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THICK VISCOUS STRUCTURES IN THE LIPID MEMBRANE OF A MODERATELY HALOPHILIC GRAM-NEGATIVE BACTERIUM

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1. Introduction

In a moderately halophilic bacterium, *Pseudomonas halosaccharolytica,* elevation of growth temperature or increase of NaCl concentration in the medium caused the promotion of cyclopropanoic acid formation from the corresponding monounsaturated fatty acids [1,2].

Electron paramagnetic resonance (EPR) may be used to study biological lipid membranes [3]. Near the surface in the lipid bilayer of intact bacterial membrane, lipid orientations are ordered, and rotational movements of lipid in intact cells are more restricted than in isolated membranes and in lipid vesicles [4–6]. We studied the membrane properties of *P. halosaccharolytica* by EPR with stearic acids labeled at the position, 5, 12 or 16 of their carbon chains [7]. The results revealed that the viscous surface regions in the membrane lipid bilayers of this bacterium were unusually thick and uniform to a depth of 12 carbon atoms of stearic acid from the hydrophilic surface. These thick restricted regions may be related to bearing the osmotic pressure at the membranes or to the Na^+ permeability of the membrane in this halophilic bacterium.

2. Materials and methods

A moderately halophilic Gram-negative bacterium, *Pseudomonas halosaccharolytica* ATCC 29429, was incubated in a medium containing 0.5 or 2.0 M NaCl as in [2]. The cells were washed with 50 mM Tris–HCl buffer (pH 7.2) containing the same NaCl concentrations as the growth media. Spin labels for EPR were suspended in the same buffer, containing appropriate concentrations of NaCl, by sonication and added to the washed cells. This suspension was incubated under reciprocal shaking for 0.5–6.0 h at the temperatures used for the growth. NaCl used for the experiments was ultrapure grade (Merck) to avoid the influence of paramagnetic impurities on EPR spectra. Spin labels, *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid, 12-ketostearic acid and 16-ketostearic acid (5 ns, 12 ns and 16 ns) were from Syva Co. (Palo Alto, CA). The EPR spectra were observed using a Hitachi 771 ESR spectrometer at various temperatures (10–50°C).

The order parameter can be expressed in terms of the observed hyperfine splittings of EPR spectra by an equation [7]:

$$S = f_a (A_{/\!/} - A_\perp)/(A_z - A_x) \quad (1)$$

where f_a is given as:

$$f_a = (A_x + A_y + A_z)/(2A_\perp + A_{/\!/}) \quad (2)$$

$A_\perp$ and $A_{/\!/}$ are the hyperfine splittings of the observed spectra and A_x, A_y and A_z are the principal values of hyperfine tensor (5.8, 5.8, 29.8 G, respectively). The rotational correlation time is given by [7]:

$$\tau_c = 3.418 \times 10^{-10}\ \Delta(0) \left\{ \sqrt{\frac{h(0)}{h(-1)}} - \sqrt{\frac{h(0)}{h(1)}} \right\} \quad (3)$$

where Δ(0) is the peak-to-peak width of the central

line in G and $h(m)$ is the peak height of the line belonging to nuclear quantum number m. The activation energy E_{vis} of rotational viscosity is given by Andrade's equation [8] for viscosity, η:

$$\eta = B \exp (E_{vis}/RT) \quad (4)$$

The viscosity is given for a spherical particle of radius r from Stokes' law by:

$$\tau_c = 4\pi\eta r^3/kT \quad (5)$$

On the other hand, the theory of rate processes shows that B in eq. (4) is a function of temperature, T, ($B \propto T^{1/2}$) [9] and the relation between τ_c and E_{vis} is given by:

$$\ln(\tau_c/T^{1/2}) = C + E_{vis}/RT \quad (6)$$

Then E_{vis} may be given by plotting $\ln(\tau_c/T^{1/2})$ against $1/T$.

3. Results and discussion

EPR spectra of the spin-labeled, moderately halophilic bacterium revealed that the spin probes were incorporated preferentially into the lipid layer of the cell wall of this bacterium, because the order parameters of this bacterium were not similar values to those of cytoplasmic membranes but of outer membranes of *Escherichia coli* [10]. The EPR spectra of spin label 5 ns had two clearly separated parallel and perpendicular hyperfine splittings, while the spectra of 12 ns and 16 ns were imperfectly separated (fig.1). The order parameters decreased slightly as elevating the measuring temperature (fig.2). The relations between correlation time and measuring temperature, and $\log(\tau_c/T^{1/2})$ and $1/T$ are shown in fig.3,4. Each correlation time of 5 ns and 12 ns gave similar value and changed similarly with measuring temperatures for this bacterium of the same growth conditions. Both correlation time curves of 5 ns and 12 ns in fig.3,4 had break points where the lipid phase altered from solid to liquid and those break point temperatures just coincided with their respective

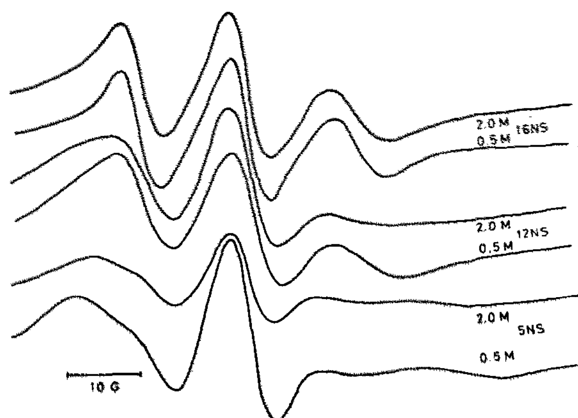

Fig.1. EPR spectra of spin labels, 5 ns, 12 ns and 16 ns, in the cell walls of *P. halosaccharolytica*. Growth and measuring temperatures were 30°C and 27°C, respectively. The media contained 2.0 or 0.5 M NaCl.

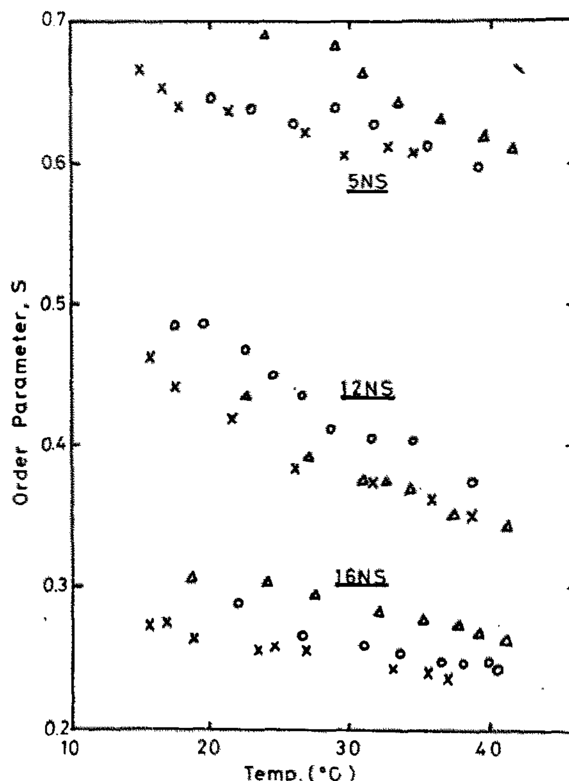

Fig.2. Relations between order parameter, S and measuring temperature. Spin labels were 5 ns, 12 ns and 16 ns. The organisms were grown in 2.0 M NaCl medium at temperatures, 20°C (X), 28°C (o) or 35°C (Δ).

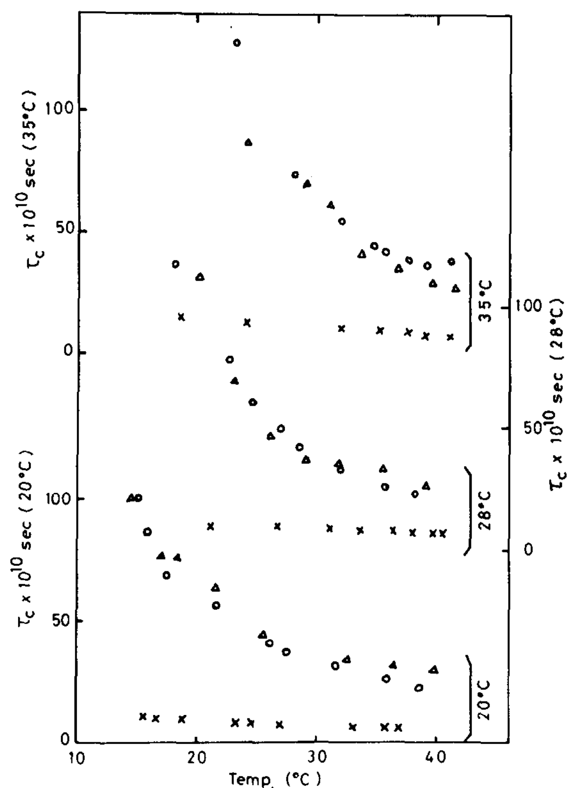

Fig.3. Correlation time–temperature relations. Growth temperatures were 20°C, 28°C and 35°C. All medium contained 2.0 M NaCl. Spin labels were 5 ns (Δ), 12 ns (○) and 16 ns (X).

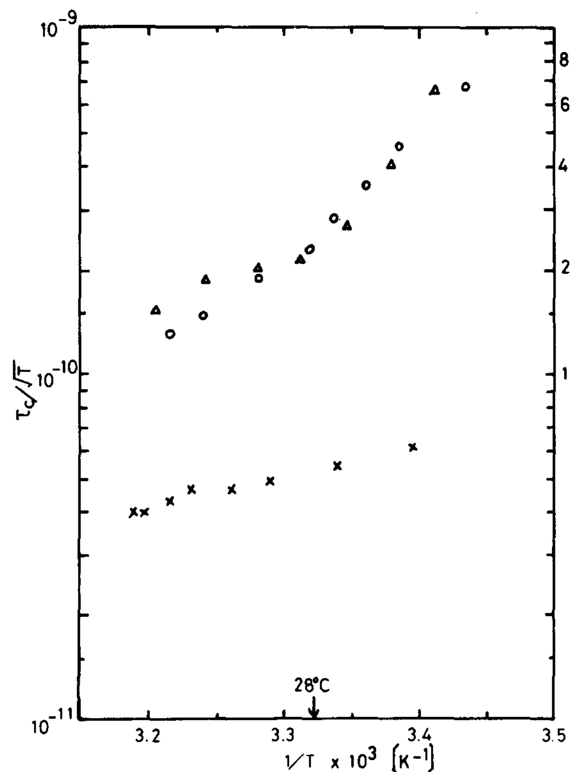

Fig.4. Relations between $\log(\tau_c/T^{1/2})$ and $1/T$. Growth temperatures were 28°C and growth media contained 2.0 M NaCl. Spin labels were 5 ns (Δ), 12 ns (○) and 16 ns (X).

growth temperatures [11]. On the contrary, it is noticed that the correlation time of 16 ns was dissimilar to those of 5 ns and 12 ns and had no break point irrespective of the same growth conditions.

Because of the difficulty to compare the correlation times of spin labels in this bacterial membrane of different growth conditions, we used the activation energy of rotational microviscosity as a marker instead of the correlation times. The activation energies of this bacterium were similar to those of various biological membranes [12]. The probes, 5 ns, 12 ns and 16 ns feel the atmospheres at the depths of 5, 12 and 16 carbon atoms of stearic acid, respectively. From table 1, the activation energies for 5 ns and 12 ns were 15–20 kcal/mol at the lower temperature and 7–10 kcal/mol at the higher temperature, indicating again that the outer region in the lipid layer would have an uniform viscosity to a depth of 12 carbon atoms and everywhere in this viscous region the lipid phase would change simultaneously from solid to liquid at its transition temperature. On the contrary, the activation energies for 16 ns were 4–7 kcal/mol and at the depth of 16 carbon atoms in the membrane the lipid phase would remain always liquid at all the measuring temperatures, because the 16 ns curves had no break points (fig.3,4).

In intact cell membranes and isolated cell membranes order parameters of 5 ns are only a little larger than those of 12 ns as described [5,10] (e.g. 5 ns, 0.75; 12 ns, 0.69), while in lipid vesicles the former are considerably larger than the latter as reported [13,14] (5 ns, 0.54; 12 ns, 0.26), whereas the order parameters of 5 ns and 12 ns in the intact cells of this bacterium were 0.6–0.7 and 0.3–0.5, respectively, indicating that the lipid orientations in this cell mem-

Table 1
Rotational activation energies (kcal/mol) determined by correlation times of spin labels in the lipid bilayer of *P. halosaccharolytica*

NaCl (M) in growth medium	Growth temp. (°C)	Spin labels					
		5 ns		12 ns		16 ns	
		LP	SP	LP	SP	LP	SP[a]
0.5	20	9.8	17.2	9.5	25.4	5.5	–
	30	10.3	17.5	7.7	14.1	7.2	–
2.0	20	7.9	15.3	8.6	14.9	5.5	–
	28	5.4	25.9	10.6	17.9	4.2	–
	35	10.6	16.1	11.2	18.1	4.6	–
0.5[b]	20	6.4	15.5	4.0	10.6	6.4	–

[a] LP and SP are liquid and solid phase values, respectively
[b] Results of sonicated liposomes of lipids extracted from this bacterium

brane would be rather random. Thus, the order of lipid orientation in the bilayer of this bacterium would decrease rapidly with increasing the depth in the membrane and finally at the central region of membranes the hydrocarbon chains would orient almost randomly.

The only effect of NaCl concentrations on the membrane properties was that the correlation time of 12 ns was as large as that of 5 ns for this bacterium grown in 2 M NaCl, whereas 12 ns was ~ 50% of 5 ns in 0.5 M NaCl bacterium. This may reflect the differences of cyclopropanoic acid contents. Thus the order parameter decreased rapidly as increasing the depth, while the microviscosity did not decrease to ~ 12 carbon atoms of stearic acid. Similar properties were also found in the EPR studies of sonicated liposomes of lipids extracted from this bacterium (table 1).

It was concluded that the lipid membranes of this bacterium would be composed of 3 regions, 2 viscous outer regions and 1 fluid central region forming a sandwich-like membrane. Since the average length of the lipid carbon chains of this bacterium was ~ 18 [2], the thicknesses of 2 outer regions and a central region were calculated to be equivalent to ~ 12 in hydrocarbon chains. If we consider that the double bond and cyclopropane ring are mainly located at 11–12 carbon atoms, border surface between outer and central regions may lay just a little inside of 11–12 carbon atoms. As high rotational correlation times were maintained in the viscous outer regions, the short range orientations should be preserved everywhere in this region, while the order parameters decreased rapidly as increasing the depth from the hydrophilic surface.

Though it has not been clarified that these thick viscous regions in membrane lipid layers of this bacterium were indispensable to its Na^+ permeability, not only cyclopropanoic acids [15] but also such thick viscous, restricted regions in the membranes would be necessary for this bacterium to grow in high NaCl concentration medium.

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