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A Dynamically Regulated Transformation of a Bacterial Bilayer Membrane to a Cross-linked 2-Dimensional Sheet during Adaptation to Unfavorable Environmental Pressures

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Abstract: To maintain an optimum dynamic range, membranes of living systems must have the ability to regulate their translational and vibrational motion in the face of environmental changes that might offset them. This is done through structural modifications of the lipids. *Sarcina ventriculi* was used as a case study to explore membrane structural reorganizations which allow some organisms to adapt to extreme environmental changes. It is capable of a variety of unusual and dramatic chemical processes including lipid alkyl chain tail-to-tail and lipid head-to-head coupling. There is also interlipid headgroup transfer or shuffling. The tail-to-tail coupling activity is capable of joining foreign (exogenously added) hydrocarbon chains to the native chains. The adaptative processes occur dynamically and instantaneously and render this organism tolerant to low and high pH, moderately high temperatures, the presence of organic solvents, and a wide spectrum of antibiotics at concentrations as high as 200 μg/mL. Chemical analyses indicate that the membrane of *Sarcina ventriculi* exists in a dynamic equilibrium somewhere between a bilayer and cross-linked bipolar monolayer. Based on the degree of cross-linking of both the alkyl chains and the headgroups, under more extreme conditions, the membranes should approach highly cross-linked, two-dimensional molecular sheets. These structural reorganizations parallel the same strategies used by organic chemists in their effort to synthesize stabilized monolayers and vesicles. Catalytic activities present in the membranes of this and similar organisms hold much potential for use in stabilizing supramolecular arrays and nano structures.

Introduction

Membranes are critical components of the cell surface of living organisms and are responsible for maintaining cellular integrity while still maintaining contact and communication with the environment and with other cells or organisms. This contact and communication include the passage of metabolites out of the cell and the passage of nutrients in. In addition, it includes the sensing and preliminary responses to stimuli. These functions often require catalytic or structural reorganizational events that require precise and very concerted molecular motion. At the same time, sufficient rigidity is required to maintain cellular integrity. Membranes, therefore, must possess very sophisticated molecular mechanisms for ensuring fluidity sufficient enough that molecular events can take place on the correct time scales. The term "homeoviscous adaptability" has been used to describe the process whereby, after a perturbation

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that changes membrane viscosity, the membrane chemistry of an organism is altered so as to restore the original viscosity or fluidity.[1] For modest changes in environmental parameters such as temperature, pressure, or pH, these changes involve modification of fatty acid chain lengths, degree of unsaturation, or modest changes in lipid composition.[2,3] There are, however, some extremophilic microorganisms which can tolerate much larger changes in environmental parameters.[4] Such conditions include high temperatures, high or low pH, high salt concentrations, and the presence of organic solvents and antibiotics. These can all destroy membrane integrity. Organisms that can adjust, survive, and even thrive in such conditions must have very dynamic, rapid, and efficient means of controlling membrane properties.

Sarcina ventriculi is a Gram-positive bacterium that is tolerant to extremes of pH (2.0 to 10) as well as moderately high temperatures, and the presence of organic solvents.[5−7] In previous studies, we demonstrated that this tolerance was linked to the production of very long, bifunctional fatty acids. We proposed that these bifunctional fatty acids span the cell membrane and are synthesized by the tail-to-tail joining of membrane lipid chains between the bilayers to form bipolar transmembrane species that stabilize membrane structure.[7−9] Hence if one knows the structures and abundances of all of the regular length fatty acids, then the structures and abundances of all of the very long bifunctional fatty acids can be accurately predicted. This was demonstrated by mathematical modeling[8] and by rigorous structural proof[9] of the actual very long chain bifunctional fatty acid species.

The synthesis of α,ω-bifunctional fatty acids is not unique to *Sarcina ventriculi*. Similar fatty acids have been reported in *Butyrivibrio* sp.,[10−13] *Thermatoga maritima*,[14] and *Thermoanaerobacter ethanolicus*[15] among others. In the case of *Butyrivibrio* sp., it was suggested that covalent tail-to-tail coupling could have occurred and a structure for one transmembrane lipid species was proposed.[13] These organisms are all acclimatized to extreme environmental conditions. These range from the low pH conditions of the stomach in the case of *S. ventriculi* to the high pressures and temperatures up to and above 100 °C in geothermally active regions at the bottom of the ocean floor in the case of *Thermatoga maritima*. A parallel membrane organization is found in the archaebacteria except that the hydrocarbon chains in these organisms have ether linkages to their headgroups and are isoprenoid in nature. Although there has been some debate to the contrary,[16] it has been proposed that the transmembrane ether lipids found in the archae might also be formed by tail-to-tail coupling.[17,18] A related adaptative response in which the proportion of transmembrane ether lipids in archae increases with increased growth temperature is also observed[19] and supports the tail-to-tail coupling mechanism. Transmembrane lipid synthesis, therefore, is an adaptative mechanism that has been adopted by a large cross section of extremophiles. There are other possible structural modifications taking place in the adaptation of membrane structure and dynamics of highly adaptable organisms. These are likely to be as spectacular as hydrocarbon chain-to-chain coupling and are further explored here.

Results and Discussion

Tail-to-Tail Coupling Is Independent of New Protein Synthesis. Since formation of transmembrane lipid species is triggered by a perturbation of environmental factors, the speed of activation of the process and the actual cellular steps leading to this activation are important features of the overall response. Whether the activity was constitutive and whether it required new protein synthesis or even new fatty acid synthesis are central issues. Attempts were therefore made to block the process by inhibiting new fatty acid synthesis with cerulenin and new protein synthesis with several antibiotics that are known to do so at different stages. Thus, treatment of cells at pH 7 and 37 °C with cerulenin, chloramphenicol, erythromycin, streptomycin, neomycin, or tetracycline, followed by a temperature shift to 45 °C, resulted in no decrease in the tail-to-tail coupling response compared to controls without antibiotics. In fact, the proportion of transmembrane fatty acids synthesized in the presence of antibiotics after the perturbation was much greater than that in the controls that lacked antibiotics. These results suggested that, somehow, antibiotics were actually promoting the formation of these transmembrane fatty acid species. This was confirmed by experiments in which the antibiotics were added but the system was not subjected to a temperature shift or any other perturbation. This led to the production of the same family of transmembrane species as was formed by a temperature shift in the absence of antibiotics (Figure 1). The levels of antibiotics used were as high as 200 μg/mL. On the basis of these results, it could be concluded that the formation of the very long bifunctional fatty acids takes place independently of new enzyme and fatty acid synthesis. This is also consistent with the observation that these transmembrane fatty acid species can be detected by GC and GC-MS immediately after the perturbing event. The adaptative process is very general and is triggered not only by pH change, temperature increase, and the presence of solvents, but also by the action of any foreign substance that may interact with the cell membrane and tend to destabilize it. This is consistent with results from the antibiotic studies, since they are either hydrophobic or neutral (in the case of chloramphenicol) and should partition into and disturb the lipid chains much like alcohols or they are polar and cationic and should bind to the lipid headgroups and disturb their arrangement. Either process should lead to destabilization of the membrane structure creating voids in the lipid packing thus increasing molecular motion and triggering the repair process.

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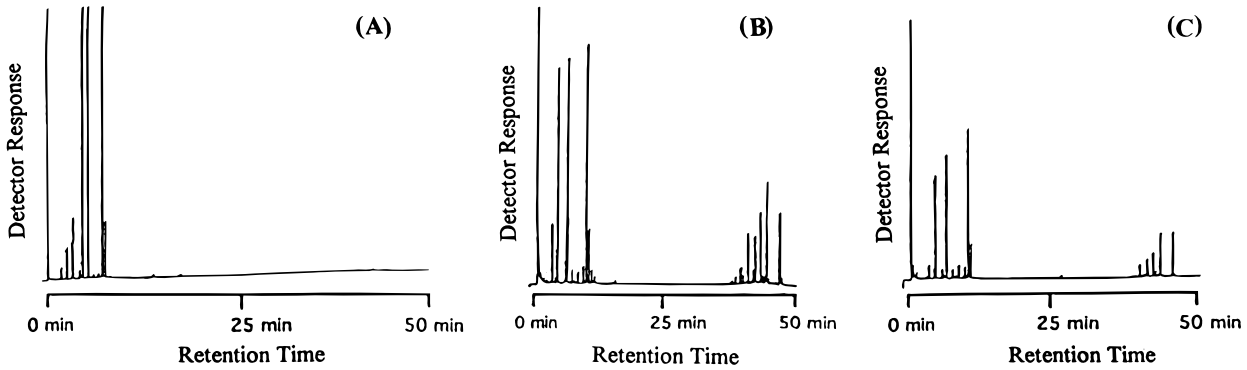

Figure 1. Gas chromatography profiles of the fatty acid methyl ester derivatives of the total membrane fatty acids of *S. ventriculi* cultured at pH 7.0 and 37 °C: (A) in the absence of antibiotics, (B) in the presence of streptomycin (100 μg/mL), (C) in the presence of chloramphenicol at the same concentration. The later eluting fatty acid methyl esters and aldehydes range from 28 to 36 carbon atoms in length.

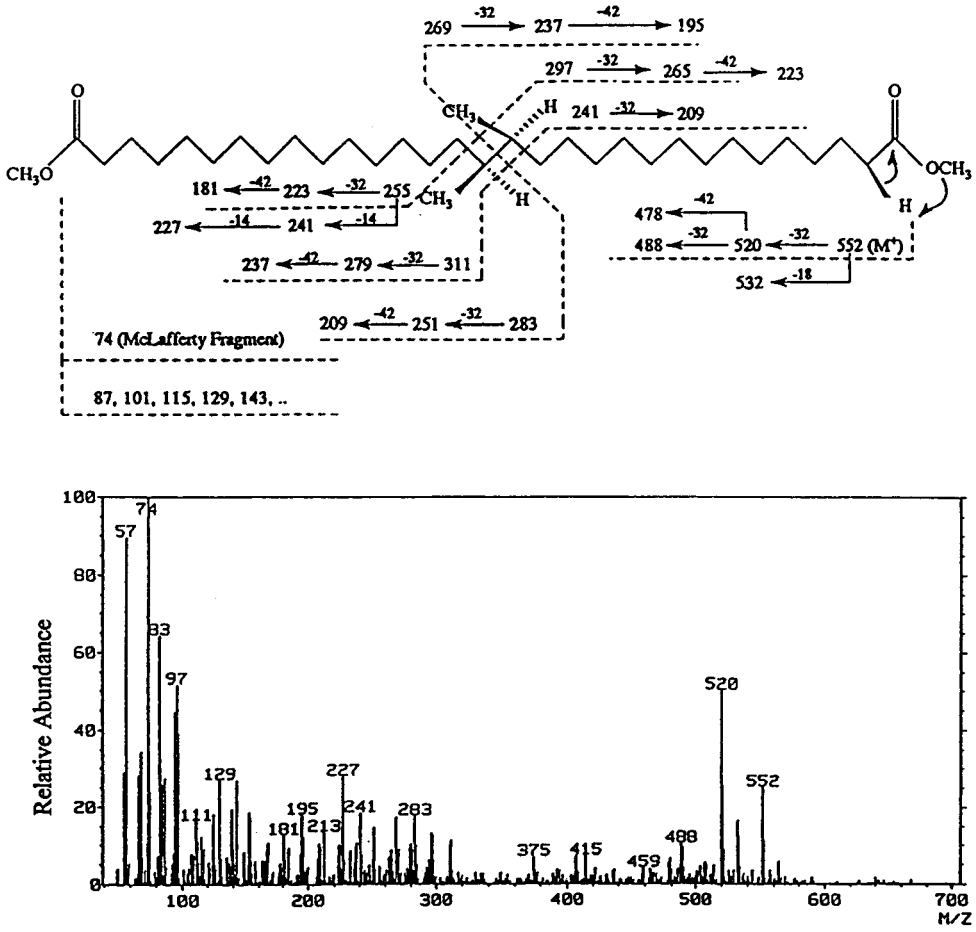

Figure 2. EI mass spectrum of one of the chimeric bifunctional fatty acid dimethyl esters formed (heptadecanoic acid) and a hexadecanoic acid residue from the *S. ventriculi* lipid component. The fragmentation pattern is indicated above the spectrum. Detailed discussions of the fragmentation modes for these molecules have been presented by Jung et al.[7,9]

It has been shown that the site-specific coupling of two unactivated alkyl chains in *S. ventriculi* is also enantiospecific.[9] This is not known in ordinary laboratory chemistry and would require a very sophisticated enzymatic system. It is clear from the antibiotic studies described above that the enzymatic activity responsible for this coupling event must be constitutive and an intact viable organism should not even be required.

Cell-Free Coupling Activity. We have concluded that no new protein (and possibly no new message) synthesis is involved in the formation of these very long bifunctional fatty acid species and that they are formed by random and indiscriminate but catalytic tail-to-tail joining of existing acyl chains. This conclusion is based on the observation that foreign, exogenously added fatty acids are taken up intact into membrane vesicles of *S. ventriculi* and incorporated into transmembrane lipid species. This was demonstrated by the addition of heptadecanoic acid, which is not normally found in membranes of *S. ventriculi*, to a growing culture of that organism at 37 °C, followed by cell lysis by French press and a temperature shift to 45 °C. This resulted in the formation of a family of chimeric fatty acid species with structures one-half of which were formed from the foreign fatty acid and the other from a native species (Figure 2). In this experiment, methyl ethyl ketone or a similar substrate had to be added as a final hydrogen acceptor presumably for the two hydrogens removed in the coupling process. In such a scenario, the ketone would be reduced to form 2-butanol in a

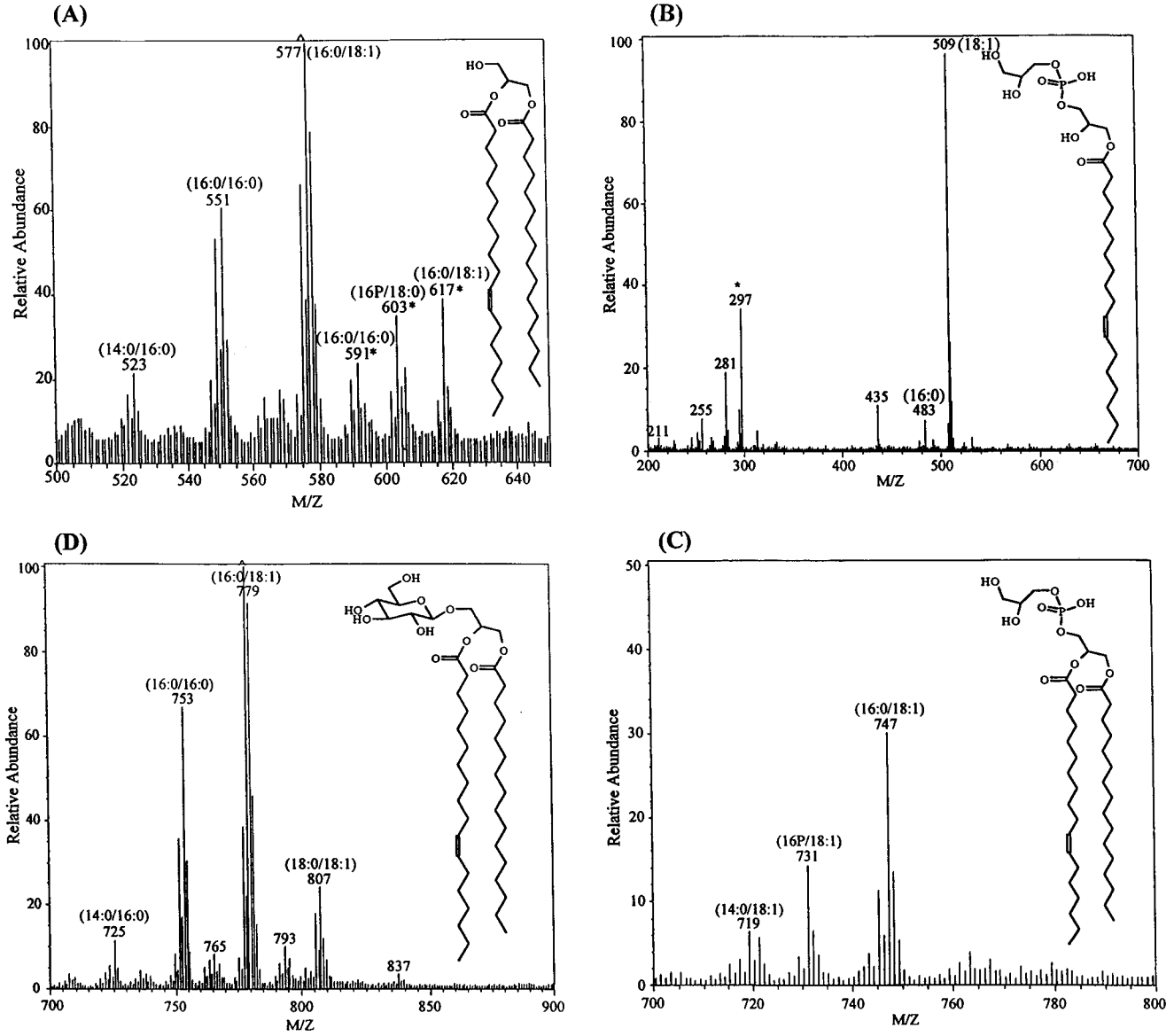

Figure 3. FAB mass spectra of the most predominant membrane lipid components of *S. ventriculi* cells grown at pH 7 and 37 °C. (A) Positive ion FAB mass spectrum of DAG. The ion at *m*/*z* 577 is due to the loss of water from the pseudomolecular ion as $[M + H - H_2O]^+$ with a hexadecanoyl and an octadecenoyl group. The ions designated by an asterisk correspond to the sodium adduct ions. (B) Negative ion FAB mass spectrum of lyso-PG with an octadecenoyl residue (*m*/*z* 509). The ion at *m*/*z* 297 is from the matrix. (C) Negative ion FAB mass spectrum of PG with a hexadecanoyl group and an octadecenoyl group (*m*/*z* 747) and lower homologues. The ion at *m*/*z* 731 is from a plamalogen with the same alkyl chain lengths and unsaturation. (D) Positive ion FAB mass spectrum of MGDG containing a hexadecanoyl and an octadecenoyl chain (*m*/*z* 779) and homologous. Similar species containing alkenyl ether groups (plasmalogens) are also observed (*m*/*z* 765 and 793). All ions correspond to the sodium adduct ions. Structures were also confirmed by FAB collisionally activated dissociation tandem mass spectrometry (FAB-CAD-MS/MS).

process catalyzed by native oxidases which would utilize reducing equivalents generated from the coupling process.

Tail-to-Tail and Head-to-Head Coupled Lipids. Proton NMR spectroscopy analysis of the aqueous fraction left after removal of the fatty acids and aldehydes from alkenyl ethers (plasmalogen) by acid hydrolysis of total lipids of cells grown at pH 7 and 37 °C revealed that glycerol, phosphoglycerol, and glucose were the only components. $^1H-^{31}P$ correlated NMR spectroscopy of the crude lipid fraction showed cross-peaks at 3.86 and 3.92 ppm on the proton axis correlating with a chemical shift of 1.41 ppm on the phosphorus axis which was attributable to phosphate in a phosphodiester linkage (data not shown). These results were confirmed by FAB/MS after isolating the individual lipid components. The predominant components of cells grown at pH 7 and 37 °C were diacylglycerol (DAG), monoglucosyldiacylglycerol (MGDG), lyso-phosphatidylglycerol (lyso-PG), and phosphatidylglycerol (PG) (Figure 3A−D). These contained palmitic acid ($C_{16:0}$), *cis*-vaccenic acid ($C_{18:1}$), and stearic acid ($C_{18:0}$) as the primary fatty acids and *n*-hexadecanal and *n*-octadecanal as 1-hexadecenyl (C_{16P}) or 1-octadecenyl ethers (C_{18P}). Lesser ions corresponding to lipids containing tetradecanoic acid, other minor fatty acids, and alkenyl ethers were also observed.[8,9] The presence of alkenyl ether (plasmalogen) molecular species was also confirmed by FAB-CAD-MS/MS.

A 2-dimensional $^1H-^{13}C$ HMQC NMR study also confirmed the mass spectrometric identification of the major lipid components from the pH 7 cells and indicated that glucose was present in the β-D-glucopyranosyl form (Figure 4A). This was indicated by the presence of a signal for the anomeric carbon of the glycosyl group at 103.5 ppm in the ^{13}C spectrum correlating with another at 4.20 ppm in the proton spectrum. A smaller signal at 104.8 ppm in the ^{13}C spectrum was assigned to a similar β-anomeric carbon appearing at a slightly different chemical shift because of different anisotropic effects caused

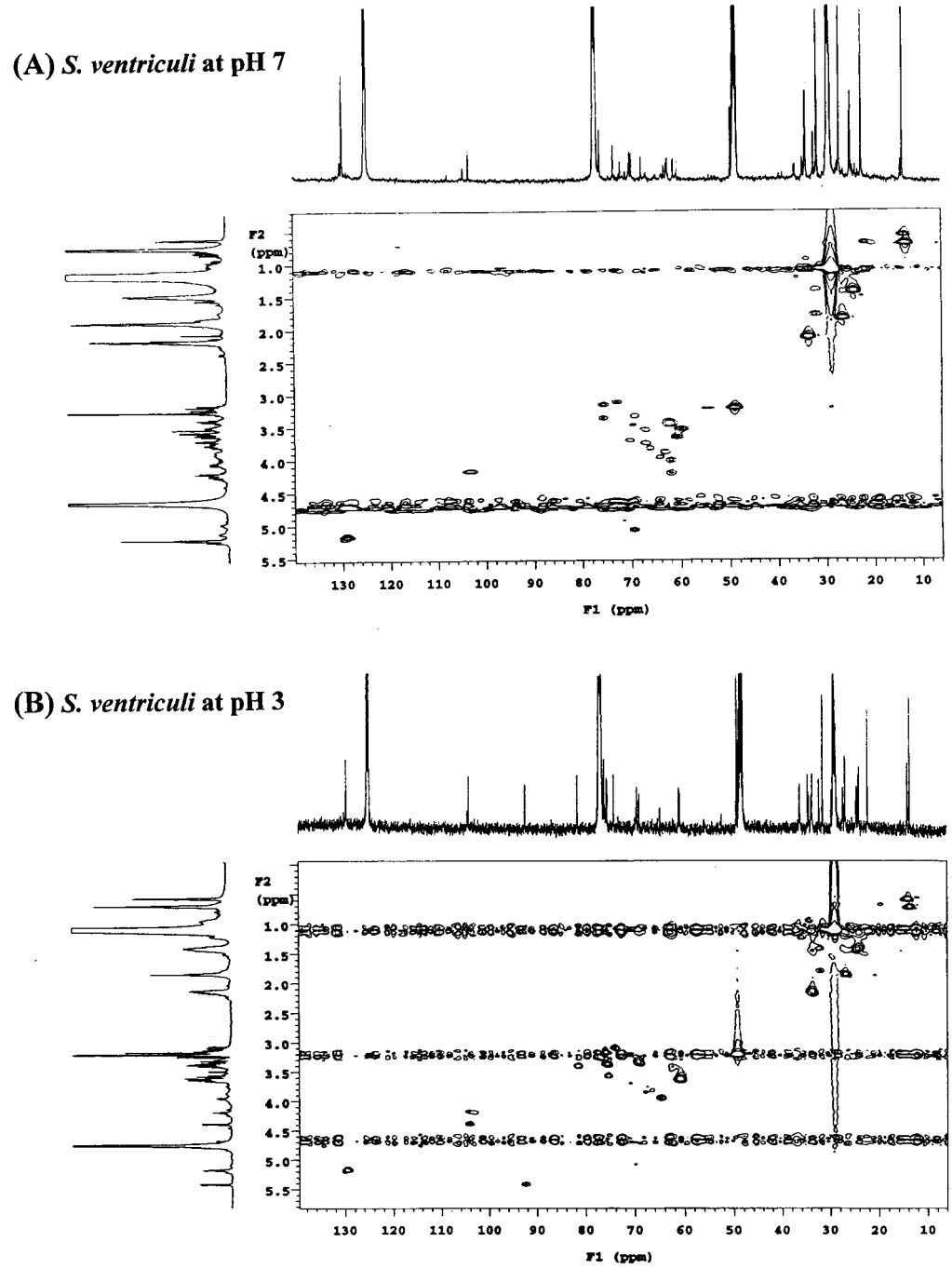

Figure 4. ^{1}H-^{13}C HMQC NMR spectrum of the total lipids from *S. ventriculi* cells grown (A) at pH 7 (B) at pH 3. The intense signals in the proton spectra at 49, 77, 126, 142 and 146 ppm are residual signals from the NMR solvent. The first two signals are due to methanol and chloroform, respectively. The others are due to pyridine.

by differing substituents on the glyceryl residue. This was most likely because either alkenyl ether (in plasmalogens) or acyl residues can occur at the 1-position of the glyceryl group. Other signals were mostly attributed to the signals from MGDG and PG. The NMR spectra of the lipids obtained from cells grown at pH 3 showed dramatic differences indicating that major modifications had occurred (Figure 4B). The doublet signal at 0.70 ppm in the proton spectrum correlating with the ^{13}C signal at 14.8 ppm was assigned to the vicinal methyl group formed by tail-to-tail coupling of alkyl chains. Two new resonances at 92.0 and 104.2 ppm in the ^{13}C NMR spectrum were correlated with two doublets ($J = 8$ Hz) at 5.44 and 4.40 ppm in the proton spectrum, respectively. These new signals were assigned to the anomeric signals from the novel glycolipid of this bacterium, β-1-*O*-acyl-β-1,2-diglucosyl glycoside.[20] Perhaps the most important new resonances appeared at 104.0 ppm in the ^{13}C spectrum correlating with a triplet ($J = 7$ Hz) at 4.20 ppm in the proton spectrum. This unusual combination of chemical shifts was readily assignable to an acetal function. The fact that the acetal proton appeared as a triplet with a 7 Hz splitting indicated that it was adjacent to a methylene group. Such an acetal group could only be possible if it were derived from a fatty aldehyde or its equivalent. This conclusion was substantiated by a proton−proton total correlated spectroscopy (TOCSY) experiment (Figure 5). It clearly showed that the signal for the new acetal proton was correlated with signals for methylene groups at 1.40 and 1.17 ppm as anticipated. The signal at 1.40 ppm is due to the methylene group adjacent to the acetal proton. Chemical proof of the acyclic acetal linkage came from the fact

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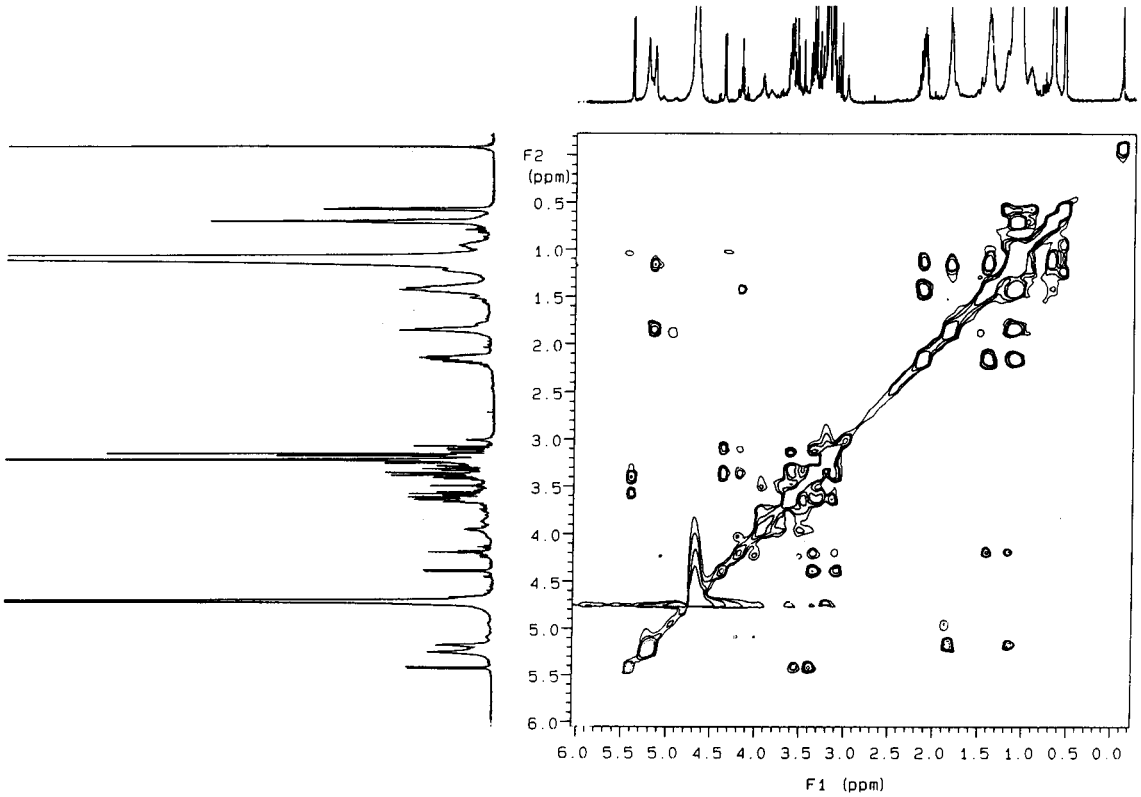

Figure 5. TOCSY NMR spectrum of the total lipids from *S. ventriculi* cells grown at pH 3.

that the NMR signal corresponding to it was lost on allowing the sample to stand in the presence of traces of acid. A new signal at 10.4 ppm, corresponding to an aldehyde proton, appeared in the proton NMR spectrum.

A simple chemical mechanism can be proposed to explain how the 2-position of a glucosyl residue of a glycolipid or the free primary alcohol function of glycerol could be involved in the formation of the acetal linkage observed in the membrane lipids of *S. ventriculi*. Acetals can be formed from the addition of alcohols to enol ethers or aldehydes. In this system, such a mechanism would simply involve the nucleophilic attack of a hydroxyl group on the enol ether moiety of a plasmalogen (1-alkenyl ether lipid) with assistance from either a proton or a metal ion. The fact that the process is so total, stereospecific, and rapid and takes place even in instances when the pH is not lowered indicates that it is probably enzymatic.

An attempt was made to isolate and further characterize some of these unusual lipids by FAB/MS and FAB/MS/MS. These structures included β-1-*O*-acyl and alkyl sophorosides (Figures 6A).[20] The mass spectra also confirmed tail-to-tail and head-to-head joined lipids including DAG/PG, DAG/DAG, DAG/MGDG, and MGDG/MGDG molecules. Their FAB mass spectra and proposed structures are presented in Figure 6, spectra B−E. In this study, data obtained by FAB mass spectrometry provide the structural information of intact transmembrane lipids. The molecular weight of the tail-to-tail joined lipid between PG and DAG having two 16:0, one 18:1, and one 18:0 chains is 1343.0, and that of the form with one 16:0, two 18:1, and one 18:0 chains is 1369.0 (Figure 6B). The FAB mass spectrum obtained in the positive mode gave *m*/*z* 1343.8 and 1369.8 as psedomolecular ions $[M + H]^+$. The ion at *m*/*z* 1327.8 corresponded to the head-to-head joined lipid between PG plasmalogen (16P/18:1) and DAG (16:0/18:1) molecules. As demonstrated in the positive FAB mass spectrum of DAG, the protonated molecular ions $[M + H]^+$ of a family of head-to-head and tail-to-tail coupled DAG molecules were absent. The highest ions corresponded to the loss of water from the psedomolecular ions (Figure 6C). In some cases the molecular masses derived from the head-to-head coupled lipids could not be distinguished from those ions of equivalent masses derived from the tail-to-tail coupled lipids. Additionally, the identities of fatty acid chains of the molecular ions were proposed on the basis of masses of different combinations of fatty acid chains involved in the tail-to-tail couplings. The FAB-CAD-MS/MS may provide a means of distinguishing between tail-to-tail and head-to-head coupled lipids without recourse to laborious separation and isolation schemes. In this study, the characterization of a large proportion of lipids formed at low pH or under other stress conditions proved to be too difficult. The different species were difficult to separate and to obtain useful spectra for. This coupled with their chromatographic properties indicated that they were larger oligomeric or polygomeric structures that were characterized by a high degree of heterogeneity.

Conclusion

Membrane lipid chemistry has always been somewhat of an enigma because of the structural complexity and, seemingly, excessive heterogeneity of lipid components. The origin and functional significance of this heterogeneity has always been elusive. This work provides a rationale for the existence and function of very long bifunctional fatty acids and ethers[7,21] found in the membrane lipids of bacteria that are adapted to a wide variety of extreme environmental conditions. The adaptative head-to-head coupling mechanism also explains the formation, presence, and significance of the unusual acetal lipids that have been observed in some extremophiles.[22,23] These include unusual acetal dimers[22] which can now be rationalized simply

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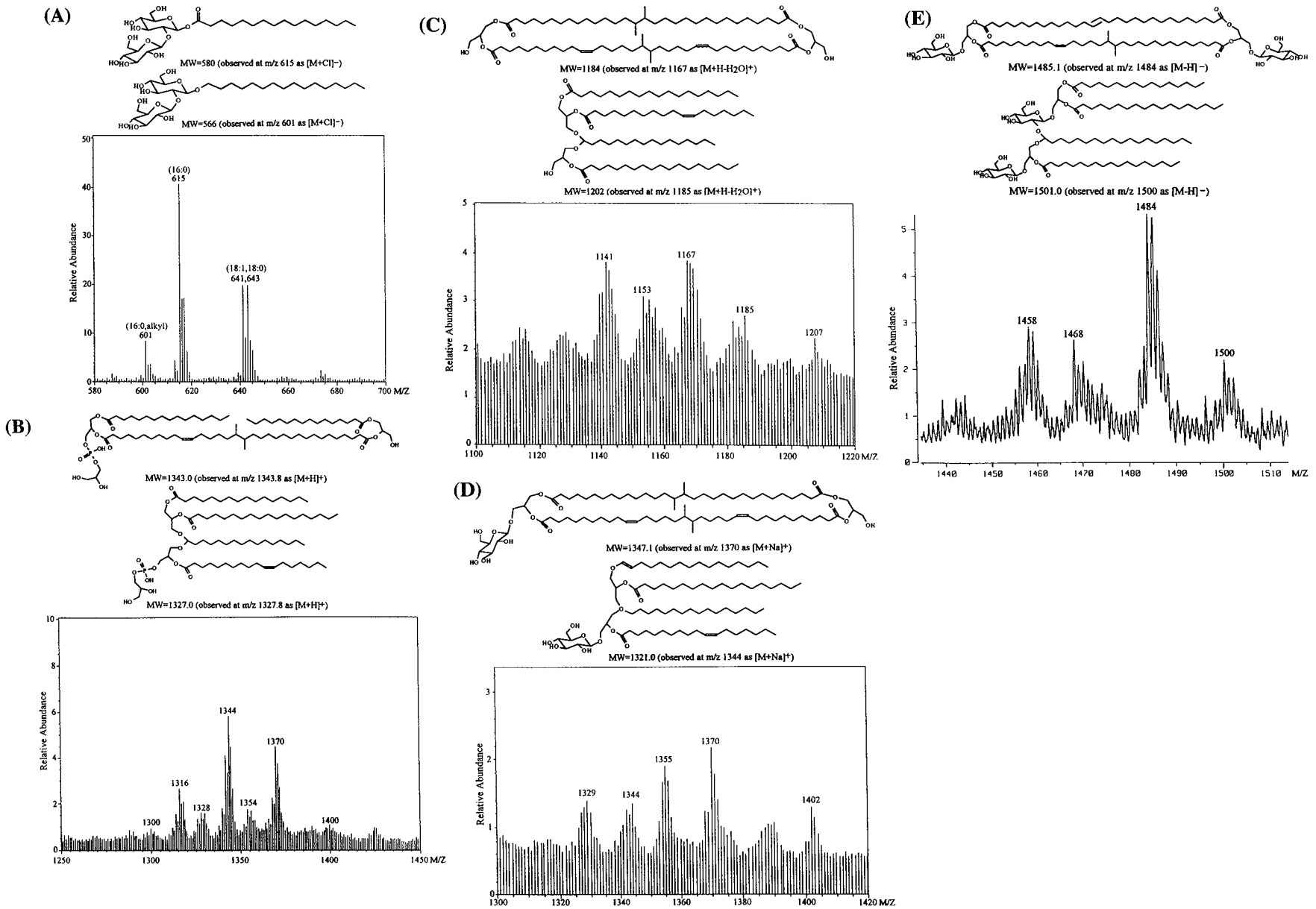

Figure 6. FAB mass spectra and their structures of the novel lipid components from *S. ventriculi* cells grown at pH 3. (A) Negative ion FAB mass spectrum and the structure of β-1-O-acyl and alkyl sophorosides. (B) Positive ion FAB mass spectrum and the structure of a family of tail-to-tail and small amounts of some head-to-head coupled lipids of DAG molecule and PG molecule. (C) Positive ion FAB mass spectrum and the structure of tail-to-tail and head-to-head coupled lipids of DAG molecules. (D) Positive ion FAB mass spectrum and structure containing cluster ions corresponding to the sodium adducts of the tail-to-tail and head-to-head coupled DAG molecule and the MGDG molecule. (E) Negative ion FAB mass spectrum showing cluster ions of a family of tail-to-tail and head-to-head coupled glycolipids with a variety of fatty acyl and fatty alkenyl chains.

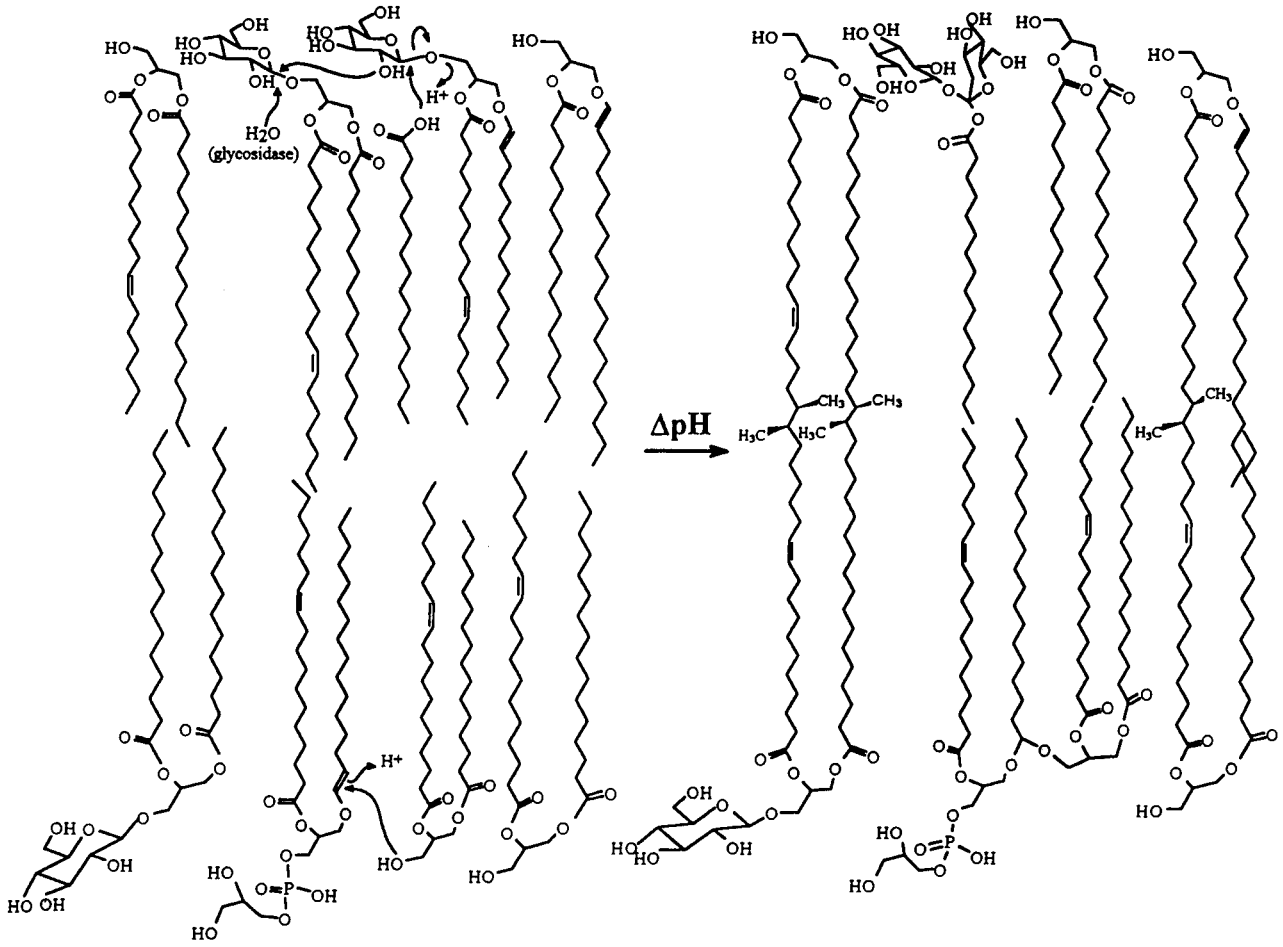

Figure 7. Proposed model showing hydrocarbon tail-to-tail coupling between the bilayer leaflets and lipid molecules partially cross-linked between the headgroups.

as head-to-head coupling between two phosphatidylethanolamine plasmalogens followed by phospholipase C cleavage of the phosphate groups. The structure of another known lipid acetal, a head-to-head dimer, can now be easily rationalized as the result of coupling between phosphatidylglycerol and the plasmalogen form of phosphatidylethanolamine.[23] In one organism, a head-to-head trimer has been tentatively identified.[24] A layer of finer adaptative control involving phospholipase and glycosidase activities that allow the disconnection of head-to-head cross-links thus affording a high degree of tunability is also evident.

It is not expected that one molecular structure will have constant dynamic properties under all physical conditions. This is especially true of temperature variations. Yet, this is exactly what seems to be the case with bacterial membranes. This has led to the theory of homeoviscous adaptation.[1] Dynamic NMR spectroscopy using spin−lattice relaxation measurements also demonstrates that this dynamic control is also present in *S. ventriculi*.[25] The results of this study suggest a very remarkable mechanism for explaining the adaptability of biomembranes to such a large variety of conditions. It embodies the concept of dynamic structure. The tremendous heterogeneity of these lipid species stems from the truly combinatorial fashion of the adaptative chemistry. We propose that there is not a particular structure per se that is required for an organism to survive but, rather, the ability of the organism to quickly shuffle the current molecular species to come up with new ones that have the correct dynamic state. If the enzymes that catalyze these processes are triggered when the correct dynamic state does not prevail, they will act on the membrane structure to re-model it until that state is reached when they will be again inactive. This is a kind of dynamic feedback control and is suggested by our NMR relaxation studies.[25] We propose that this response is highly influenced by proximity effects. A minimum of structural reorganization is then necessary thus ensuring the swiftest response. This combinatorial response to generate a continuum of structures over a continuum of environmental conditions is illustrated in Figure 7. For example in the top layer, when a glycosidase acts on a glycosidic linkage during a perturbation event, reaction with water competes with attack from a nearby sugar hydroxyl group. In the first case a free monosaccharide is formed and in the second case a disaccharide linked to diacylglycerol is formed. In another scenario, the nucleophile could be the carboxylate of a fatty acid that was released by an esterase. The product would be a 1-acyl hexose similar to the ones demonstrated earlier.[20] In the bottom layer, a well-positioned hydroxyl group from diacyl glycerol could form an acetal linkage by addition to an enol ether (plasmalogen). These and other structures are described here and a myriad of others are possible. In cases of extreme perturbations, over 80% of the fatty acids are transformed to the trans-membrane type. The molecules we have identified here represent a modest proportion

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of the structural diversity we know to be present. Many of the molecules are too large and intractable to allow complete characterization. Some appear to be polymeric. This type of response explains the remarkable structural complexity and high dynamic fidelity of membrane systems.

Experimental Section

Bacterial Cultures and Lipid Extraction. *S. ventriculi* was grown and harvested as described previously.[7,9] The cells (20 g) were extracted with 200 mL of chloroform/methanol/water (15:3:2 ratios in volume) at 40 °C for 2 h. The slurry was filtered and then the cell debris was extracted again with 100 mL of chloroform/methanol (5:1 ratios) at 40 °C for 1 h. The organic layer was taken to dryness on a rotary evaporator and the product was redissolved in $CHCl_3$/MeOH (1:1) for further analyses.

Tail-to-Tail Coupling of Foreign Fatty Acids. Cells (200 mL) were cultured anaerobically in the absence of the exogenous fatty acid at 37 °C and pH 7. They were then harvested anaerobically by centrifugation, resuspended in 2 mL of oxygen free medium, and lysed by French press under anaerobic conditions. Hexadecanoic acid (1 mg) and methyl ethyl ketone (0.2 mL) were then added to the mixture. The mixture was incubated at 45 °C for 3 h and then the fatty acid composition was determined by GC/MS after conversion to methyl ester derivatives.

Isolation and Purification of Individual Lipids. Lipids were separated and isolated by preparative thin-layer chromatography (TLC) on silica gel by using a solvent system composed of chloroform/methanol/ammonia/water (3:3:1:0.1:0.05 by volume). Bands were removed from the layers by scraping and extracting with $CHCl_3$/MeOH (1:1). The eluants were brought to dryness under nitrogen and redissolved in $CHCl_3$/MeOH (1:1).

Fatty Acid Analyses. The whole cells were methanolyzed with 5% HCl in MeOH at 72 °C for 24 h.[9] After concentration to dryness under nitrogen, samples were partitioned between chloroform and water and the aqueous layer was washed with chloroform. The combined chloroform solutions were brought to dryness and redissolved in chloroform.

Gas Chromatography and Mass Spectrometry. The components were analyzed by fast atom bombardment mass spectrometry (FAB/MS). FAB mass spectra were recorded on a JEOL HX 110 double focusing mass spectrometer in both positive and negative modes with nitrobenzyl alcohol or glycerol as the matrix. Collisionally activated dissociation tandem mass spectrometry (CAD-MS/MS) was conducted by scanning the electric sector and magnetic sector in a fixed ratio (B/E linked scan). Helium was used as the collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the abundance of the precursor ion by 50%. The fatty acid methyl esters were carried out on a gas chromatograph on a 25m J&W Scientific DB1 capillary column with helium as the carrier gas. The temperature was programmed from 150 to 300 °C (holding 30 min) at 3 °C/min. GC/MS analysis was carried out with a JEOL JMS-AX505H mass spectrometer interfaced with a Hewlett-Packard 5980A gas chromatograph.

NMR Spectroscopy. NMR spectra were recorded on a Varian VXR-500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). The NMR solvent system was prepared as a mixture of pyridine-d_5/deuterium chloride in deuterium oxide/methanol-d_4/chloroform-*d* in a volume ratio of 1:1:2:10, respectively.[19] Chemical shifts are quoted relative to the chloroform resonance taken at 7.24 ppm for proton and 77 ppm for ^{13}C measurements. For the heteronuclear multiquantum coherence (HMQC) experiments, the spectral width of the ^{13}C dimension was 3927 Hz. A total of 32 transients were acquired at 1024 points each. A total of 512 data sets were acquired. The total correlated spectroscopy (TOCSY) experiment was performed by using a total of 512 data sets with 8 transients at 2048 data points each. A mixing time of 60 ms was used.

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