On the Unusual Homeoviscous Adaptation of the Membrane Fatty Acyl Components against the Thermal Stress in *Rhizobium meliloti*

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In order to maintain the optimal fluidity in membrane, microorganism genetically regulates the ratio of the unsaturated fatty acids (UFAs) to saturated ones of its biological membrane in response to external perturbing condition such as the change of temperature.[1,2] The remodelling of fatty acyl chain composition is the most frequently observed response to altered growth temperature. It is reflected in the elevated proportions of unsaturated fatty acid (UFAs) at low temperature. Because *cis* double bonds, normally positioned at the middle of fatty acyl chains, introduce a kink of approximately 30° into acyl chain, UFAs pack less compactly and exhibit lower melting points than their saturated homologues. Thus, enrichment of membranes with UFAs offsets, to a significant degree, the increase in membrane order caused by a drop in temperature. This is so called homeoviscous adaptation of the membrane fatty acyl chains against thermal stress.[3,4] Membrane maintains the optimal viscosity using homeoviscous adaptation.

We investigated the adaptive response in a microorganism where unusual fatty acyl component appears in its membrane. The Lipid A moiety of *Rhizobium meliloti* was known to contain a 27-hydroxyoctacosanoic (C_{28}) fatty acyl component, of which chain length is unusually very long comparing with normal components ($C_{14\text{-}18}$) in the bilayer membrane.[5] The adaptive response of *Rhizobium meliloti* was compared with that of *Enterobacter aerogenes or E. coli* with normal membrane fatty acyl components. Figure 1 showed the Gas Chromatographic analysis of the membrane fatty acyl components in *Enterobacter aerogenes.* The decrease in temperature (low temperature shock) greatly enhanced the ratio of UFAs to saturated ones up to about 280% after the thermal stress from 37 °C to 25 °C as a result of homeoviscous adaptation. Similar phenomenon was also observed in *E. coli* (data not shown). However, a soil microorganism, *Rhizobium meliloti* with an unusual C_{28} fatty acyl component did not show any observable difference in membrane fatty acid compositions before and after thermal stress as shown in Figure 2. Table 1 compares the ratio of UFAs to saturated ones for *Rhizobium meliloti* and *Enterobacter aerogenes.* As homeoviscous adaptation is regarded as a general mechanism to maintain the optimum fluidity of the membrane against low temperature shock, it is surmised that there may be another novel mechanism for *Rhizobium meliloti* to adapt to thermal stress.

As it is possible that the molecular order in membrane also

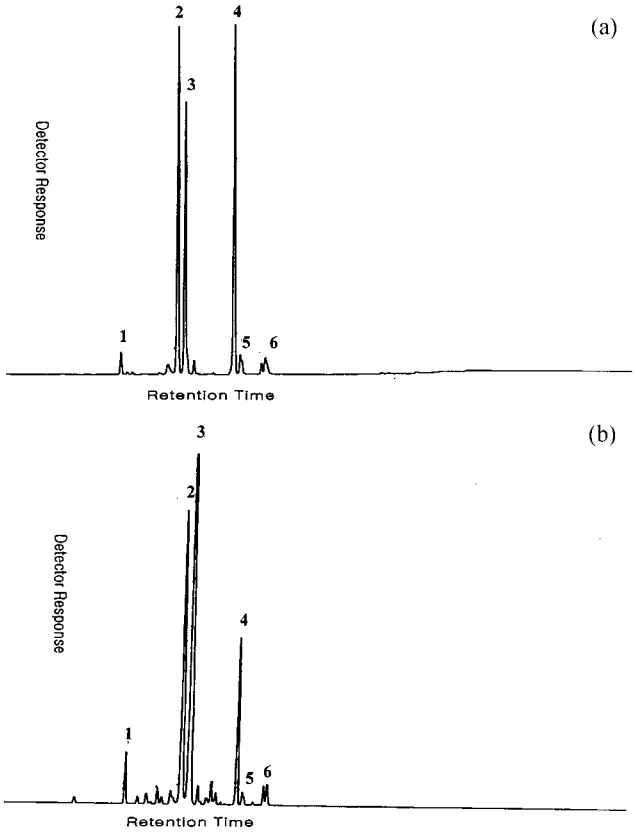

Figure 1. Gas Chromatographic analyses of major fatty acyl components in the membrane of *Enterobacter aerogenes* shocked at 25 °C (a) *versus* 37 °C (b). Total fatty acids within the membrane were analyzed as fatty acid methyl ester derivatives after methanolysis. 1. $C_{14:0}$ carboxylic acid methyl ester (28.9 min), 2. $C_{16:1}$ carboxylic acid methyl ester (34.1 min), 3. $C_{16:0}$ carboxylic acid methyl ester (34.8 min), 4. $C_{18:1}$ carboxylic acid methyl ester (39.3 min), 5. $C_{18:0}$ carboxylic acid methyl ester (39.9 min), 6. $C_{19:0}$ cyclopropane carboxylic acid methyl ester (42.2 min). The characteristic ratio of the relative amount of the saturated per unsaturated fatty acids changed from 0.5 at 25 °C to 1.3 at 37 °C. All the exact structures were confirmed with GC/MS analysis. The value in parenthesis indicates the retention time for the GC analysis.

depends on the conformational change of its component as well as the compositional change, we tried to investigate all the possible conformational changes over a wide range of temperature with computer simulation. In order to search all the possible conformations of an unusual C_{28} fatty acyl component, simulated annealing molecular dynamics - minimi-

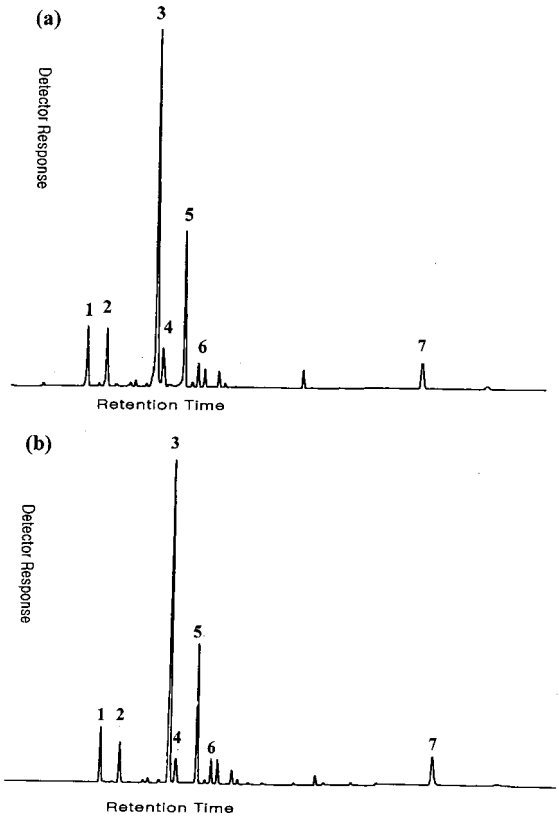

Figure 2. Gas Chromatographic analyses of major fatty acyl components in the membrane of *Rhizobium meliloti* shocked at 25 °C (a) versus 37 °C (b). Total fatty acids within the membrane were analyzed as fatty acid methyl ester derivatives after methanolysis. 1. C_{15} 3-OH carboxylic acid methyl ester (32.9 min), 2. $C_{16:0}$ carboxylic acid methyl ester (34.7 min), 3. $C_{18:1}$ carboxylic acid methyl ester (39.3 min), 4. $C_{18:0}$ carboxylic acid methyl ester (39.9 min), 5. $C_{18:0}$ carboxylic acid methyl ester (41.9 min), 6. $C_{18:0}$ 3-OH carboxylic acid methyl ester (43.2 min), 7. C_{28} 27-OH carboxylic acid methyl ester (64.0 min). The characteristic ratio (0.7) of the relative amount of the saturated per unsaturated fatty acids did not change at both temperatures. All the exact structures were confirmed with GC/MS analysis. The value in parenthesis indicates the retention time for the GC analysis.

zation calculation was performed with the temperature changed between 300 K and 1000 K five times at intervals of 50 K. After heavy searching process of possible conformational states of a 27-hydroxyoctacosanoic acid (C_{28}), we obtained the 10 conformations within 3 kcal/mol of the lowest energy minimum categorized. We made a further classification to three conformational classes as shown in Figure 3. There are folded forms with (Figure 3(a)) or without hydrogen bond (Figure 3(c)), and unfolded forms (Figure 3(b)). As molecular order of membrane rapidly decrease against low temperature shock as indicated by homeoviscous adaptation, we speculated that the conformational changes of the unusual fatty acyl components might be involved in the regulation of the membrane fluidity.

Table 1. Effect of thermal stress on chain length and the degree of saturation of the fatty acids in *E. aerogenes* and *R. meliloti*

Fatty acids[a]	*Enterobacter aerogenes*		*Rhizobium meliloti*	
	25 °C	37 °C	25 °C	37 °C
Saturated	32.8	56.6	41.0	39.9
Unsaturated	67.2	43.4	59.0	60.1
Ratio of S/U[b]	0.5	1.3	0.7	0.7

[a]Relative amounts of unsaturated, saturated fatty acids were determined by the calculation of integrated peak areas on GC analysis at each condition. [b]Saturated fatty acids/Unsaturated fatty acids.

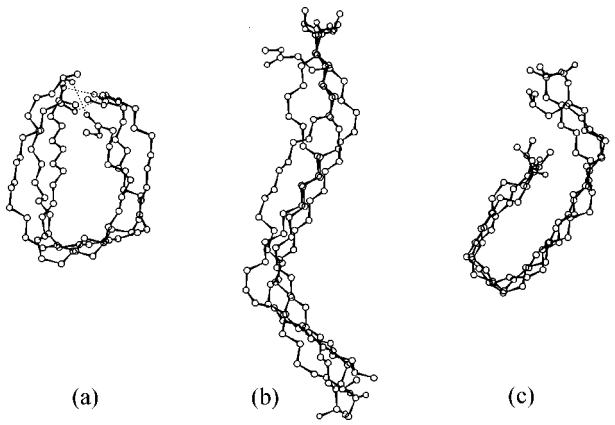

Figure 3. The conformations within 3 kcal/mol of the lowest energy minima were categorized into three classes. The conformations in each class were superimposed and represented as a ball-stick model; (a) three folded conformations with hydrogen bond, (b) four unfolded conformations (c) three folded conformations without hydrogen bond. Hydrogen bonds are displayed as dashed lines.

If we consider the *Boltzman distribution* to determine the probability of finding a molecule with energy ΔE_i, the probability P_i is,

$$P_i = \frac{e^{-\Delta E_i/RT}}{\sum_j e^{-\Delta E_j/RT}}$$

, where R is the gas constant (8.314 J/mol·K) and T is the absolute temperature (K).

So, define P_{fold} and P_{unfold} as below;

$$P_{fold} = \Sigma P_{fold,i} \text{ and } P_{unfold} = \Sigma P_{unfold,i}$$

When *Boltzman distribution* is applied to the 10 conformations of the lowest energy minima obtained in the simulation, $(P_{fold}/P_{unfold})_{298K} < (P_{fold}/P_{unfold})_{310K}$ (Table 2). As the applied temperature increases, the relative proportion of folded forms to unfolded ones increases. It may suggest that the low temperature shock can induce the conformational changes from folded forms to unfolded ones. If the folded forms are more thermodynamically accessible than the unfolded ones at elevated temperature, the low temperature shock in *Rhizobium meliloti* may induce an unfolded conformation as a less ordered form. Based on this speculation, the C_{28} fatty acyl component could be involved in regulation of the membrane order by changing its conformation from a folded form to an unfolded one. This adaptation is comparable to that of *Enterobacter aerogenes* in which the less

Table 2. Energy of 10 conformations within 3 kcal/mol of the lowest energy minimum among 141 structures (frames) obtained from the MD simulation

Rank	Frame	ΔE (kcal/mol)	Conformation	H-bond[b]
1	45	0[a]	folded	yes
2	125	0.56	folded	yes
3	26	1.99	unfolded	no
4	87	2.49	unfolded	no
5	15	2.64	folded	yes
6	1	2.69	unfolded	no
7	138	2.76	folded	no
8	58	2.94	unfolded	no
9	120	2.94	folded	no
10	114	2.98	folded	no

[a]The lowest energy value is 1.62 kcal/mol. [b]There are three classes; folded forms with or without hydrogen bond, and unfolded forms.

ordered UFAs are induced against low temperature shock.[4] Since a novel 27-hydroxyoctacosanoic acid in the membrane of *Rhizobium meliloti* was first reported in 1989,[5] its unusual structure induced interesting questions on biochemical functions correlated with legumes-*Rhizobium* symbiosis,[8,9] most of which still remain to be answered. Our present work suggests that conformational changes of an unusual C_{28} fatty acyl component possibly plays an important role in maintaining the optimum fluidity of the membrane of the *Rhizobium meliloti* in response to the low temperature shock.

Lacking direct experimental evidences for this conformational shift at present stage, heavy searching of all the possible conformations over a wide range of temperature by simulated annealing molecular dynamics - minimization strongly pointed out weighted distribution of a folded form relative to an unfolded form. Sterol, inside the animal membrane, acts as a 'fluidity buffer' around the phase transition temperature of lipids with its own conformation.[12] It is a similar interesting case on the membrane adaptation induced without compositional changes of other fatty acyl components against the thermal stress.

Therefore, based on the present results we suggest the conformational changes of an unusual 27-hydroxyoctacosanoic fatty acyl component inside the membrane of *Rhizobium meliloti* may play an important role in the adaptive response of external thermal stress.

Experimental Sections

Thermal stress on the microorganisms.[5,6] Different temperature (25, 37 °C) shocks for 3 h were treated at the late log phase of the *Rhizobium meliloti* 2011 cells grown in broth culture in GMS (Glutamine-Mannitol-Salt) medium at 30 °C. As control bacterial strains *Enterbacter aerogenes* or *E. coli* were used for temperature shift experiments.

Structural analysis of fatty acyl components of membrane. Experiments were performed on harvested whole cells or isolated membrane fractions by treatment with methanolic HCl to prepare fatty acid methyl esters. Cells (1-5 mg) suspended with 0.3 mL of chloroform and 1.5 mL of 5% methanolic HCl solution were sealed in a Teflon-lined screw-capped vial and heated in a water bath or oven at 72 °C for 24h. Chloroform (3 mL) was added every 8h, followed by mild sonication for 5 min. After concentration to dryness under nitrogen gas, samples were partitioned between water and chloroform, and the aqueous layer was washed several times with chloroform or hexane. The combined solutions were filtered through glass wool. Prepared fatty acid methyl esters were subjected to gas chromatography analysis on a 25 M J&W scientific DB1 column using nitrogen as the carrier gas and a temperature program of 50 °C initial temperature, 0.00 min hold time, and 10 °C/min rate to a temperature of 100 °C, and 3 °C/min rate to a temperature of 150 °C. A third ramp of 4 °C/min was then immediately started until the final temperature of 300 °C was obtained. This temperature was held for 30 min. The relative proportion of lipid components were calculated from the integrated peak areas. The fatty acid identification and molecular weight were determined using a GC/MS (Gas Chromatography/Mass Spectrometry) analysis using a JEOL JMS-AX505H spectrometer interfaced with a Hewlett-Packard 5890A gas chromatograph.

Conformational search of 27-hydroxyoctacosanoic acids by simulated annealing molecular dynamics minimization simulation. Conformational search of 27-hydroxyoctacosanoic acid over a wide range of temperature was performed by simulated annealing molecular dynamics full minimization strategy. In the simulated annealing molecular dynamics (MD) simulation, the temperature was changed between 300 K and 1000 K five times at intervals of 50 K. At each temperature, MD simulation was performed for 2.5 ps: 0.5 ps of equilibration phase and 2 ps of production phase. One structure was saved from the end of each production phase. Total MD simulation time was 352.5 ps. Molecular dynamics calculation was performed with DISCOVER program (version 97.0, Molecular Simulations Inc.) using consistent valence force field.[7] No cutoff was imposed on the calculation of non-bonded interactions. NVT MD calculation was performed using the leap-frog algorithm[11] with a 1 fs time step. Temperature was controlled by velocity scaling in equilibration phase and Berendsen algorithm[12] in production phase with a coupling constant of 0.2 ps. Dielectric constant was set to 1. After the MD simulation, all the 141 structures were fully energy-minimized: 100 iterations of steepest descent minimization and conjugate gradient minimization until the maximum derivative reached below 0.001 (typically 10,000-20,000 iterations).

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