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Effect of Fatty Acids on the Membrane Potential of an Alkaliphilic *Bacillus*

Hisami Furusawa, Noriyuki Koyama

Department of Chemistry, Faculty of Science, Chiba University, Yayoi, Chiba 263-8522, Japan

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Abstract. Effect of various fatty acids on the membrane potential of an alkaliphilic *Bacillus*, YN-2000, was examined. Addition of unsaturated fatty acids such as palmitoleic acid, oleic acid, linoleic acid, and linolenic acid at 30 μM caused the instantaneous depolarization of the membrane potential of the bacterium, which appears to result in the drastic decrease of viability. On the other hand, no depolarization was detected by the addition of saturated acids such as palmitic acid, stearic acid, and 12-hydroxystearic acid even at 1 mM.

It has been suggested that unsaturated fatty acids exhibit the inhibitory effect on the growth of various bacteria [9, 10]. Remarkable decreases of viability and morphological changes such as loss of cell shape and disruption of cell membranes have been induced in several bacteria by incubation with unsaturated fatty acids [2, 3, 4]. These facts are based on the results obtained by the exposure of bacteria to unsaturated fatty acids for 1 h or longer duration. In *Helicobacter pylori*, for instance, it has been suggested that incubation with oleic acid and linoleic acid for 24 and 48 h led to alteration of the phospholipid composition of the bacterium, which was related to the membrane destruction [3].

In this study, it was found that unsaturated fatty acids caused an instantaneous depolarization of the membrane potential of an alkaliphilic *Bacillus*, YN-2000, which appears to result in the drastic decrease of viability. The toxicity of unsaturated fatty acids for the bacterium seems to be induced immediately after the incubation.

Materials and Methods

Culture and preparation of the membrane vesicles. The alkaliphilic *Bacillus*, YN-2000, was aerobically grown as described previously [6]. The cells were collected at the late logarithmic phase, washed, and suspended in 20 mM Tris-HCl buffer (pH 8.2) containing 150 mM NaCl, 0.5 mM $MgCl_2$, and 0.2 M sucrose.

From freshly cultured cells, the right-side-out membrane vesicles were prepared according to the procedure described previously [6, 11] and were suspended in 20 mM phosphate buffer (pH 8.2) containing 150 mM NaCl and 1 mM $MgCl_2$.

Correspondence to: N. Koyama; *email:* nkoyama@scichem.s.chiba-u.ac.jp

Fluorescence measurement. The membrane potential across membranes of whole cells or the membrane vesicles was monitored by the change of fluorescence of rhodamine 6G, which was measured with excitation at 520 nm and emission at 550 mm as described previously [5–7].

The assay for whole cells was conducted at 25°C by adding 50 μL of the cell suspension (approx. 13 mg/mL) to 2 mL of 20 mM H_3PO_4-Tris buffer (pH 9) containing 150 mM NaCl, 1 mM $MgCl_2$, 5 mM glucose, 0.2 M sucrose, and 1 μM rhodamine 6G.

As for the membrane vesicles, the assay was conducted at 25°C in 20 mM Tris-HCl buffer (pH 8.5) containing 100 mM NaCl and 0.5 mM $MgCl_2$. To 2 mL of the buffer containing 1 μM rhodamine 6G, 50 μL of the membrane vesicles (approx. 1 mg) was added. After 2 min of incubation, 1.2 mM ascorbate and 0.03 mM tetramethylphenylenediamine (TMPD) were added as energy source for the generation of the membrane potential.

Assay of viability. Various concentrations of fatty acids were added to each bacterial suspension in the growth medium (approx. 2×10^5 CFU/mL). After 30 s of incubation at room temperature, an aliquot (50 μL) of the suspension was transferred to 2 mL of new growth medium to dilute the fatty acid, and then 100 μL of the suspension was spread on the agar plate, the composition of which was the same as that of the growth medium. The plate was incubated at 37°C overnight, and the number of colonies was counted. Viability was estimated by the ratio of colony forming units of the treated cells to those of the cells without treatment as a control.

Protein determination. Protein concentration was determined according to the method of Lowry et al. [8] and Gornall et al. [1] with bovine serum albumin as the standard.

Results and Discussion

When the bacterium YN-2000 was added to the reaction mixture containing rhodamine 6G, fluorescence of the

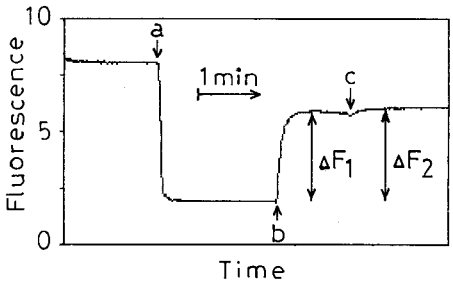

Fig. 1. Effect of oleic acid on the membrane potential of YN-2000. To the reaction mixture containing rhodamine 6G, 50 μL of the cells, 30 μM oleic acid, and 0.1 μM gramicidin A were added at the times indicated by the arrows a, b and c, respectively. Fluorescence intensity as the ordinate is expressed by an arbitrary unit.

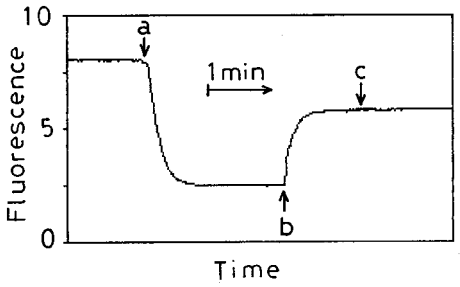

Fig. 2. Effect of oleic acid on the membrane potential of the membrane vesicles of YN-2000. To the reaction mixture containing the membrane vesicles and rhodamine 6G, ascorbate plus TMPD, 30 μM oleic acid, and 0.1 μM gramicidin A were added at the times indicated by the arrows a, b and c, respectively. Fluorescence intensity as ordinate is expressed by the arbitrary unit.

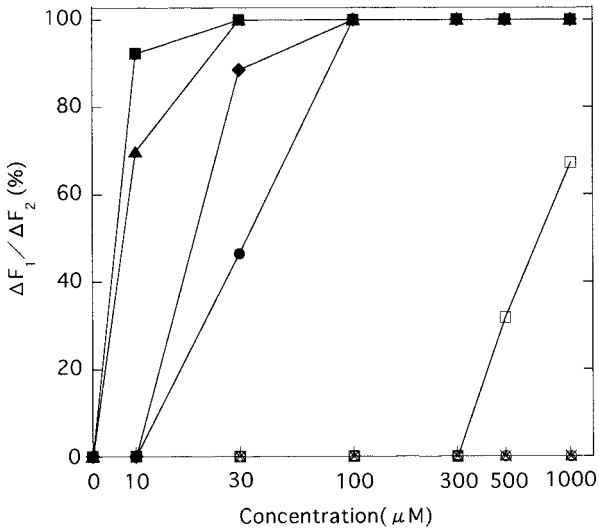

Fig. 3. Concentration effect of various fatty acids on the membrane potential of YN-2000. After the fluorescence quenching of rhodamine 6G in the reaction mixture containing the cells as observed in Fig. 1, the increases of fluorescence intensity were measured by the addition of palmitic acid (○), palmitoleic acid (●), stearic acid (△), 12-hydroxystearic acid (×), oleic acid (▲), ricinoleic acid (□), linoleic acid (■), linolenic acid (◆), followed by the addition of gramicidine A. The extent of depolarization of the Δψ is expressed by $\Delta F_1/\Delta F_2$, where ΔF_1 and ΔF_2 were increases of the fluorescence intensity by the additions of fatty acids and gramicidin A, respectively, as shown in Fig.1. Values are means from at least three replicate experiments. Standard deviation was less than 6%.

dye was rapidly quenched, which is ascribed to the membrane potential (Δψ; negative, inside) [6, 8, 9]. Upon addition of 30 μM oleic acid, unsaturated fatty acid (C18:1), the fluorescence intensity instantaneously increased and reached a stationary level within 30 s, suggesting the depolarization of the Δψ of the cells (Fig. 1). Further increase of fluorescence was not induced by the addition of gramicidin A, a sodium ionophore [5, 7]. This fact suggests that the addition of 30 μM oleic acid caused a complete dissipation of the Δψ of the cells.

To confirm the depolarization effect of oleic acid, we examined whether the Δψ of the membrane vesicles of YN-2000 was affected by oleic acid. When ascorbate and TMPD as energy source were added to the reaction mixture containing the membrane vesicles and rhodamine 6 G, the rapid quenching of the fluorescence was also observed, reflecting the generation of the Δψ (negative, inside) (Fig. 2). The fluorescence was increased immediately after the addition of 30 μM oleic acid and reached a stationary level within 30 s. Addition of gramicidin A caused no more change of the fluorescence level. This strongly supports that the fluorescence quenching observed in the whole cells by the addition of oleic acid resulted from the depolarization of the Δψ.

Effect of various fatty acids on the Δψ of YN-2000 was examined at different concentrations (Fig. 3). Addition of unsaturated fatty acids such as palmitoleic acid (C16:1), oleic acid, linoleic acid (C18:2), and linolenic acid (C18:3) completely depolarized the Δψ at 100 μM. Oleic acid and linoleic acid were effective even at 10 μM. However, no depolarization was detected by saturated fatty acids such as palmitic acid (C16:0), stearic acid (C18:0), and 12-hydroxystearic acid even at 1 mM. It is noteworthy that ricinoleic acid (C18:1:12-hydroxy) caused no depolarization at a concentration lower than 300 μM.

It has been suggested that the exposure of bacteria to unsaturated fatty acids for 1 h caused a drastic decrease of viability [2, 4]. Since the Δψ was depolarized immediately after the addition of unsaturated fatty acids, we examined whether a short incubation of YN-2000 with fatty acids affected the viability (Fig. 4). When the bacterium was incubated for 30 s with unsaturated fatty acids such as palmitoleic acid, oleic acid, linoleic acid, and linolenic acid at 30 μM, the viability was almost completely lost. Ricinoleic acid, which exhibited a low

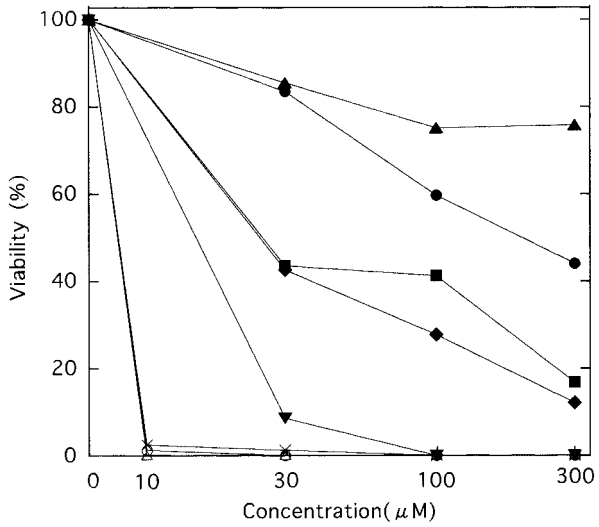

Fig. 4. Concentration effect of various fatty acids on the viability of YN-2000. The bacterium was treated with the indicated concentrations of palmitic acid (●), palmitoleic acid (○), stearic acid (▲), 12-hydroxystearic acid (■), oleic acid (△), ricinoleic acid (◆), linoleic acid (▼), and linolenic acid (×), respectively. The relative viability is expressed by the ratio of colony forming units of the treated cells to those of the cells without treatment as a control. Values are means from at least three replicate experiments. Standard deviation was less than 8%.

depolarization effect, decreased viability at 30 μM by only 40% of the control. The result suggests that depolarization of the $\Delta\psi$ by unsaturated fatty acids results in the loss of viability. Although saturated fatty acid such as palmitic acid, stearic acid, and 12-hydroxystearic acid caused no depolarization at 1 mM, they caused significant decreases of viability even at 30 μM. Thus, the decrease of viability appears to result not only from the depolarization of the $\Delta\psi$, but also from other unknown reasons. One possible explanation might be the function of fatty acids as proton conductors.

An instantaneous depolarization of the $\Delta\psi$ of the whole cells and membrane vesicles by unsaturated fatty acids may suggest that the effect is ascribed to the insertion of an unsaturated carbon chain to the lipid layer of the membrane. For the existence of a 12-hydroxy group, ricinoleic acid might be difficult to insert the full length of carbon chain into the hydrophobic lipid layer, which resulted in a much lower effect as compared with oleic acid. Unsaturated fatty acids are bent and less flexible because of the double bond and exhibit much lower melting points when compared with saturated fatty acids. It is possible that the insertion of unsaturated fatty acids makes the membrane leaky by producing a distortion or affecting its fluidity, although further study will be needed to confirm this.

Literature Cited

1. Gornall AG, Bardwill CS, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 179:751–766
2. Hazell SL, Graham DY (1990) Unsaturated fatty acids and viability of *Helicobacter* (*Campylobacter*) *pylori*. J Clin Microbiol 28: 1060–1061
3. Khulusi S, Ahmed HA, Patel P, Mendall MA, Northfield TC (1995) The effect of unsaturated fatty acids on *Helicobacter pylori in vitro.* J Med Microbiol 42:276–282
4. Knapp HR, Melly MA (1986) Bactericidal effects of polyunsaturated fatty acids. J Infect Dis 154:84–94
5. Koyama N (1989) Na^+-dependence of the stability under alkaline conditions of the membrane of an alkaliphilic *Bacillus.* FEMS Microbiol Lett 61:7–10
6. Koyama N, Nosoh Y (1985) Effect of potassium and sodium ions on the cytoplasmic pH of an alkaliphilic *Bacillus.* Biochim Biophys Acta 812:206–212
7. Koyama N, Wakabayashi K, Nosoh Y (1987) Effect of K^+ on the membrane functions of an alkaliphilic *Bacillus.* Biochim Biophys Acta 898:293–298
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
9. Maczulak AE, Dehority B., Palmquist DL (1981) Effects of long-chain fatty acids on growth of rumen bacteria. Appl Environ Microbiol 42:856–862
10. Thompson L, Cockayne A, Spiller RC (1994) Inhibitory effect of polyunsaturated fatty acids on the growth of *Helicobacter pylori:* a possible explanation of the effect of diet on peptic ulceration. Gut 35:1557–1561
11. Wakabayashi K, Koyama N, Nosoh Y (1988) Leucine transport system in a facultatively alkalophilic *Bacillus.* Arch Biochem Biophys 262:19–26