ACCESS MICROBIOLOGY

RESEARCH ARTICLE

Nakase *et al.*, *Access Microbiology* 2022;4:000397

DOI 10.1099/acmi.0.000397

MICROBIOLOGY SOCIETY

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Development of skin sebum medium and inhibition of lipase activity in *Cutibacterium acnes* by oleic acid

Keisuke Nakase*, Misato Momose, Tomoko Yukawa and Hidemasa Nakaminami

Abstract

Cutibacterium acnes is associated with the exacerbated inflammation of acne vulgaris, which occurs through the immune induction and pathogenicity factor production. Sebum, which is not present in the growth medium currently used to study acne, is present in acne pustules in differing concentrations among the pathological stages, such as the initial formation and inflammatory phase. To evaluate the effect of *C. acnes* on inflammation exacerbation in acne pustules *in vitro*, we developed an skin sebum medium containing artificial sebum and studied the growth and pathogenicity factor production of *C. acnes* in the skin sebum medium.

The growth and lipase activity of *C. acnes* ATCC11828 were tested using skin sebum medium containing different sebum concentrations. Only lipase activity decreased in the skin sebum medium culture containing 0.5% sebum when compared with that without sebum, while both growth and lipase activity decreased in cultures with 1.0% sebum. Therefore, the growth and lipase activity of *C. acnes* changed in the presence of sebum. Furthermore, when the growth and lipase activity of *C. acnes* were tested in skin sebum medium containing sebum components, unsaturated fatty acids, such as oleic acid and triolein, led to a decrease in lipase activity without inducing a change in growth. In the presence of oleic acid, *C. acnes* lipase activity decreased noncompetitively in a concentration-dependent manner.

Our data showed that *C. acnes* growth and lipase activity changed upon sebum addition to the skin sebum medium, and acne inflammation caused by *C. acnes* needs to be studied under conditions similar to those in acne pustules.

INTRODUCTION

Cutibacterium acnes, a skin bacterium, exacerbates acne vulgaris. *C. acnes*, which overgrows as a result of follicular occlusion, is associated with inflammation and cytotoxicity because it induces the production of inflammatory cytokines and produces enzymes such as lipase, hyaluronidase and protease [1–3]. Notably, *C. acnes* lipase has been studied as a target for acne treatment because it produces fatty acid-inducing inflammatory cytokines by hydrolysing triglycerides in the sebum [4]. Acne pustules, which store sebum, show different conditions than healthy follicles [5]. Acne pathology is known to involve comedo formation, sebum storage in follicles, inflammation induction and cicatrization by cellular necrosis [6]. *C. acnes* in acne pustules have also been reported to produce biofilms, which are related to the avoidance of antimicrobials and to the excessive production of the haemolysin Christie–Atkins–Munch–Peterson (CAMP) factor [7].

Human sebum consists of squalene, triglycerides, fatty acids and wax esters [5]. Although the sebum contributes to maintaining moisture on the skin surface, excessive fatty acids, especially oleic acid, lead to the development of acne by hyperkeratinization [8]. *C. acnes* strains with lipase activity metabolize triglycerides, producing fatty acids that play a role in inducing inflammation. Therefore, *C. acnes* pathogenicity has been evaluated as a contributory factor to acne vulgaris, was used to evaluate *C. acnes* pathogenicity [9, 10]. However, the *in vitro* culture medium is rich in nutrients, whereas the skin environment is poor in nutrients and sebum. Additionally, the sebum concentration in acne pustules is unknown and is estimated to change according to the pathological stage. Sebum was digested by *C. acnes* lipase to free fatty acids, which causes a failure of the skin barrier function by hyperkeratinization and induces an increase in inflammatory cytokines [11]. Although *C. acnes* has multiple genes encoding

Received 05 July 2021; Accepted 09 September 2022; Published 03 October 2022

Author affiliations: [1]Department of Clinical Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan.

***Correspondence:** Keisuke Nakase, nakase@toyaku.ac.jp

Keywords: Acne vulgaris; *Cutibacterium acnes*; lipase; oleic acid; sebum.

Abbreviations: LNB, low-nutrient broth; 4-MUO, 4-methylumbelliferyl oleate; SLST, single-locus sequence typing.

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extracellular lipases, *gehA* is most related to *C. acnes* lipase activity [12]. Its expression differs among *C. acnes* strains classified into subspecies or by phylogenetic typing [9].

To understand the *C. acnes* phenotypes in acne pustules at each pathological stage, we developed a skin sebum medium as an *in vitro* acne pustule model, which is poor in nutrients and sebum, and used it to study *C. acnes* growth and lipase activity. Here, we studied the growth and pathogenicity factor production of *C. acnes* in our skin sebum medium.

METHODS

Development of the skin sebum medium

Skin sebum medium was developed from low-nutrient broth (LNB) and artificial sebum, because acne pustules are poor in nutrients, including sebum. LNB [15.0 g/L tryptone (Oxoid, Hampshire, UK), 3.0 g/L yeast extract (Oxoid), and 2.0 g/L NaCl] was modified from the minimal medium used by Kishishita *et al.* for a sugar metabolism test [13]. Artificial sebum [12.5 w/w % squalene, 2.5 w/w % jojoba oil, 45.0 w/w % triolein, and 17.0 w/w % oleic acid] was prepared based on a report by Wertz *et al.* [14]. Jojoba oil (used as wax ester), squalene, oleic acid, and triolein were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Artificial sebum was added at 0.05–20% (final concentration) in the skin sebum medium; there is currently no report on the optimum sebum concentration that should be used in culture media.

Analysis of growth and lipase activity in *C. acnes*

C. acnes subsp. *defendens* ATCC11828 (single-locus sequence typing [SLST], K9, type strain), which showed elevated lipase activity, was used in all of these studies. *C. acnes* subsp. *acnes* ATCC6919 (SLST A1, type strain), which is frequently isolated from healthy skin and acne pustules, and *Staphylococcus aureus* JCM2874 as type strains, were used in a portion of these studies [15]. *C. acnes* was grown in brain heart infusion broth (Oxoid) and inoculated at approximately 10^5 c.f.u./ml in skin sebum medium or LNB-containing sebum components. After 72 hr of anaerobic incubation, the bacterial population and lipase activity were measured. For the sebum components, palmitic acid and linoleic acid were purchased from FUJIFILM Wako Pure Chemical, while stearic acid and palmitoleic acid were purchased from Tokyo Chemical Industries (Tokyo, Japan).

Quantification of biofilm formation

Biofilm formation was quantified using a microtitre plate assay, as described in our previous study [16]. *C. acnes* was incubated anaerobically in brain heart infusion broth (BHI, Oxoid) for 72 hr. Bacterial cultures were adjusted to an OD of 0.2 in 150 µl of skin sebum medium containing a prepared sebum concentration, and inoculated into a 96-well flat-bottom plate (Thermo Fisher Scientific, MA, USA). After incubation for 72 hr at 37°C, supernatant was removed, and biofilms were washed with PBS and was stained with 0.5% crystal violet. Planktonic crystal violet was washed with PBS, and biofilms were subsequently extracted with 160 µl of 30% acetic acid. Biofilm quantification was performed by reading OD at 600 nm using a MULTISKAN microplate reader (Thermo Fisher Scientific). Results are shown as means±SD of three independent tests.

Measurement of lipase activity and quantification of mRNA for *gehA*

Lipase activity was measured using the method reported by Nakase *et al.* [10]. 4-Methylumbelliferyl oleate (4-MUO, Sigma-Aldrich, St. Louis, USA) was used as a lipase fluorescent substrate, and fluorescence intensity was measured using Varioskan Flash 2.4 (Thermo, MS, USA) with an excitation filter of 320 nm and an emission filter of 450 nm. The expression of the *gehA* gene encoding extracellular lipase was analysed using the method reported by Nakase *et al.* [10]. After anaerobic incubation of *C. acnes* ATCC11828 for 72 hr, total RNA was extracted using a High Pure RNA Isolation Kit (Roche). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Gene expression was analysed by real-time PCR using THUNDERBIRD™ SYBR qPCR MIX (TOYOBO). qPCR analysis was conducted using the StepOne Real-Time PCR System (Thermo) set to 40 cycles of 95 °C for 15 sec and 60 °C for 40 sec. The amplification primers for *gehA* and endogenous control genes *oxc* were as follows: *gehA*-F, ACAGCCCCGACGGTATTC *gehA*-R;, GCTTGACCTTGCAGGAGAA *oxc*-F;, CTTGTCATCGGCGTATTCG; and *oxc*-R, CACTTCAAGCGGAAGGTGA. The results are averages of three independent experiments.

Enzyme inhibition assay on bacterial lipase

Bacterial lipase solution was used as the supernatant, and the bacterial culture was centrifuged at 3000 ×***g*** for 10 min and sterilized using a 0.45 µm pore size filter (ADVANTEC, Tokyo, Japan). *C. acnes* and *Staphylococcus aureus* were cultured in Gifu anaerobic (GAM) broth, modified Nissui (Nissui Pharmaceutical, Tokyo, Japan) containing sodium thioglycolate, hemin, and vitamin K_1 in addition to basic nutrients under anaerobic conditions for 72 h, and tryptone soya broth (Oxoid, Hampshire, UK) under aerobic conditions for 24 hr, respectively. *S. aureus* strain JCM2874 was used as the strain type in this study. Bacterial lipase solutions of *C. acnes* and *S. aureus* (10 mM final concentration) were added to sebum components and incubated for 30 min at 37 °C. After centrifugation at 15,000 r.p.m. for 1 min, the lipase activity was measured [10]. The fatty acids elaidic acid (Cayman

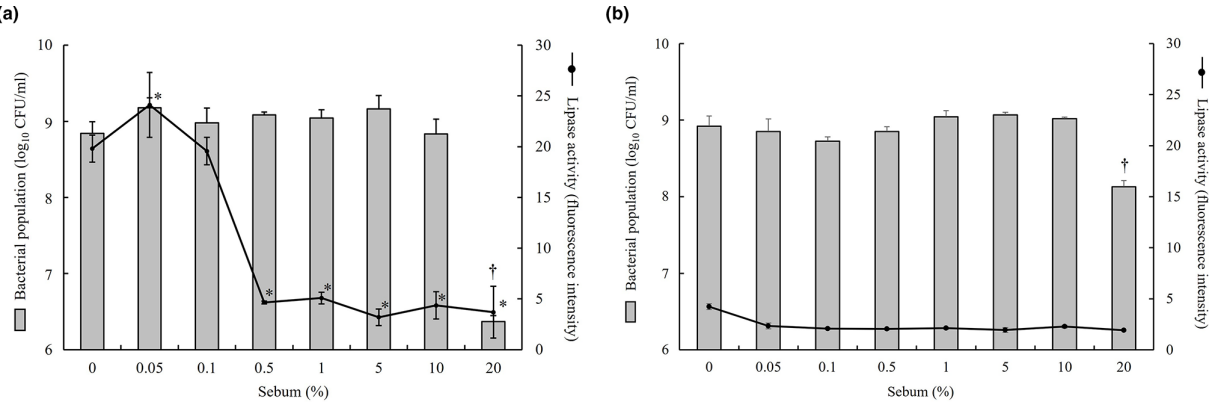

Fig. 1. Growth and lipase activity of *C. acnes* in skin sebum medium containing different sebum concentrations. The bacterial population and lipase activity were analysed using bacterial solutions and supernatants incubated for 72 h in LNB containing different sebum concentrations in (a) ATCC11828 and (b) ATCC6919. Bars and error bars represent means and SEM, respectively. The 0.05% artificial sebum contained 0.15 mM squalene, 0.02 mM jojoba oil, 0.25 mM triolein and 0.3 mM oleic acid. * and † indicate that lipase activity and bacterial population, respectively, showed statistically significant differences compared to their controls using Welch's *t*-test ($P<0.05$).

Chemical, Michigan, USA), erucic acid (Tokyo Chemical Industries), and sodium oleate (Tokyo Chemical Industries) were used as compounds similar to oleic acid and as sebum components. $CaCl_2$ and $BaCl_2$ (10 mM final concentration) were used as suppressors of lipase inhibitors [17].

Determination of the reaction rate of lipase in *C. acnes*

The inhibition pattern of oleic acid was evaluated using a Michaelis–Menten plot and a Lineweaver–Burk plot. Fivefold diluted lipase solution containing oleic acid was incubated for 30 min at 37 °C. The reacted solution was mixed with 0.6–80 µM 4-MUO, and the lipase activity was measured. Lipase for Biochemistry (FUJIFILM Wako Pure Chemical) was used to create a standard curve to calculate the reaction rate. Lipase activity values (fluorescence intensity) were converted to enzyme amounts (units) in the region of concentrations showing linearity. Data are shown as the mean±SEM from three experimental replicates.

Statistical analysis

All data (figures and tables) are presented as means±SEM of three experimental replicates. Two different groups were compared using Welch's *t*-test as a parametric test.

RESULTS

Lipase activity and growth of *C. acnes* in the presence of sebum and sebum components

The skin and acne pustules are low in nutrients and contain sebum, but limited quantities of sugar [18]. To evaluate *C. acnes*-induced exacerbated inflammation under the same conditions found in acne pustules *in vitro*, we developed skin sebum medium containing modified minimum medium and artificial sebum. *C. acnes* growth and lipase activity were tested in skin sebum medium containing different concentrations of sebum, because it is predicted that the sebum concentration changes based on the acne vulgaris pathology stages (Fig. 1a and b). In the culture grown in skin sebum medium containing 0.05% sebum, no change in growth of *C. acnes* ATCC11828 was observed, while bacterial lipase activity was *significantly increased* relative to that in skin sebum medium without sebum ($P<0.05$) (Fig. 1a). Similarly, in medium containing 0.5–10% sebum, only lipase activity was significantly decreased. Furthermore, in medium containing 20% sebum, both growth and lipase activity decreased. By contrast, no change in lipase activity was observed in *C. acnes* ATCC6919, which showed lower lipase activity than ATCC 11828, and growth was decreased in medium containing 20% sebum (Fig. 1b). Therefore, *C. acnes* growth and lipase activity varied with sebum concentration and differed among strains.

The sebum components causing changes in growth and lipase activity of *C. acnes* ATCC11828 were evaluated using *C. acnes* cultured in skin sebum medium containing sebum components (Fig. 2a to h). Growth and lipase activity did not vary in the culture on skin sebum medium containing jojoba oil and stearic acid compared to those without sebum components. However, adding 10 mM (highest tested concentration) palmitic acid, palmitoleic acid or linoleic acid to cultures decreased both growth and lipase activity. By contrast, adding oleic acid or triolein (10 mM) to cultures decreased lipase activity, but not growth.

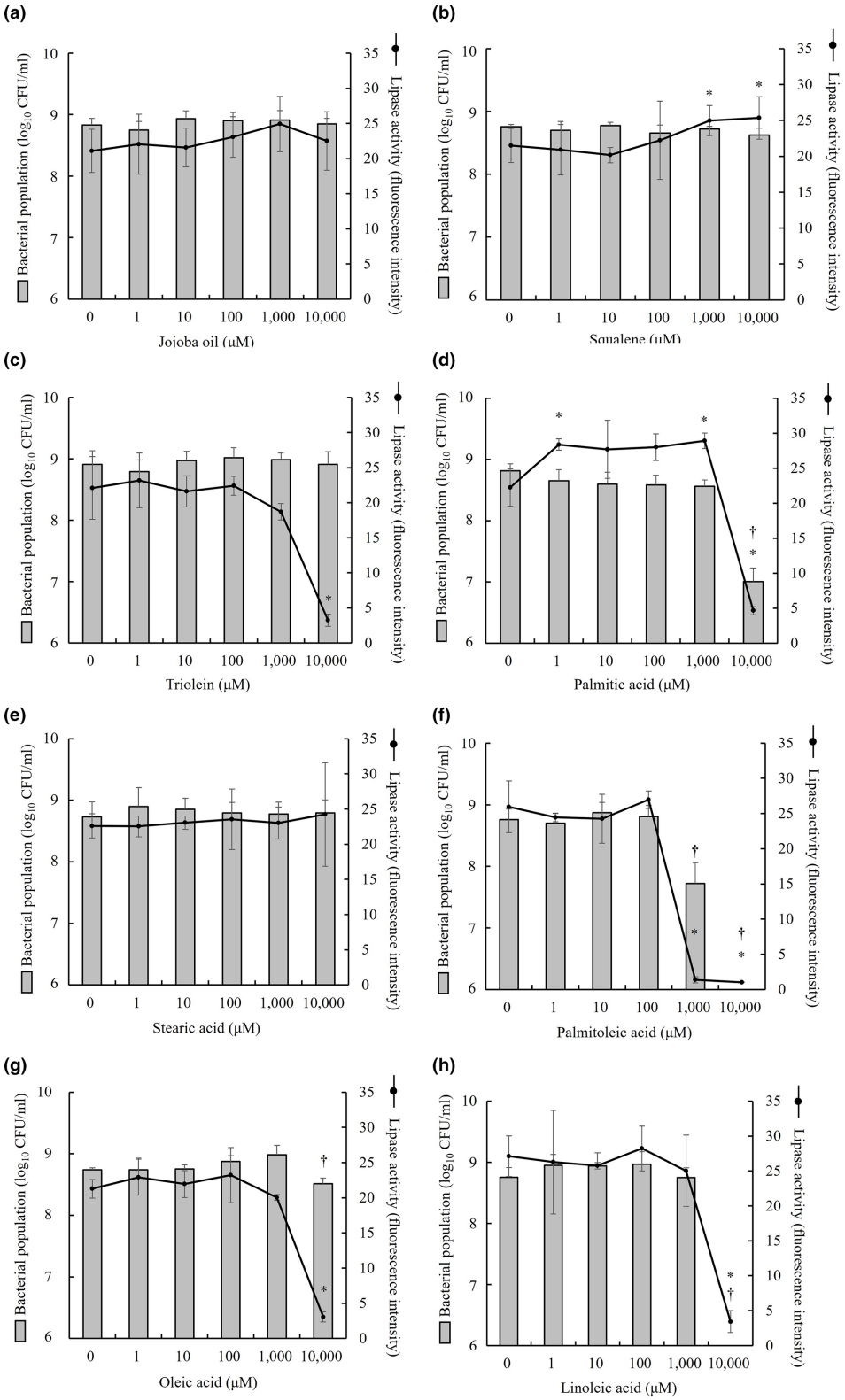

Fig. 2. Growth and lipase activity of *C. acnes* ATCC11828 in the presence of sebum components. (a) Jojoba oil, (b) Squalene, (c) Triolein, (d) Palmitic acid (C16:0), (e) Stearic acid (C18:0), (f) Palmitoleic acid (C16:1)(n-7), (g) Oleic acid (C18:1)(n-9), (h) Linoleic acid (C18:2)(n-6). Bacterial populations and lipase activity were analysed using bacterial solutions and supernatants incubated for 72 h in LNB containing different concentrations of sebum components. Bars and error bars represent means and SEM, respectively. * and † indicate that lipase activity and bacterial populations, respectively, showed statistically significant differences compared to their controls using Welch's *t*-test ($P<0.05$).

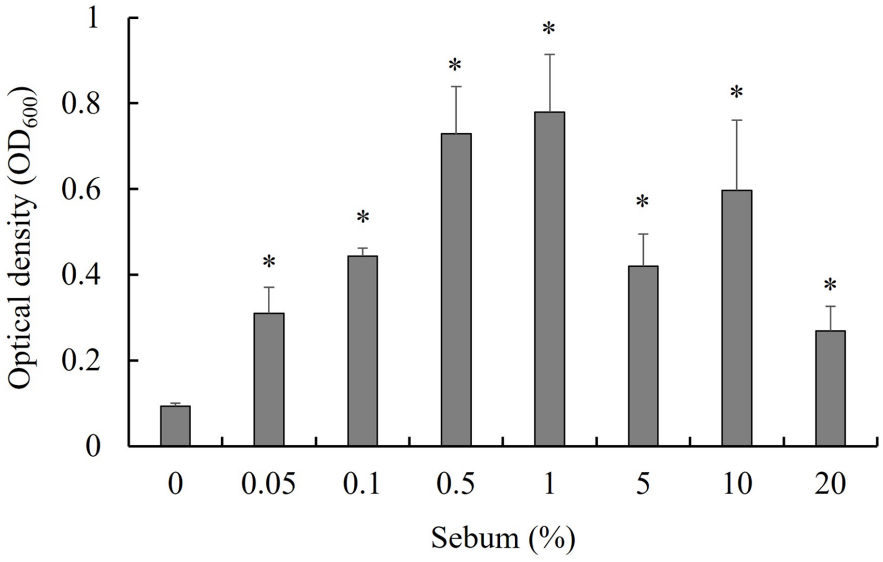

Fig. 3. Biofilm quantification in skin sebum medium. Bars and error bars represent means and SEM, respectively. * indicates a significant difference (P<0.05) compared to media without sebum (0%) using Welch's t-test.

Biofilm formation was evaluated using *C. acnes* ATCC11828 in skin sebum medium, and varied with varying sebum concentrations (Fig. 3). Biofilm formation was enhanced in the presence of sebum relative to its rate of formation in its absence (P<0.05). The greatest amounts of biofilm were observed in skin sebum medium containing 1% sebum.

Inhibition of *C. acnes* lipase activity by oleic acid

An exacerbation of acne vulgaris by *C. acnes* may be prevented by the development of novel medicines that selectively inhibit lipase activity. It is suggested that triolein acts as an oleic acid because it is decomposed to oleic acid and glycerol. To analyse the mechanism by which oleic acid decreases lipase activity, the expression of *gehA* encoding extracellular lipase was measured (Fig. 4). No difference in the expression level of this gene upon the addition of oleic acid and triolein was found.

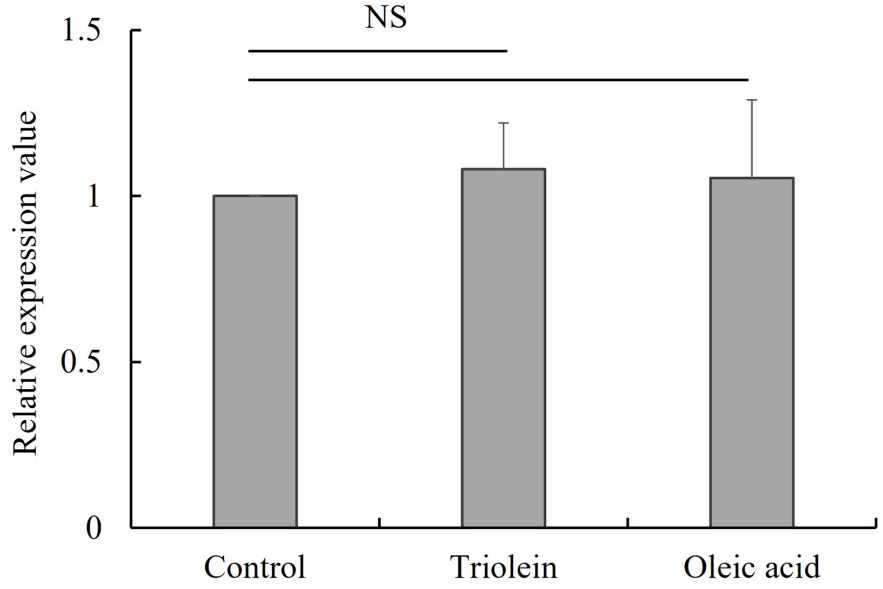

Fig. 4. Quantitation of *C. acnes gehA* gene expression in the presence of oleic acid and triolein. NS, not significant. Total RNA was extracted from *C. acnes* cells incubated for 72 h in skin sebum medium with or without 10 mM oleic acid or triolein. Bars and error bars represent means and SEM, respectively. Statistical analysis was performed using Welch's t-test.

Table 1. *C. acnes* lipase inhibition by sebum components and fatty acids

Compound	Relative lipase activity (%)	
	C. acnes	***S. aureus***
Jojoba oil	126.7±9.6*	97.5±5.5
Squalene	116.2±16.7	97.5±3.3
Triolein	67.7±6.1*	102.9±1.5
Palmitic acid (C16:0)	90.4±2.9*	100.2±5.3
Stearic acid (C18:0)	96.4±15.2	99.3±6.0
Palmitoleic acid (C16:1)(n-7)	87.7±13.4*	108.4±6.6
Oleic acid (C18:1)(n-9)	36.1±12.9*	100.6±6.3
Linoleic acid (C18:2)(n-6)	72.0±8.3*	104.8±3.1
Elaidic acid (C18:1)(n-9)	73.3±11.8*	103.7±4.5
Erucic acid (C22:1)(n-9)	46.0±12.1*	102.8±5.6
Sodium oleate	3.4±1.1*	6.5±3.6*
Sodium oleate + $BaCl_2$	6.9±3.3*	103.5±5.9
Sodium oleate + $CaCl_2$	4.3±0.2*	101.8±4.8

Lipase activity was evaluated relative to activity in the absence of compound, which was defined as 100%. Elaidic acid and erucic acid, which do not exist on the skin, were used as structural isomers of oleic acid. $BaCl_2$ and $CaCl_2$ were used to inhibit lipase and oleic acid.
* $P < 0.05$ vs. without any compound (positive control, 100%).

Subsequently, the direct reaction of *C. acnes* lipase and oleic acid led to a significant decrease in lipase activity (Table 1). Similarly, lipase activity decreased when triolein, palmitic acid, palmitoleic acid, and linoleic acid were added. In contrast, no change was observed in lipase activity of *S. aureus,* which is the most populous pathogenic skin bacterium, upon the addition of oleic acid (P=0.443). Therefore, we suggest that oleic acid directly reacts with *C. acnes* lipase and decreases its activity (Fig. 5). Oleic acid analogues were further tested to clarify the structures of compounds capable of inhibiting *C. acnes* lipase activity (Table 1). *C. acnes* lipase activity decreased upon addition of elaidic acid, a trans-isomer of oleic acid; erucic acid, a longer unsaturated fatty acid; and sodium oleate. Because sodium oleate mediated lipase inhibitory activity against *Pseudomonas fraji* decreases in the presence of bivalent metallic iron [17], when the reactivation experiment was conducted in the presence of bivalent metallic iron, reactivation of *C. acnes* lipase was not observed in the presence of $CaCl_2$ and $BaCl_2$. Therefore, oleic acid apparently inhibits *C. acnes* lipase via a different mechanism than that for the lipases of *P. fraji* and *S. aureus*.

Mode of inhibition of *C. acnes* lipase by oleic acid

The inhibition of lipase activity by oleic acid showed Michaelis–Menten kinetics. *C. acnes* lipase activity showed concentration-dependent inhibition (Fig. 6a, b). The inhibition levels were observed to be the same between 1 mM and 10 mM oleic acid, and 10 mM oleic acid was considered an excess amount. When the *C. acnes* lipase solution was prepared for the measurement of enzyme inhibition activity, a fivefold dilution solution that showed a linear correlation plot was determined as the appropriate concentration. The measured *C. acnes* lipase activity was converted to a unit of lipase based on a standard curve obtained from the activity of the lipase reference standard. From Michaelis–Menten kinetics, lipase activity (Vmax, 2.51 mUnit/min; Km, 3.55) was calculated in the absence of oleic acid (no inhibitor) (Fig. 6a). By contrast, lipase activity was calculated in the analysis with 0.01 (Vmax, 2.07 mUnit/min; Km, 3.09) and 10 mM (Vmax, 1.07 mUnit/min; Km, 3.17) of oleic acid. Vmax values decreased with oleic acid addition in a concentration-dependent manner, while no change in Km values was observed either with or without oleic acid. Similarly, in the analysis of Lineweaver–Burk kinetics, *C. acnes* lipase activity was also inhibited in a non-competitive and concentration-dependent manner upon oleic acid addition (Fig. 6b).

DISCUSSION

There are many experimental models of acne vulgaris. Although models using keratinocytes or sebaceous cells are known *in vitro*, they have conditions different from the actual conditions in acne pustules [19, 20]. Similarly, anaerobic growth media, such as reinforced clostridial medium and Brucella broth, are very nutrient rich. Animal models developed for assessing *C. acnes*-induced exacerbated inflammation *in vivo* are difficult to evaluate, because the method causing acne vulgaris in animals has not been

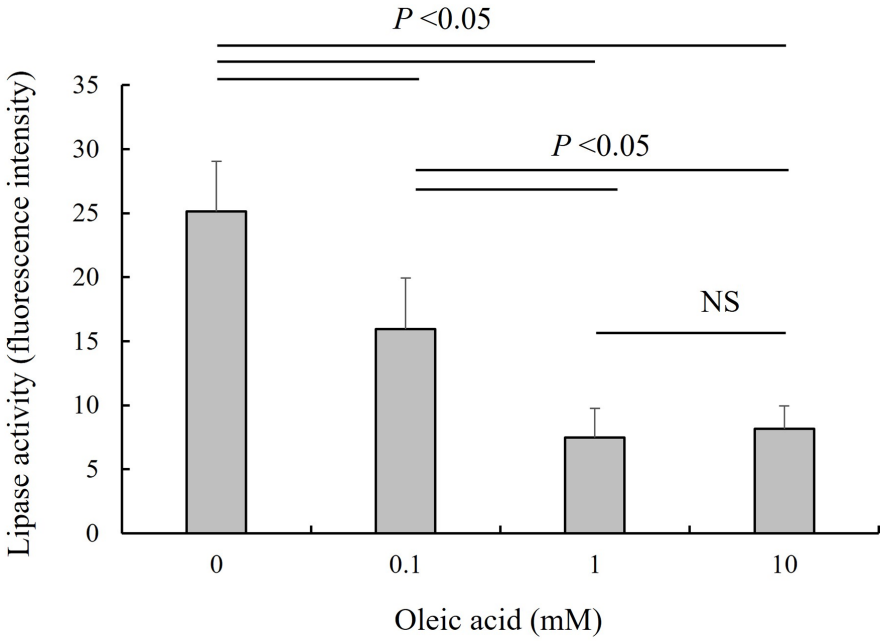

Fig. 5. Inhibition of *C. acnes* lipase activity by different concentrations of oleic acid. Bars and error bars represent means and SEM, respectively. Lipase activity was analysed in bacterial supernatants directly mixed with oleic acid. * indicates a significant difference (P<0.05) compared with the control using Welch's *t*-test.

established, and there are no reports of animals that have *C. acnes* as a skin microbe. Therefore, to study the phenotype of *C. acnes* in acne pustules, we developed a skin sebum medium that models the nutrient conditions of acne pustules. *C. acnes* biofilm formation and pathogenicity factor production were evaluated using an *in vitro* acne model by Spittales *et al.* [21]. However, it is difficult to evaluate *C. acnes* phenotypes other than biofilm formation in the semi-solid medium used. Our developed medium allows variation in the sebum concentration, and evaluation of phenotypes including growth, lipase activity, and biofilm formation.

Using skin sebum medium, *C. acnes* growth, lipase activity and biofilm formation were observed to change based on the different sebum concentrations added. When *C. acnes* was cultured in skin sebum medium containing 0.5–10% sebum, a

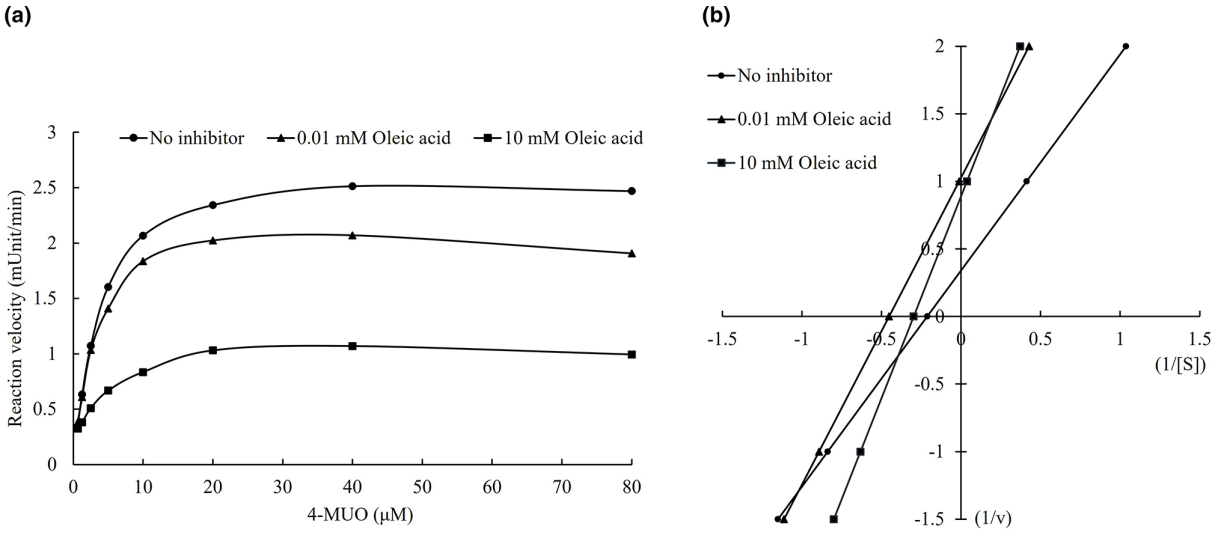

Fig. 6. Inhibition of *C. acnes* lipase activity by oleic acid. Enzyme activity analysis for lipase of *C. acnes* in a (a) Michaelis–Menten plot and (b) Lineweaver–Burke plot. A curve was created from the calculated values using the mean from three experimental replicates.

range which is assumed to reflect the sebum concentration of acne during the initial formation stage, *C. acnes* growth was not inhibited, but lipase activity significantly decreased. Furthermore, upon addition of 20% sebum, which is assumed to mimic the later stage of acne, both *C. acnes* growth and lipase activity were significantly inhibited. Consequently, *C. acnes* is related to the exacerbation of inflammation by actively growing during the initial stages of acne vulgaris. Our data suggest that it is possible to use skin sebum medium to evaluate the *C. acnes* phenotype found in acne pustules *in vitro*. To evaluate *C. acnes* phenotypes in acne pustules, we considered that skin sebum medium containing 5% sebum, which does not inhibit growth or lipase activity, is preferential. The pathogenicity of *C. acnes* in acne pustules can be investigated and clarified by using different phylogenetic types of *C. acnes* strains in this medium. One limitation of this study is that only *C. acnes* ATCC6919 and ATCC11828, which were classified as subsp. *acnes* and subsp. *defendens*, respectively, were selected as candidates for this study. Because the lipase activity of *C. acnes* ATCC11828 was higher than that of ATCC6919 in BHI broth, this strain was used in this study. For further study, phenotypic changes in clinical isolates need to be studied.

Pantothenic acid (vitamin B_5) inhibits lipase activity without causing growth inhibition; however, its mechanism is not understood. Our data are the first to show that oleic acid directly inhibits *C. acnes* lipase activity noncompetitively. Similarly, triolein inhibited *C. acnes* lipase. The active component within triolein is postulated to be oleic acid digested by *C. acnes* lipase; however, triolein itself may inhibit *C. acnes* lipase. Sebum composition is different for each report, but it is mainly composed of triglycerides, fatty acids, wax esters and squalene [5, 22, 23]. In fatty acids, palmitic acid, palmitoleic acid and oleic acid are abundant in sebum [24]. Both oleic acid and other unsaturated fatty acids decreased lipase activity in our study. We tested both *cis*- and *trans*-unsaturated fatty acids to identify which chemical structure inhibited *C. acnes* lipase and both decreased lipase activity. In the study of the epidermal model, oleic acid caused a failure of the skin barrier function by hyperkeratinization and induced an increase in inflammatory cytokines [11]. Excessive unsaturated fatty acids, including oleic acid, are considered to be related to the development of acne vulgaris, because a similar pathology is observed in acne lesions. Therefore, topical application of unsaturated fatty acids may adversely affect the skin. Topical and oral antimicrobials are used for acne treatment, and the emergence of antimicrobial-resistant *C. acnes* has become a critical problem [15]. We believe that the development of novel medicines that inhibit only *C. acnes* lipase activity without antimicrobial activity, based on unsaturated fatty acids, may lead to decreased antimicrobial use and contribute to preventing the emergence of antimicrobial-resistant bacteria. Therefore, identification of the lipase inhibitory active site in oleic acid warrants further study.

Our data showed that the *C. acnes* phenotype changes in the presence of sebum and suggest that the effect of acne inflammation by *C. acnes* can be satisfactorily evaluated using skin sebum medium.

Funding information
This work was supported by the Matching Fund Subsidy for Private Schools in Japan.

Acknowledgements
We would like to thank Editage (www.editage.com) for English language editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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