Systematic Analysis of Selective Bactericidal Activity of Fatty Acids against Staphylococcus aureus with Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

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Abstract: Bacterial flora on the skin surface contains Staphylococcus aureus (S. aureus) and Staphylococcus epidermidis (S. epidermidis) which causes rough skin and atopic dermatitis and enhances innate immunity, respectively. In this study, minimum inhibitory concentration (MIC) was evaluated for six saturated fatty acids and two unsaturated fatty acids against S. aureus and S. epidermidis. The antimicrobial behavior in the liquid medium was categorized into three groups. The first was the selective antibacterial activity group comprising myristic acid (C14:0 fatty acid), palmitoleic acid (C16:1 fatty acid), and oleic acid (C18:1 fatty acid) and preferentially displayed antimicrobial activity for S. aureus (group 1). C16:1 fatty acid displayed high antimicrobial activity only for S. aureus. The second was the non-selective antibacterial activity group which displayed antibacterial activity for both Staphylococci (group 2). Caprylic acid (C8:0 fatty acid), capric acid (C10:0 fatty acid), and lauric acid (C12:0 fatty acid) comprised group 2. The third was the non-antibacterial activity group which did not show significant antimicrobial activity (group 3). Bactericidal activities were confirmed for C12:0 fatty acid and C16:1 fatty acid by evaluating the minimum bactericidal concentration (MBC) on the agar medium. C12:0 fatty acid displayed non-selective bactericidal behavior against S. aureus and S. epidermidis when the fatty acid concentration was above 250 μg mL⁻¹. These findings suggest that C16:1 fatty acid has the potential to be used as a detergent in skin care and medical products because it can selectively kill only S. aureus.

Key words: selective bactericidal activity, fatty acid, Staphylococcus, MIC, MBC

1 INTRODUCTION

Bacteria live on human skin surface and play crucial roles in various skin conditions. The microbiota on healthy skin surfaces contains many kinds of bacteria, such as Corynebacterium, Staphylococcus, and Propionibacteriaceae. Staphylococcal bacteria, including Staphylococcus aureus (S. aureus) and Staphylococcus epidermidis (S. epidermidis) are present in the nasal cavity or sebaceous glands. S. aureus causes inflammation of atopic dry skin as well as skin roughness and food poisoning. In contrast, S. epidermidis enhances the innate barrier immunity. Therefore, many dermatologists and cosmetic chemists have focused on the harmful (S. aureus) and beneficial bacteria flora (S. epidermidis) for the development of bactericidal agents to control the floral composition.

Fatty acids and their derivatives demonstrate selective bactericidal activities. Nakatsuji et al. found that lauric acid (C12:0 fatty acid) shows a stronger bactericidal activity against S. aureus than S. epidermidis. Chao-Hsuan et al. also reported that oleic acid (C18:1 fatty acid) has bactericidal activities that preferentially kill S. aureus over other bacteria. Moran et al. showed that sapienic acid has selective antibacterial activity against S. aureus. Additionally, we have reported the bactericidal phenomenon...
of various fatty acids\textsuperscript{16–19}. In particular, palmitoleic acid metal salt killed \textit{S. aureus} and \textit{Propionibacterium acnes} which cause rough skin and acne; however, it did not kill \textit{S. epidermidis} which keeps the epidermis healthy.

Minimum inhibitory concentration (MIC), which is the minimum drug concentration to suppress the growth of bacteria is widely used to demonstrate antibacterial activity. However, there are few reports on the evaluation of MIC of fatty acids against \textit{S. aureus} and \textit{S. epidermidis} under the same conditions. In this study, MICs of C8:0~C18:1 fatty acids were systematically evaluated. The minimum bactericidal concentration (MBC), which is the minimum drug concentration for killing all bacteria were evaluated for C16:1 and C12:0 fatty acids.

2 EXPERIMENTAL

2.1 Materials

Caprylic acid (C8:0 fatty acid, CH\(_3\)(CH\(_2\))\(_7\)COOH, ≥ 98\%), capric acid (C10:0 fatty acid, CH\(_3\)(CH\(_2\))\(_9\)COOH, ≥ 99\%), lauric acid (C12:0 fatty acid, CH\(_3\)(CH\(_2\))\(_11\)COOH, ≥ 99\%), palmitoleic acid (C16:1 fatty acid, CH\(_3\)(CH\(_2\))\(_{15}\)CH=CH(CH\(_2\))\(_{7}\)COOH, 99\%), stearic acid (C18:0 fatty acid, CH\(_3\)(CH\(_2\))\(_{16}\)COOH, 99\%), oleic acid (C18:1 fatty acid, CH\(_3\)(CH\(_2\))\(_{17}\)CH=CH(CH\(_2\))\(_{7}\)COOH, ≥ 99\%), and 0.1 mol L\(^{-1}\) phosphate buffer solution (pH 6) were purchased from Fujifilm Wako Pure Chemical Industries Ltd. (Osaka, Japan). Myristic acid (C14:0 fatty acid, CH\(_3\)(CH\(_2\))\(_{14}\)COOH, ≥ 99\%) and palmitic acid (C16:0 fatty acid, CH\(_3\)(CH\(_2\))\(_{16}\)COOH, ≥ 99\%) were purchased from Sigma-Aldrich Co. LLC (St. Louis, USA). Water was purified using a Demi-Ace Model DX-15 demineralizer (Kurita Water Industries Ltd., Tokyo, Japan). Ethanol was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Beef extract was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Polypeptide was purchased from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan). Sodium chloride was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Agar powder was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). \textit{S. aureus} (NBRC13276) and \textit{S. epidermidis} (NBRC12993) were obtained from National Institute of Technology and Evaluation (Tokyo, Japan).

2.2 Evaluation of antibacterial activities and bactericidal activities of fatty acids against \textit{S. aureus} and \textit{S. epidermidis}

Before cultivation of \textit{S. aureus} and \textit{S. epidermidis} and evaluation of antibacterial activities, all media and buffer were sterilized in an autoclave at 121°C for 20 min. The medium for cultivation contained beef extract (0.15 g), polypeptide (0.30 g), sodium chloride (0.15 g) and water (30 mL). The cultures of \textit{S. aureus} and \textit{S. epidermidis} were prepared by shaking 30 mL of the medium containing one colony for 24 h at 37°C (145 rpm).

MIC was defined as the minimum concentration at which the turbidity did not increase when the fatty acid was added to the bacterial dispersion. The fatty acid ethanol solutions (400 μg mL\(^{-1}\)) were diluted to prepare sample solutions (3.13~350 μg mL\(^{-1}\)). MIC was not evaluated above 400 μg mL\(^{-1}\) because fatty acid crystals precipitated in the medium.

To prepare the sample for the MIC evaluation, 1000 μL of liquid medium containing beef extract (0.01 g), polypeptide (0.02 g), and sodium chloride (0.01 g), 940 μL of phosphate buffer (pH 6), 40 μL of fatty acid ethanol solution and 20 μL of bacterial dispersion (3 × 10\(^{6}\)~2 × 10\(^{8}\) CFU mL\(^{-1}\)) were mixed in a test tube. The optical density of the prepared sample (100 μL) was evaluated by a 96-well True Line Cell Culture Plate (Japan Genetics Co., Ltd., Tokyo, Japan) and an absorption grating microplate reader SH-1200 Lab (Corona Electric Co., Ltd., Ibaraki, Japan). We confirmed that 2wt% of ethanol does not affect bacterial growth in preliminary tests. The evaluated conditions were as follows: wavelength = 660 nm and number of flashes = 10. The \textit{S. aureus} (\textit{S. epidermidis}) medium was shaken at 1000 rpm by a microplate shaker PSU-2T (Waken B Tech Co., Ltd., Kyoto, Japan) during incubation for 24 (48) h at 37°C.

Subsequently, 100 μL of transparent sample medium after antibacterial evaluation was smeared on agar medium or 48 h at 37°C. The turbidity did not increase when the fatty acid contained palmitoleic acid and \textit{S. aureus} containing palmitoleic acid and \textit{S. epidermidis} at 37°C. The MBC was defined as the concentration at which no growth of bacteria was observed on all agar medium. The medium was prepared so that the final concentrations of fatty acid were 18.8~400 μg mL\(^{-1}\).

3 RESULTS and DISCUSSION

The optical density at 660 nm (OD\(_{660}\)) was measured after the liquid medium including \textit{S. aureus} or \textit{S. epidermidis} was added to the various fatty acids and incubated for 24 or 48 h at 37°C. The turbidity did not increase when the concentration of some fatty acids was above a critical concentration, while the liquid medium became turbid if the medium did not contain a fatty acid. Figures 1(a) and (b) display the temporal change in turbidity of liquid media containing palmitoleic acid and \textit{S. aureus} (or \textit{S. epidermidis}). In the case of \textit{S. aureus}, the turbidity gradually increased to about 1.0 when the fatty acid concentration was less than 18.8 µg mL\(^{-1}\). In contrast, the turbidity increased for all palmitoleic acid (C16:1 fatty acid) concentrations for \textit{S. epidermidis}. These results indicate that the MIC is 18.8 µg mL\(^{-1}\) for \textit{S. aureus} and >400 µg mL\(^{-1}\) for \textit{S. epidermidis}. Figures 1(c) and (d) show the turbidity of liquid
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media containing lauric acid and *S. aureus* or *S. epidermidis*. The turbidity increased in the 12 to 24 (48) hours period when the fatty acid concentration was less than 18.8 μg mL⁻¹. These results indicate that MIC of lauric acid is 18.8 μg mL⁻¹ for both *Staphylococci*.

Figure 2 shows MIC of eight fatty acids against *S. aureus* and *S. epidermidis*. According to the systematic evaluation, the fatty acids which exhibited significant antibacterial activity for *S. aureus* were C8:0, C10:0, C12:0, C14:0, C16:1, and C18:1. In contrast, fatty acids exhibiting antibacterial activity against *S. epidermidis* were C8:0, C10:0, and C12:0 and their MIC was 18.8 to 400 μg mL⁻¹. These 8 fatty acids were classified into 3 groups based on their antibacterial activity. The first group is the selective antibacterial activity group in which C14:0, C16:1, and C18:1 fatty acids inhibited *S. aureus* growth (group 1). Interestingly, C16:1 fatty acid exhibited high antimicrobial activity only for *S. aureus*. The second is the non-selective antibacterial activity group in which fatty acids inhibited the growth of both *Staphylococci* (group 2). For C8:0, C10:0, and C12:0 fatty acids, MIC was almost the same for both *Staphylococci*. The third group is the non-antibacterial active group that did not significantly inhibit bacterial growth (group 3). The C16:0 and C18:0 fatty acids did not inhibit growth against *S. aureus* and *S. epidermidis* in the range of ≤ 400 μg mL⁻¹. These results reveal that MIC of saturated fatty acids decreases as the alkyl chain length increases and attains the minimum value at C12:0 fatty acid. However, only C12:0 fatty acids displayed no selectiv-

**Fig. 1** Inhibitory effects of bacterial growth. (a) C16:1 fatty acid; *S. aureus*, (b) C16:1 fatty acid; *S. epidermidis*, (c) C12:0 fatty acid; *S. aureus*, (d) C12:0 fatty acid; *S. epidermidis*. Symbols are as follows: OD₆₆₀ at 3 h[( ], 6 h[○ ], 12 h[● ], 24 (or 48) h[□ ], after addition of *S. aureus* (or *S. epidermidis*) dispersion. Gray-zone suggests the turbidity at 0 h when fatty acid was not added in the medium.
were dispersed, was smeared on agar and incubated at 37°C for 24 hours. In addition, this liquid medium contained 18.8 – 400 μg mL⁻¹ of fatty acids. This concentration was above MIC. When the bacterial fluid containing C16:1 fatty acid was smeared, S. epidermidis grew under all conditions, but S. aureus did not grow at a concentration of 37.5 μg mL⁻¹ and above. The bactericidal behavior of the C12:0 fatty acid was similar to the C16:1 fatty acid: S. aureus did not grow at ≥ 37.5 μg mL⁻¹ and S. epidermidis growth was inhibited at ≥ 250 μg mL⁻¹. Both C16:1 and C12:0 fatty acids exhibited selective bactericidal activity against S. aureus, although C12:0 fatty acid killed both S. aureus and S. epidermidis non-selectively under high concentration.

We demonstrated that the antibacterial activity is enhanced as the alkyl chain length increases. In addition, the maximum antibacterial activity was achieved at C12:0 fatty acid. These results suggest that the significant biological activity is observed when the fatty acid has the suitable alkyl chain. The C16:0 fatty acid did not exhibit bactericidal activity while the C16:1 fatty acid displayed the greatest activity. Such dependency of the bactericidal activity in this group. MIC for S. aureus depends on the alkyl chain length and shows the lowest value if the surfactants contains C12:0 alkyl chain[22–24]. Tamura et al. predicted that penetration of fatty acid into the cell membrane occurs when the lipid solubility is suitable for cell surface hydrophobicity of bacteria[25]. Maeda et al. demonstrated that the bactericidal and bacteriostatic activities of alkyl pyridinium iodide against Escherichia coli are related with the hydrophobicity of the drug molecule[26]. In addition, the minimum cell destruction concentration (minimum vesicular concentration) at which cell membranes are destroyed and vesicles are formed is related to the length of the alkyl group of pyridinium iodide. Some experimental data of quartz crystal microbalance and fluorescence microscopy suggested that the addition of C12:0 fatty acid induce the deformation and destruction of lipid membranes[27,28].

The selectivity of bactericidal activity is interesting and useful for designing cosmetic and medical products. Although the mechanism is still unclear, related phenomena have been previously reported. For example, Moran et al. reported that membrane polarization, which is induced by the addition of fatty acid, causes the antibacterial activity[25,26]. The polarization is inhibited by the production of ammonia for resistance against fatty acid. The production pathway is different between these two Staphylococci. S. aureus has a defense mechanism characterized by the synthesis of staphyloxanthin which contributes to membrane stabilization and the upregulation of genes causing a reduction against acid stress[29,31]. These findings suggest that elucidating the difference in defense mechanisms of Staphylococci against fatty acids will lead to better understanding of selective bactericidal activities.

### 5 CONCLUSION

The antimicrobial behavior of C8 – 18 fatty acids was divided into 3 groups as a result of systematic analysis of MIC. The first group (selective antibacterial activity) made up of C14:0, C16:1, and C18:1 fatty acids exhibited preferential antimicrobial activity for S. aureus (group 1). The C16:1 fatty acid displayed high antimicrobial activity (MIC = 18.8 μg mL⁻¹) only for S. aureus. The second is the non-selective antibacterial activity group which exhibited antibacterial activity for S. aureus and S. epidermidis (group 2). C8:0, C10:0, and C12:0 saturated fatty acids belong to this group. MIC of C12:0 fatty acid, which showed the maximum antibacterial activity against Staphylococci was 18.8 μg mL⁻¹. The third is the non-antibacterial activity group which did not show clear antimicrobial activity (group 3). Additionally, bactericidal activity was evaluated using MBC revealed that C16:1 fatty acid selectively sterilized only S. aureus (MBC = 37.5 μg mL⁻¹). C12:0 fatty acid displayed selective bactericidal activity for S. aureus; however, it showed non-selective bactericidal activity at a concentration of ≥ 250 μg mL⁻¹. These findings suggest that C16:1 fatty acid has the potential to be used as a detergent for skin care and medical products because it can selectively sterilize only S. aureus.
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