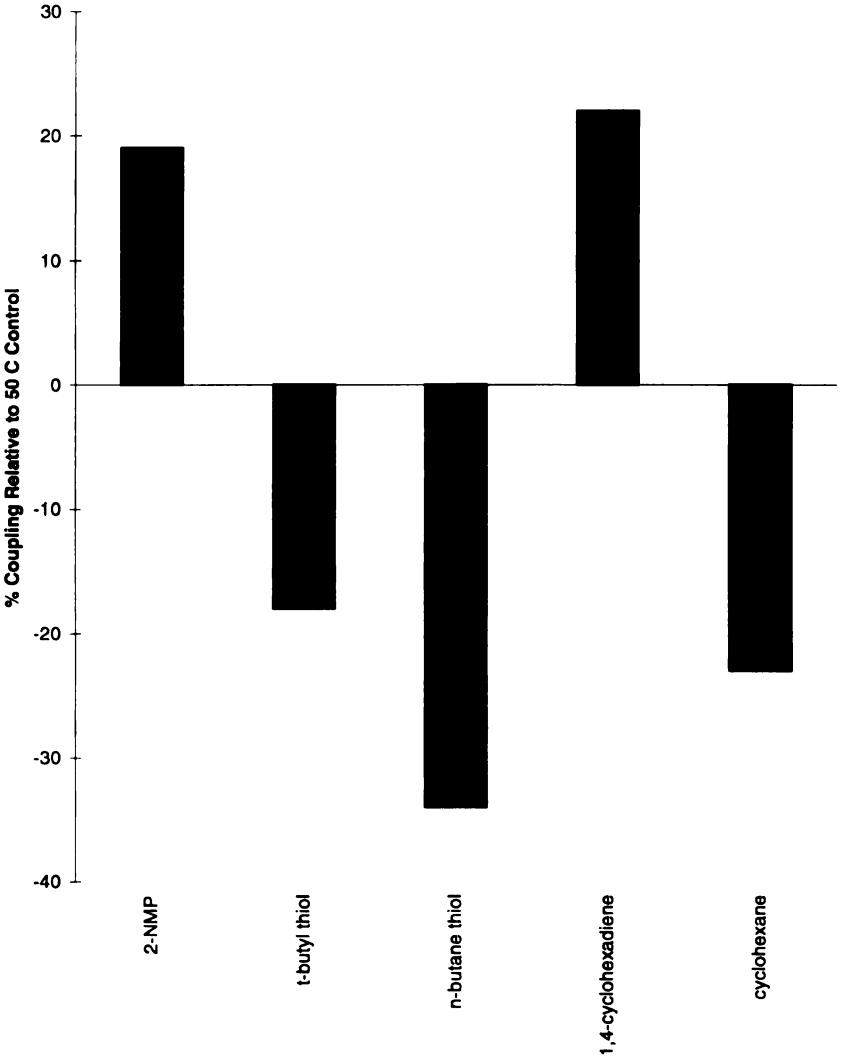


Figure 5.8: Results from radical trap/scavenger experiments indicating the percent of tail-to-tail coupling of *S. ventriculi* membrane lipids normalized to the 50°C controls. 2-Nitroso-2-methylpropane and 1,4-cyclohexadiene enhanced coupling, while the two thiols and cyclohexane inhibited the process.

a center and not the presence of an accessible radical center. To further clarify this point, 1,4-cyclohexadiene was used as a radical scavenger. This is a radical-sensitive diene that should have no effect on iron-sulfur centers. A similar result to the one seen with 2-nitroso-2-methylpropane was obtained. The levels of cross linking actually rose (Figure 5.8). It appeared that 2-nitroso-2-methylpropane and 1,4-cyclohexadiene were not reacting with radicals but were further fluidizing membrane. 1,4-Cyclohexadiene is flattened and will not stack well between the membrane lipid chains. Cyclohexane, on the other hand, has a chair conformation and will stack well between the lipid chains. Since it would reduce the alkyl chain mobility, it could be expected to reduce the level of coupling considerably. This is exactly what was observed (Figure 5.8). The reduction of alkyl chain mobility by cyclohexane has also been characterized by fluorescence spectroscopy [18].

The relative effects of 1,4-cyclohexadiene and cyclohexane on hydrocarbon chain dynamics was investigated using NMR spectroscopy. The test system used was dipalmitoyl phosphatidylcholine. This is a comparatively rigid molecular system and the signals for the methyl and methylene groups at ~ 0.7 and 1.2 ppm are broad with very low intensities because of very short spin spin and spin lattice relaxation times. In the NMR (Figure 5.9) cyclohexane (spectra B and C) does not increase the fluidity of the lipids (rigidifies) as compared to the control (spectrum A). The methylene and terminal methyl peaks at 1.19 ppm and 0.72 ppm are still barely visible in all three spectra indicating no increase in T_1 or T_2. 1,4-Cyclohexadiene, however, shows a marked affect on the lipids. Both the methylene and terminal methyl groups show increased mobility as seen by the increase in intensity of

Figure 5.9: ^{1}H NMR spectra showing the effect of cyclohexane and 1,4-cyclohexadiene on the lipid packing arrangement of dipalmitoyl phosphatidylcholine. (A) dipalmitoyl phosphatidylcholine control, (B) 5μl cyclohexane, (C) 10μl cyclohexane, (D) 5 μl 1,4-cyclohexadiene, an (E) 10 μl 1,4-cyclohexadiene. Signals for the methylene protons (1.19 ppm) and methyl groups (0.72 ppm) are indicated.

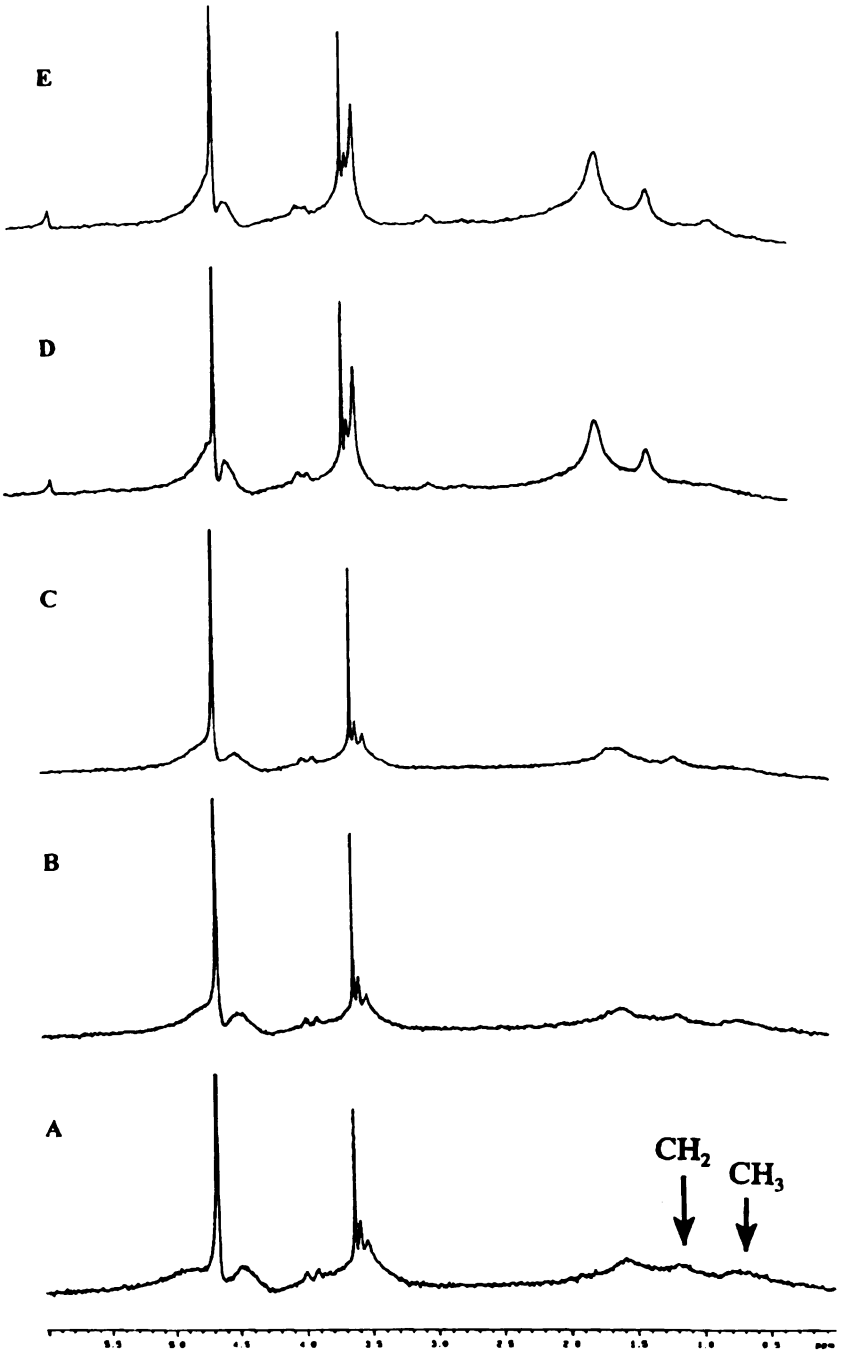


Figure 5.9

the peaks at 1.19 ppm for the methylene (seen in both spectra D and E) and 0.72 ppm for the methyl groups (which is more prominent in spectrum E), respectively.

All of the results obtained here, therefore, indicate the putative hydrocarbon radicals are caged and therefore inaccessible to any external agents. It is clear that thiols are inhibiting cross-linking via another mechanism and not by radical scavenging. One possibility is that they are extruding iron-sulfur centers from the necessary cofactors such as ferredoxin [17], thus breaking the electron transfer chain and stopping the recycling of the hydrogen acceptor. The involvement of iron-sulfur centers is also consistent with the oxygen lability of the coupling process.

The hydrocarbon chain coupling phenomenon demonstrated has no known precedent. The coupling conditions are mild and the regio- and stereospecificity is extremely high. It is independent of the nature of the functional groups on the distal ends of the carbon chains. Other systems containing this activity will, for sure, be identified and knowing how to optimize and control it will have important implications for synthetic organic chemistry. The stereoselective formation of carbon-carbon bonds between unactivated centers is viewed as the last frontier in carbon-carbon bond formation.

ACKNOWLEDGMENTS

I would like to acknowledge Seunho Jung, a past member of our laboratory, who did

the cell-free coupling of the heptadecanoic acid to *S. ventriculi* membrane fatty acids. I would also like to acknowledge Luc Bérubé's, another past member of our laboratory, contributions to this work as well. He did the alkaline pH and D_2O experiments. He also did the experiments in which *S. ventriculi* was cultured in the presence of cyclohexane, 1,4-cyclohexadiene, and *tert*-butyl thiol.

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CHAPTER VI

SUMMARY AND PERSPECTIVES

The ability of procaryotes to survive in, or adapt to, many extreme environments is impressive. The fact that they can not only withstand such extremes of temperatures, pH, pressure, salinity, and high concentrations of detrimental chemical agents, but thrive under these conditions is extraordinary. One of the most commonly observed cellular change, however, in response to all of the different environmental perturbations is alteration of membrane acyl (or alkyl) chain structures. As the cell envelope membrane(s) is in constant contact with the environment, it makes sense that rapid alterations in membrane structure are necessary as conditions change.

We have shown in this work that the extent of membrane acyl chain length heterogeneity observed response to environmental perturbations is significant. The selection of the acyl chain length distribution is a random process, in which the mean and variance of the distribution curve depends on the available thermal energy spectrum. We know that many plants and animals also make similar changes in their membrane acyl chains in response to temperature as well, for instance fish adapt to cold temperatures by a similar spectrum of membrane chemistry changes. This, then, seems to be a general response to temperature changes. Thus aspects of the relationship between temperature and acyl chain length distributions are likely to be universal. From an ecology and paleontology perspective, the relationship between fatty acid chain length heterogeneity of a given organism and temperature could be used basically as a temperature stamp. The fatty acid compositions of bacteria isolated from different soil depths (or from other organisms like fish) that have been preserved in some fashion (whether or not they are still viable), like the

Siberian permafrost bacteria, could give indications of global climate changes or catastrophic events that have occurred in a particular region as the earth has evolved.

The fact that there is also a temperature equivalent for solvent and antibiotics effects is another important finding. The possible relationship between temperature, solvent concentration, and growth rate could be used to extend the industrial utility of microorganisms. Using a three-dimensional graph like the one shown in chapter two, the external operation temperature for microorganisms could be extended in both directions, by adding the appropriate concentration of a fluidizing or rigidifying solvent. It may also be possible to overcome drug resistance of some bacteria by determining the appropriate combination of temperature and drugs to kill the bacteria. It has been shown, in fact, that several bacterial species show temperature dependent sensitivity to certain antibiotics. Several *Pseudomonas* strains were more resistant to gentamicin at 20°C and 30°C, but became more susceptible at 37°C and 42°C [1]. In another study, all *Yersinia kristensenii* strains were more resistant to β-lactam antibiotics at 22°C than at 37°C [2]. The reason for the increased resistance to antibiotics at lower temperatures is probably due to the increased rigidity of the membranes such that antibiotics are less effective in intercalating into the membrane and so there is less uptake of the drug. Recent evidence for this was shown in a study in which strains of *Stenotrophomonas maltophilia* that were more resistant to aminoglycoside antibiotics at lower temperatures all had more rigid membranes than strains that were susceptible at those temperatures as shown by ESR spectroscopy [3]. It may also be feasible to kill cancer cells using the right concentrations of chemotherapy drugs and

localized heating of the tumor with lasers or probes.

Many researchers use the profile of fatty acid compositions of microorganisms as an indication of the species, but these organisms are typically cultured under different conditions in the laboratory than found in nature. However, we have shown that the balance between metabolic pathways, for instance carbohydrate versus amino acid metabolism, could have a significant impact on the membrane acyl chain structures. This balance is determined by the available heat (kinetic energy) needed to carry out chemical processes. At lower temperatures the amount of maintenance energy increases dramatically and cells need to use carbon sources that require less energy to catabolize and yet yield more energy for maintaining the necessary cellular functions. These shifts in metabolism directly affect the types of fatty acid chains produced by changing the available acyl-CoA primer pool for fatty acid synthesis. This finding also supports the randomness of membrane acyl structures, since the cell has to use the primers available, a random selection process would favor whatever is the most prominent primer in the pool. Therefore it would be more appropriate to demonstrate that organisms produce the same spectrum of fatty acids under different conditions. This is preferable to basing the relatedness of organisms on similar fatty acid profiles when the organisms were most likely cultured under two very different conditions. Also since it is expected that organisms that are adapted to subzero climates would most likely use triacylglycerol stores for their primary energy source at low temperatures, as is shown by large amounts of triacylglycerol found in Arctic strain 45-3 at 4°C, they should have lipases which are very active at low temperatures. Isolation of these enzymes would

have many uses in industry. Work in our laboratory is currently being done to characterize the activity of lipases in Arctic strain 45-3.

The findings that Arctic strain 45-3 does not produce compatible metabolites in response to cold temperatures indicates that it is not clear that there is link between extreme salinity and freezing. Also the current models on the ability of compatible solutes to confer cryotolerance as well are certainly not general. As such more serious physico-chemical models and theories for explaining the interactions between water and biological surfaces need to be developed. The model we propose, in which cell surface alterations are sufficient to provide cryoprotection, should also be expanded and subjected to a more physico-chemical analysis in more systems.

The advantages of carbon-carbon bond formation at unactivated centers by *S. ventriculi* has many potential synthetic organic chemistry and industrial applications. The coupling of fatty acids in this manner could be used to make biodegradable plastics, liquid-crystalline devices, and allow the grafting of materials onto polymers to help in the biocompatibility of artificial implants. While the entire functioning enzymatic system will probably not be able to be isolatable from the cells, since its regulation in the membrane is likely very complex, characterizing aspects of the process would aid in understanding ways of optimizing the system to effect the coupling of the desired lipid species. Initial work done in isolating potential enzymes involved in this coupling process is presented in Appendix A.

A lot of work in studying adaptative responses of microorganisms to environmental perturbations seems to result in "catalogs" of types of responses, the levels of the responses, and in which organism they occur. Since these fine details of the adaptations seem to vary so much in bacteria, little (if any) general conclusions are drawn from these observations. In this work, we have hopefully provided new insights and theories as to the fundamental principles involved in adapting to fluctuations in the environment. Bacteria, and even higher organisms, have certain cellular functions which must be maintained regardless of the environment. The manner in which organisms adapt to perturbations vary based on the types of cellular processes which they are able to carry out. However, all of these chemical processes rely on the available thermal energy (and thus kinetic energy) spectrum. There is a circular relationship, though, between this available energy, the balance of metabolic processes, and membrane structure. But, by "tuning" the membranes through continual alterations in membrane structures to propagate the maximum of this available energy, an optimal balance of metabolic pathways can be achieved to maintain necessary cellular functions. The process by which organisms adapt to other environmental perturbations, other than temperature, results from similar perturbations in the relationship of kinetic energy, metabolism, and membrane structure. These changes are again detected by disturbances in membrane phase equilibria, similar to temperature effects, which is why similar adaptative responses are observed. So organisms typically have a general set of changes they can make in response to many stress conditions, rather than a specific mechanism of adaptation for a given type of environmental change. Thus the idea of specific receptors, channels, and pumps for individual antibiotics or other chemical species is highly unlikely. Hopefully the

work presented here will elicit new views, ideas, and models as to the generality of adaptation mechanisms to environmental perturbations and how they are tied to the types of cellular processes than can be carried out by the cell. Also the application of the principles we have developed for the adaptation and adaptability of bacteria to extreme environments, along with the use of certain enzymatic functions of extreme adapted organisms has a great deal of potential use in research and industry.

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APPENDIX A

PREPARATION OF THE RADIOLABELED PHOTOAFFINITY PROBE FOR IDENTIFYING PUTATIVE CROSS-LINKING ACTIVITY

We have begun work to try to isolate the enzymes involved in the tail-to-tail fatty acid cross-linking mechanism in *Sarcina ventriculi* characterized in chapter 5. Below is described the synthesis of a membrane ^{14}C-labeled photoaffinity probe, which was then incorporated into growing cultures of *Sarcina ventriculi*. Preliminary results of this experiment are then shown.

Synthesis of a ^{14}C-labeled photoaffinity probe

A ^{14}C-labeled photoaffinity probe was synthesized as outline in Figure A.1. (1) The probe was made by combining 50 mg of the C_{15}-iodofatty acid with 40 µl (40 µCi) ^{14}C-phenol and 20 mg cold phenol (final concentration of 1 µCi/mg phenol), plus 50 mg anhydrous potassium carbonate and 0.5 ml acetone. The reaction was stirred overnight. The reaction was stopped by adding 1 ml of water and glacial acetic acid until effervesence ceased. The product was extracted with chloroform and dried under a stream of nitrogen. The product was dissolved in 2 ml acetic acid and the solution cooled in ice while stirring. One milliliter of concentrated sulfuric acid was then added followed by the slow addition of 100 µl fuming nitric acid. The reaction became very dark orange/brown in color. After about 10 minutes it was diluted with about 10 g of crushed ice, and then 15 ml chloroform was added. The mixture was stirred, and the chloroform layer was then removed, washed with cold water, and evaporated to dryness. The nitrated product was then hydrogenated to form the amino derivative. This was done by taking up the product in 50 ml of absolute ethanol and adding a few drops of water and acetic acid. Ten percent of palladium on carbon (~20 mg) was added while passing nitrogen over the reaction. Hydrogen was supplied by

attaching two hydrogen filled balloons to the system. The reaction mixture was allowed to stir for over 8 hours until the yellow color had disappeared. The palladium on carbon was removed by vacuum filtration through methanol washed celite. The product mixture was evaporated to dryness. The product was dissolved in 10 ml of 2 M sulfuric acid and 10 ml of absolute ethanol and cooled in a salt/ice water bath. Sodium nitrite (0.2 g) was slowly added. The reaction then continued to stir for 2 hours more. From this step on, the reactions were protected from light. Sodium azide (0.2 g) was slowly added, and then the reaction was stirred for one hour at room temperature, followed by a half hour at 60°C. The product mixture was evaporated only long enough to remove the ethanol, leaving the product in about 5 ml of water. The ^{14}C-photoaffinity probe, containing the azide groups, were extracted three times with ethyl acetate and evaporated to dryness. The product was taken up in 5 ml of ethyl acetate and 50 µl was counted on the scintillation counter. The product was then dried again and stored at -20°C. The specific activity of the photoaffinity probe was about 55 mCi/mole.

Growth of *Sarcina ventriculi* with the ^{14}C-photoaffinity probe

The ^{14}C-photoaffinity probe was dissolved in ethanol and half of it was split between two exponentially growing cultures of *Sarcina ventriculi* (50 ml) at 37°C. The final ethanol concentration per culture was 0.2 M, and the cultures were wrapped in aluminum foil. Continued 37°C incubation for 6 hours. Cells were protected from light at all times. Cells were harvested by centrifugation, and washed with Dulbecco's phosphate buffered saline (10 mM phosphate, 0.15 M NaCl, pH 7.0) containing 0.9 mM $CaCl_2$, 2.7 mM KCl, and 0.5 mM

$MgCl_2$. Cells were lysed by passage through a French pressure cell (American Instruments Co., Inc., Silver Spring, MD) and by freeze/thawing several times. The probe was then cross-linked membrane components by exposing the lysate (in a quartz cuvette) to a high intensity UV lamp for two minutes.

Isolation of potential protein involved in the cross-linking mechanism

Half of the ^{14}C-lysate (1 ml) was separated by using a Sephadex G-75 column. The amount of radioactivity for the first 100 fractions was determined. Fractions containing the most radioactivity were lyophilized.

SDS-PAGE gels and Western transfers

The radioactive Sephadex G-75 fractions were lyophilized and separated on duplicate 12% SDS-PAGE gels using Laemmli's discontinuous buffer system [1] on 12% polyacrylamide slab mini-gels containing 0.32% bisacrylamide, 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The stacking gel contained 4% polyacrylamide, 0.1% bisacrylamide, 0.125 M Tris-HCl (pH 6.8), and 0.1% SDS. Electrophoresis was carried out at 200 V for about 1 hour. One gel was stained in a solution of 0.1% Coomassie blue in 40% methanol/10% acetic acid, and destained in a solution of 40% methanol/10% acetic acid. The other gel was Western transferred to Problott™ (Applied Biosystems) nylon membrane, which is suitable for protein sequencing, following manufacturer instructions: The nylon membrane was briefly wetted with methanol and then soaked in electroblotting buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid {CAPS}, pH 11 in 10% methanol). The gel was

soaked in electroblotting buffer for 5 minutes. The transblotting sandwich was assembled according to Bio-Rad manufacturer's instructions, and the transfer was carried out at 100V on ice for 1.5 hours. The nylon membrane was Coomassie stained with 0.1% Coomassie Blue R-250 in 40% methanol/1% acetic acid for less than one minute and then destained with 50% methanol and rinsed well with deionized water. The membrane was air-dried and then exposed to a phosphoimager.

PRELIMINARY RESULTS

Currently, work is being done to isolate components of the enzymatic system for tail-to-tail coupling of membrane fatty acids. A ^{14}C-labeled fatty acid photoaffinity probe was synthesized (Figure A.1) such that the reactive azide group would be positioned in the middle of the membrane bilayer. After growing *S. ventriculi* in the presence of this probe, the probe was cross-linked using UV light to membrane components. Since the cross-linking occurs in the center of the membrane, it is reasonable to expect that enzymes involved in this process must span the central portion of the membrane. Phosphoimages of Western blots containing proteins from the Sephadex column fractions containing the highest amounts of radioactivity show that only three of the void volumn fractions contain a detectable labeled protein (Figure A.2). The other fractions may also contain labeled proteins, but either not enough protein was loaded onto the gel, or there is too little radioactivity to detect. In these three void volumn fractions, a single 15 kDa protein is detected. Since the fractionation range of Sephadex G-75 is 3 - 80 kDa, this protein was likely a large aggregate as it passed

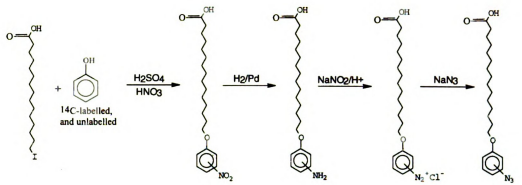

Figure A.1: Synthesis of the ^{14}C-labeled photoaffinity probe.

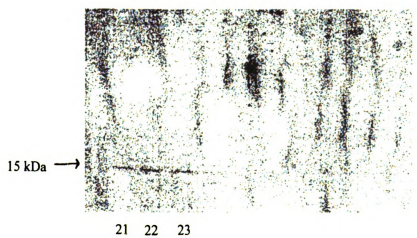

Figure A.2: Phosphoimage of the nylon membrane containing proteins from several G-75 column fractions. Only lanes containing proteins from column fractions 21, 22, and 23 had a radioactively labeled protein detectable by phophoimaging. The other lanes probably did not contain enough protein and/or enough radioactivity to detect.

through the column. This would be expected as integral membrane proteins are usually hydrophobic and thus they would tend to aggregate. This protein has been submitted for sequencing, though no results have been obtained to date.

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