Isolated hair bacteria reveal different isolation possibilities under various conditions

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Abstract:	Microorganisms are assumed to inhabit various environments and organisms, including the human body. The presence of more than 700 bacterial species on scalp hair has been reported through rRNA amplicon analysis. However, the biological properties of bacteria on the scalp hair (hair bacteria) and their functions are poorly understood as few hair bacteria have been isolated from hair in previous studies. This study aimed to isolate hair bacteria using standard media under 24 different conditions (including medium components, component concentrations, gelling agents, and atmospheric environments). Furthermore, we evaluated the possibility of isolating strains under these isolation conditions and examined the carbon metabolic ability of several predominantly isolated strains. A total of 65 bacterial species belonging to 26 genera were isolated from hair under 24 isolation conditions. The predominant bacterial species isolated from human hair in this study showed different carbon metabolic capabilities than those of the reference strains. In addition, “isolation possibility” was newly proposed to systematically evaluate the number of isolation conditions that could cultivate a bacterial species. Based on isolation possibility, the isolates were categorized into groups with a high number of isolation conditions (e.g., ≥25%; such as Staphylococcus) and those with a low number (e.g., ≤25%; such as Brachybacterium). These findings indicate the existence of "easily isolated microorganisms" and "difficultly isolated microorganism" from human hair.
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Cover Letter

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January 22, 2024

Dear Editor,

I am submitting a paper entitled **"Isolated hair bacteria reveal different isolation possibilities under various conditions"** for the publication in *Journal of Bioscience and Bioengineering* as an original article.

This paper has not been published in another journal, and is original work of the authors.

This paper is co-authored by **Azusa Yamada, Yuri Nishi, Mei Noguchi, Kota Watanabe, Mugihito Oshiro, Kenji Sakai, and Yukihiro Tashiro.** First and corresponding authors are Azusa Yamada and Yukihiro Tashiro, respectively. We all agree to submit the work to *Journal of Bioscience and Bioengineering*. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The key points of this paper are: 1. We isolated a total of 65 bacterial species belonging to 26 genera by various isolation conditions (medium components, component concentrations, gelling agents, atmospheric environments) using standard media from hair. 2. We elucidated different carbon metabolic capabilities of isolated predominant hair bacteria compared to reference strains. 3. We evaluated a new parameter of isolation possibilities of hair bacteria, categorized into groups with a high number of isolation conditions (e.g., ≥25%; *Staphylococcus* et al.) and those with a low number (e.g., ≤25%; *Brachybacterium* et al.), and proposed a new the existence of "easily isolated bacteria" and "difficultly isolated bacteria" on human hair. We believe that the outcomes of this study would support the development of isolation methods to improve the isolation possibility for difficultly isolated bacteria and the understanding of the underlying mechanisms for microbial isolation research in the future. We strongly believe that *Journal of Bioscience and Bioengineering* is the most appropriate journal in which our paper should be published and that your journal will provide our paper with the broad distribution.

We would like to consider Dr. Yoshiteru Aoi at Hiroshima University as an appropriate editor for this paper because he has published many papers on isolation of environmental microorganisms. This manuscript has already been checked by a language editing service prior to submission. We also consent the transfer of copyright from us to the Society of Biotechnology, Japan (SBJ). We hope that our manuscript meets the high standards of your journal. We are looking forward to receiving a favorable response from you regarding the acceptance of our manuscript.

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Yukihiro Tashiro, Dr. 

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We would list two potential reviewers, who are experts on the key topics covered in our paper.

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January 2021

Manuscript title: **Isolated hair bacteria reveal different isolation possibilities under various conditions**

Author(s): Azusa Yamada, Yuri Nishi, Mei Noguchi, Kota Watanabe, Mugihito Oshiro, Kenji Sakai, and Yukihiro Tashiro

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January 2021

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Title: **Isolated hair bacteria reveal different isolation possibilities under various conditions**

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Title

Isolated hair bacteria reveal different isolation possibilities under various conditions

Running title: Distinct isolation possibilities for hair bacteria

Azusa Yamada[1], Yuri Nishi[1], Mei Noguchi[1], Kota Watanabe[2], Mugihito Oshiro[1], Kenji Sakai[1,3], and Yukihiro Tashiro[1,3*]

Division of Systems Bioengineering, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Motooka 744, Nishi-ku,Fukuoka 819-0395, Japan,[1] Department of Fermentation Science, Faculty of Applied Biosciences, Tokyo University of Agriculture, Sakuragaoka 1-1-1, Setagaya-ku, Tokyo 156-8502, Japan,[2] Center for International Education and Research of Agriculture, Faculty of Agriculture, Kyushu University, Motooka 744, Nishi-ku, Fukuoka 819-0395, Japan[3]

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Keywords: hair bacteria; isolation possibilities; isolated microorganisms; standard media;

carbon metabolic capabilities

ABSTRACT

Microorganisms are assumed to inhabit various environments and organisms, including the human body. The presence of more than 700 bacterial species on scalp hair has been reported through rRNA gene amplicon analysis. However, the biological properties of bacteria on the scalp hair (hair bacteria) and their functions are poorly understood as few hair bacteria have been isolated from hair in previous studies. This study aimed to isolate hair bacteria using standard media under 24 different conditions (including medium components, component concentrations, gelling agents, and atmospheric environments). Furthermore, we evaluated the possibility of isolating strains under these isolation conditions and examined the carbon metabolic ability of several predominantly isolated strains. A total of 64 bacterial species belonging to 26 genera were isolated from hair under 24 isolation conditions. The predominant bacterial species isolated from human hair in this study showed different carbon metabolic capabilities than those of the reference strains. In addition, “isolation possibility” was newly proposed to systematically evaluate the number of isolation conditions that could cultivate a bacterial species. Based on isolation possibility, the isolates were categorized into groups with a

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INTRODUCTION

Microorganisms have been considered to inhabit various environments by adapting to different conditions, such as temperature, oxygen level, pH, salinity, and growth matrices (1–3). They play a role in the biological cycle of elements in nature and affect the lifestyle of other organisms, including humans (4,5). Recently, many studies have attempted to identify the microbiota in different environments using rRNA gene amplicon analysis and metagenomics using next-generation sequencing (NGS) without isolating the microorganisms (6,7). Recently, metagenome-assembled genomes (MAGs) has attracted attention as a non-culture method to be reconstructed from metagenome data, and this method would give understanding of microbiota and their interactions under a targeted environment (8). However, it would be difficult to elucidate actual functions and roles of environmental microorganisms by this method (9). Thus, the isolation of microorganisms is essential for elucidating their ecology and function in various environments (10). In addition, the acquisition of microorganisms can be applied to establish novel biotechnologies, including the chemical, pharmaceutical, and food industries, as well as for environmental conservation (3).

Microorganisms have been isolated from the environment using solid media since the end of the 19th century (10). Solid media are classified into two types: natural media or synthetic media that mimic the natural environment (11,12) and standard media such as

61 nutrient medium (NM) and Luria-Bertani (LB) medium (10,13). Standard media have
62 been considered advantageous for the isolation of many types of microorganisms and the
63 isolation processes require extensive investigation of isolation conditions (media
64 (component concentration and composition) and culture conditions (such as pH,
65 temperature, and aerobic or anaerobic conditions)), which would result in labor- and time-
66 consuming work. Most microorganisms are unisolated or uncultured using standard
67 media, even after extensive investigation under many isolation conditions. The number
68 of microorganisms cultured or uncultured in a targeted environment has been evaluated
69 based on a quantitative parameter “culturability”, defined as the viable count expressed
70 as a percentage of the microscopically determined total count of cells for the isolation
71 sources used in that particular cultivation experiment (14). However, few studies have
72 reported the number of isolation conditions that can cultivate an isolated microorganism.
73 Here, we proposed a novel parameter; “isolation possibility”, because a systematic
74 evaluation of isolation possibility using standard media would improve the efficiency of
75 isolating useful and targeted microorganisms.
76 Understanding the resident bacterial ecosystem in the human body is crucial for
77 clarifying its impact on human health (15,16). To date, the function of skin bacteria and
78 the interactions between skin bacteria and human hosts have been elucidated using

79 microorganisms isolated from the human body (17). Notably, skin bacteria have drawn
80 attention to the skin's barrier function, immunomodulation, and their implications for
81 dermatological condition (18,19). However, we recently reported on a unique bacterial
82 community structure of human hair from root to tip（hair bacteria), consisting of
83 predominant bacterial phyla including Actinobacteria, Firmicutes, and Proteobacteria
84 by16S rRNA gene amplicon analysis using NGS (20–23). Furthermore, several types of
85 strains related to the predominant hair bacterial species indicated by 16S rRNA gene
86 amplicon analysis have been reported to influence human keratinocyte cellular activity,
87 which suggests the health of the scalp and hair, including repair of the damaged scalp and
88 hair growth by hair bacteria (24). Thus, the isolation of hair bacteria would be significant
89 not only to elucidate their characteristics and functions in human hosts but also for human
90 scalp/hair health. In addition, only a few reports are available on evaluating the growth
91 characteristics of hair bacteria; therefore, the ability of hair bacteria to metabolize
92 substrates remains unclear.
93 The objectives of this study were to isolate hair bacteria under various isolation
94 conditions using standard media, evaluate the possibility of isolating strains, and elucidate
95 the metabolic ability of the carbon sources of several isolated strains.
96

MATERIALS AND METHODS

Samples and collection

Scalp hair shaft samples were collected from 18 healthy individuals (10 females and 8 males) ranging in age from 20 to 40 years who consented to participate in the study. Scalp hair shaft samples were cut using sterile scissors with nitrile gloves. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2013. The participants provided written informed consent with the approval of the Ethics Committee of the Graduate School of Bioscience and Biotechnology at Kyushu University (Authorized No. 104). The methods were carried out in accordance with approved guidelines.

Isolation culture conditions

The isolation procedure was based on the standard operating procedure SOP-GG-02-00 (25), with hair replacing the source material in this study. The standard media used were Luria-Bertani medium (LB), nutrient broth medium (NM), trypticase soy medium (TS), casein-peptone soy meal-peptone medium (CASO), Columbia Blood Medium (Col), Gifu Anaerobic Medium (GAM), and pig fat medium (PF). The compositions of the

115 respective media were as follows: LB with 1 g/L of tryptone (Thermo Fisher Scientific,
116 Tokyo, Japan), 5 g/L of yeast extract (BD), and 10 g/L of NaCl; NM with 5.0 g/L of
117 peptone (Becton, Dickinson and Company (BD), NJ, USA) and 3 g/L of meat extract
118 (Nacalai Tesque, Kyoto, Japan); TS with 30 g/L of trypticase soy broth (BD); CASO with
119 8 g/L of tryptone (BD), 15 g/L of yeast extract (BD), 5.0 g /L of peptone from soymeal
120 (Nacalai Tesque), and 5 g/L of NaCl; Col with peptone from casein (BD) 10.0 g/L,
121 peptone from meat (BD) 5.0 g/L, heart extract (BD) 3.0 g/L, yeast extract (BD) 5.0 g/L,
122 starch 1.0 g/L, NaCl 5.0 g/L, defibrinated sheep blood (BD) 5.0 g/L and agar 13.0 g/L.;
123 GAM with 59 g/L of GAM Broth (Nissui Pharmaceutical Co., Ltd., Tokyo, BD); PF with
124 5 g/L of pig lipid extract, 0.05 g/L NH_4NO_3, 2 g/L yeast extract (BD); and non-pig fat
125 (PFn) with 0.05 g/L of NH_4NO_3, 2 g/L yeast extract (BD); and an AnaeroPack (Mitsubishi
126 Gas Chemical Co., Inc., Tokyo, Japan) was used in the anaerobic jar for anaerobic
127 cultivation. LB, NM, TS, CASO, Col, PF, and PFn were incubated at 30°C and GAM at
128 37°C. LB, NM, TS, CASO, Col, and PF were cultured under both aerobic and anaerobic
129 conditions. Gelling agents, agar, and agarose were used for LB, NA, TS, and CASO
130 assays. Low nutrient concentrations and 10% dilution medium conditions for LB, NM,
131 TS, CASO, and GAM were also used. The culture conditions are listed in Table 1.
132

Colony purification and DNA extraction

Colonies were obtained by placing hair on a plate and incubating under each culture condition. Single colonies were inoculated into a novel plate twice for purification. Single colonies were suspended in TE solution. The mixture was heated at 100°C for 10 min and centrifuged at 13,000 rpm for 20 min. The supernatant was the DNA extraction product and was stored at -20°C until use.

PCR and 16S rRNA gene sequence analysis

The region of the 16S rRNA gene in each sample was amplified using universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3′), 1492R (5'-TACGGYTACCTTGTTACGACTT-3′). PCR was performed using Premix Taq (TaKaRa, Shiga, Japan): 5 min at 94°C, 30 cycles of denaturation 94°C for 1 min, annealing 55°C for 45 s, elongation at 72°C for 2 min, and final elongation at 72°C for 10 min. To purify the PCR products, the target bands were extracted by 1.5% agarose gel electrophoresis and purified using the FastGeneTM Gel/PCR Extraction Kit (Nippon Genetics, Japan). DNA analysis was performed using a Sanger sequencing platform performed by GENEWIZ (Tokyo, Japan), and the obtained sequences were used to identify the bacterial species using EzBioCloud (https://www.ezbiocloud.net/) based on a similarity score

(≥98.7%) with those of the type strain. Phylogenetic analysis employed the neighbor joining method with 1000 bootstrap replicates, and phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (version X) software (https://www.megasoftware.net/). The sequences of 64 identified hair bacterial isolates were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC800711–LC800727~~5~~, LC800727-LC800774, and LC8192434.

Evaluation of metabolized carbon sources by hair bacteria

The carbon metabolism capacity was exploited by the API®50CH kit (Biomerieux Japan, Tokyo). The target bacterial strains evaluated were six species identified in previous studies as having a high prevalence in the scalp environment: *Cutibacterium acnes* subsp. *defendens, Cutibacterium acnes* subsp. *acnes, Staphylococcus epidermidis, Staphylococcus caprae* and *Micrococcus luteus.* Target bacterial isolates were incubated and purified. Colonies were suspended in the pre-culture medium for bacterial adaptation, using the API®20A medium (Biomerieux Japan, Tokyo) for *Cutibacterium acnes* subsp. *defendens* and *Cutibacterium acnes* subsp. *acnes*, and API®50CHL medium for *Staphylococcus epidermidis*, *Staphylococcus caprae*, and *Micrococcus luteus*. The cultures were inoculated into API®50CH plates and incubated at 30°C for 48 h.

169 *Cutibacterium* spp. plates were assayed with the BCP reagent. The carbon metabolism
170 capacity of bacterial type strain was obtained from the APIWEB (Biomerieux Japan Ltd,
171 https://apiweb.biomerieux.com/identIndex) and compared with the isolates.
172
173 **RESULTS**
174 **Comparison of aerobically isolated bacteria using undiluted and diluted standard**
175 **media**
176 Hair bacteria were isolated from LB, NM, TS, CASO, and GAM plates using undiluted
177 and 10% diluted standard media. Totally 41 species were isolated from human hair and
178 identified under each condition (Fig. 1). The numbers of isolated bacteria on both
179 undiluted and diluted media were eight species belonging to six genera: *Dermacoccus*
180 *nishinomiyaensis* (NM and GAM), *Bacillus subtilis* sbsp. *subtilis* (LB, NM, TS, and
181 CASO), *Staphylococcus epidermidis* (NM, CASO and GAM), *Staphylococcus warneri*
182 (LB, NM, and CASO), *Brevibacterium casei* (CASO and GAM), *Brevundimonas nasdae*
183 (NM, TS), *Moraxella osloensis* (LB, NM, TS, and GAM), and *Roseomonas mucosa* (LB,
184 NM, and CASO). The numbers detected solely on undiluted media were 16 species
185 belonging to 10 genera,: *Brachybacterium muris* (TS), *Corynebacterium aquatimens*
186 (LB), *Corynebacterium senegalense* (NM and TS), *Cutibacterium acnes* subsp. *defendens*

(GAM), *Gulosibacter faecalis* (LB), *Staphylococcus argenteus* (TS), *Staphylococcus caprae* (LB and NM), *Staphylococcus haemolyticus* (NM), *Endobacter medicaginis* (NM), *Mesobacillus boroniphilus* (CASO), *Methylobacterium longum* (NM), *Rothia kristinae* (LB), *Sphingomonas mali* (NM), *Sphingomonas palmae* (CASO), *Sphingomonas panni* (LB), and *Sphingomonas* sp. (LC800757) ~~*phyllosphaerae*~~ (CASO). The numbers detected solely on 10% diluted media were 16 species belonging to seven genera: *Calidifontibacter indicus* (LB), *Microbacterium aerolatum* (LB), *Microbacterium foliorum* (NM), *Microbacterium maritypicum* (NM), *Microbacterium endophyticus* (GAM), *Lederbergia wuyishanensis* (GAM), *Staphylococcus capitis* subsp. *capitis* (CASO), *Staphylococcus capitis* subsp. *urealyticus* (CASO), *Staphylococcus hominis* subsp. *novobiosepticus* (CASO), *Staphylococcus lugdunensis* (LB and NM), *Staphylococcus saccharolyticus* (CASO), *Brevibacterium sediminis* (NM), *Brevibacterium vesicularis* (NM), *Pseudomonas oryzihabitans* (CASO), *Sphingomonas* sp. (LC800754) ~~*morindae*~~ (CASO), and *Sphingomonas* sp. (LC800758) ~~*rubra*~~ (LB). These results showed that similar numbers of different bacterial species were obtained under undiluted (16 species) and diluted media (16 species) conditions and that the use of both standard nutrient media and diluted media would be efficient in isolating hair bacteria.

205

206 **Comparison of isolated bacteria under aerobic and anerobic conditions**

207 Hair bacteria were isolated via aerobic or anaerobic cultivation on LB, NM, TS, CASO,
208 Cal, and PF plates. Totally 38 species were isolated from human hair and identified under
209 each condition (Fig. 2). The number of bacteria isolated in both aerobic and anaerobic
210 cultures was four species belonging to three genera: *Staphylococcus caprae* (LB, NM,
211 and CASO), *Staphylococcus epidermidis* (NM and CASO), *Moraxella osloensis* (LB,
212 NM, TS, and PF), and *Roseomonas mucosa* (NM and PF). The number of isolated bacteria
213 solely in aerobic cultivation was 22 species belonging to 12 genera,: *Bacillus subtilis*
214 subsp. *subtilis* (LB, TS, CASO, and PF), *Brachybacterium muris* (TS), *Corynebacterium*
215 *aquatimens* (LB), *Corynebacterium senegalense* (NM and TS), *Dermacoccus*
216 *nishinomiyaensis* (NM and PF), *Gulosibacter faecalis* (LB), *Rothia kristinae* (LB),
217 *Staphylococcus argenteus* (TS and PF), *Staphylococcus haemolyticus* (NM),
218 *Staphylococcus hominis* subsp. *hominis* (PF), *Staphylococcus warneri* (LB and CASO),
219 *Brevibacterium casei* (CASO), *Brevibacterium nasdae* (LB, TS), *Endobacter medicaginis*
220 (NM), *Mesobacillus boroniphilus* (CASO), *Methylobacterium brachiatum* (PF),
221 *Methylobacterium longum* (NM), *Sphingomonas mali* (NM), *Sphingomonas palmae*
222 (CASO), *Sphingomonas panni* (LB), and *Sphingomonas* sp. (LC800757) ~~*phyllosphaerae*~~

(CASO). The number of bacteria isolated solely in anaerobic cultivation was 11 species belonging to five genera: *Brevundimonas huaxiensis* (PF), *Cutibacterium acnes* subsp. *acnes* (LB, NM, TS, CASO, and Col), *Cutibacterium acnes* subsp. *defendens* (LB, NM, TS, CASO, and Col), *Cutibacterium namnetense* (CASO and Col), *Janibacter* sp. (LC819243) ~~*limosus*~~ (PF), *Microbacterium algeriense* (PF), *Staphylococcus* ~~*aureus* sub~~sp. (LC800761) ~~*aureus*~~ (PF), *Staphylococcus capitis* subsp. *urealyticus* (LB), *Staphylococcus lugdunensis* (Col), *Staphylococcus saccharolyticus* (LB, CASO, and PF), and *Staphylococcus saprophyticus* subsp. *saprophyticus* (LB). These results indicate that more species of hair bacteria can colonize under aerobic conditions than under anaerobic conditions.

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Comparison of isolated bacteria using different gelling agents with agar and agarose

Hair bacteria were isolated on LB, NM, TS, and CASO plates using agar and agarose as gelling agents. Totally 38 species were isolated from human hair and identified under each condition (Fig. 3). The number of isolated bacteria on both agar and agarose was eight species belonging to five genera: *Dermacoccus nishinomiyaensis* (LB, NM, and CASO), *Moraxella osloensis* (LB, NM, and TS), *Roseomonas mucosa* (NM, TS, and CASO), *Sphingomonas panni* (NM and CASO), *Sphingomonas* sp. (LC800757)

~~phyllosphaerae~~ (LB), *Staphylococcus argenteus* (NM), *Staphylococcus caprae* (LB, NM, TS, and CASO), and *Staphylococcus epidermidis* (LB, TS, and CASO). The number of isolated bacteria on solely on agar was 16 species belonging to 10 genera: *Bacillus subtilis* subsp. *subtilis* (CASO, LB, and TS), *Brachybacterium muris* (TS), *Brevibacterium casei* (CASO), *Brevundimonas nasdae* (LB and TS), *Corynebacterium aquatimens* (LB), *Corynebacterium senegalense* (NM and TS), *Endobacter medicaginis* (NM), *Gulosibacter faecalis* (LB), *Mesobacillus boroniphilus* (CASO), *Methylobacterium longum* (NM), *Rothia kristinae* (LB), *Sphingomonas mali* (NM), *Sphingomonas palmae* (CASO), *Staphylococcus haemolyticus* (NM), and *Staphylococcus warneri* (LB and CASO). The number of bacteria isolated solely on agarose was 16 species belonging to nine genera: *Janibacter hoylei* (NM), *Kocuria arsenatis* (TS), *Micrococcus endophyticus* (TS), *Micrococcus luteus* (LB and NM), *Moraxella tetraodonis* (TS and CASO), *Paracoccus panacisoli* (LB and NM), *Sphingomonas* sp. (LC800765) *~~abaci~~* (CASO), *Sphingomonas aquatilis* (NM), *Sphingomonas pseudosanguinis* (TS), *Staphylococcus capitis* subsp. *capitis* (CASO), *Staphylococcus hominis* subsp. *hominis* (CASO), *Staphylococcus hominis* subsp. *novobiosepticus* (TS), *Staphylococcus saccharolyticus* (TS), *Stenotrophomonas bentonitica* (LB), *Stenotrophomonas geniculata* (CASO), and *Tianweitania* sp. (LC800759)*~~ediminis~~* (TS). The results showed that the bacterial species

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colonizing on agar and the agarose plates were different and the number of isolates was obtained without bias.

Comparison of isolated bacteria using media with and without pig fat addition

Hair bacteria were isolated by adding pig fat, similar to human fat, to standard media for aerobic and anaerobic cultivation. In total, 17 species were isolated from human hair and identified under each condition (Fig. 4). The number of bacteria isolated on media with and without pig fat were three species belonging to three genera: *Dermacoccus nishinomiyaensis* (aerobic PF and PFn), *Staphylococcus ~~aureus subs~~*sp. (LC800761) *~~aureus~~* (aerobic PFn and anaerobic PF), and *Moraxella osloensis* (aerobic PF and PFn). The number of isolated bacteria on media solely with the pig fat was 10 species belonging to 7 genera: *Bacillus subtilis* subsp. *subtilis* (aerobic PF), *Janibacter ~~limosu~~*sp. (LC819243) (anaerobic PF), *Microbacterium algeriense* (anaerobic PF), *Staphylococcus argenteus* (aerobic PF), *Staphylococcus epidermidis* (anaerobic PF), *Staphylococcus hominis* subsp. *hominis* (aerobic PF), *Staphylococcus saccharolyticus* (anaerobic PF), *Brevundimonas huaxiensis* (anaerobic PF), *Methylobacterium brachiatum* (aerobic PF), and *Roseomonas mucosa* (aerobic and anaerobic PF). The number of isolated bacteria from media without pig fat was four species belonging to four genera: *Dermacoccus* sp.

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(LC800771) ~~*barathri*~~ (aerobic PFn), *Micrococcus endophyticus* (anaerobic PFn), *Rhizobium qilianshanense* (aerobic PFn), and *Sphingomonas* sp. (LC800772) ~~*molluscorum*~~ (aerobic PFn). Our results showed that more hair bacteria could be isolated using pig fat-supplemented media than using media without pig fat.

Assessment of the carbon metabolism of hair-isolated bacteria.

We analyzed the carbon metabolism of several isolated bacteria using API 50 (Table 2). The predominant hair bacteria, including *Cutibacterium acnes* subsp. *defendens, Cutibacterium acnes* subsp. *acnes, Staphylococcus epidermidis, Staphylococcus caprae,* and *Micrococcus luteus,* showed differences in carbon metabolism between the type strains (*Cutibacterium acnes* subsp. *defendens* JCM 6473^T, *Cutibacterium acnes* subsp. *acnes* NBRC 107605^T, *Staphylococcus epidermidis* NBRC 100911^T, *Staphylococcus caprae* DSM 20608^T and *Micrococcus luteus* NBRC 3333^T) and isolates obtained in this study. The isolated strains *Cutibacterium acnes* sbsp. *defendens* and *Cutibacterium acnes* sbsp. *acnes* could metabolize mannitol. *Staphylococcus caprae* did not show the ability to metabolize lactose or trehalose. *Micrococcus luteus* showed the ability to metabolize D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, and D-sucrose. In addition, the major isolated strains consistently showed the ability to metabolize D-glucose, D-fructose,

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295 and D-mannose. The results showed that the predominant bacterial species inhabiting the
296 isolated hair had carbon metabolic capabilities that were different from those of the
297 reference strains.
298
299 **Evaluation of isolation possibility of hair bacteria under various conditions**
300 Hair bacteria were classified into 26 genera and 64 species were isolated from human
301 hair under 24 isolation conditions, including medium components, component
302 concentrations, gelling agents, and atmospheric environments (Table S1, Fig. S1). To
303 systematically evaluate the number of species that can be cultivated under an isolation
304 condition and the number of isolation conditions that can cultivate a bacterial species, the
305 numbers of isolated bacteria from human hair were counted, based on 24 isolation
306 conditions (Figs. 5A) and 26 genera (Figs. 5B, 5C and 5D) Four isolation conditions (Nos.
307 1, 5, 6, and 16) obtained a large number (≥9 species); while low number (≤2 species) of
308 hair bacteria were isolated by several isolation conditions (Nos. 10, 11, 15, 19, and 24)
309 (Fig. 5A). Conversely, 11 genera were isolated by only one isolation condition:
310 *Brachybacterium* sp. (No. 9), *Calidifontibacter* sp. (No. 2), *Gulosibacter* sp. (No. 1),
311 *Kocuria* sp. (No. 12), *Rothi*a sp. (No. 1), *Lederbergia* sp. (No. 20), *Endobacter* sp. (No.
312 5), *Mesobacillus* sp. (No. 13), *Pseudomonas* sp. (No. 14), *Rhizobium* sp. (No. 23), and

313 *Tianweitania* sp. (No. 12) (Fig. 5B). In total, 49 *Staphylococcus* spp., 15 *Moraxella* spp.,
314 and 12 *Sphingomonas* spp. were isolated under 20, 15, and 10 isolation conditions,
315 respectively (Figs. 5D, and 5C). In addition, the possibility of isolation, dividing the
316 number of isolation conditions for each genus by 24 of the total isolation conditions, was
317 calculated to evaluate the ease or difficulty of isolating each genus (Fig. 5D). Five genera
318 (*Dermacoccus*, *Microbacterium*, *Staphylococcus*, *Moraxella*, and *Shingomonas*) showed
319 high isolation possibilities (>25 %), whereas lower isolation possibilities (≤25%) were
320 observed in the other 21 genera. Watanabe et al. previously reported bacterial abundance
321 in hair using 16S rRNA gene amplicon analysis (22). The correlation between the number
322 of isolation conditions for each genus and its relative abundance in human hair (22) was
323 investigated (Fig. 6 and S2). The correlation coefficient was only 0.1086 (Fig. S2), which
324 suggested that it would not be possible to attribute the isolation of the target bacteria to
325 their abundance on the scalp hair. These results indicate that hair bacteria can be classified
326 into two groups: a high isolation possibility group, which is easy to isolate under various
327 conditions, and a low isolation possibility group, which is difficult to isolate and requires
328 specific conditions.
329
330 **DISCUSSION**

331 To understand the functionality of bacteria in the environment, individual bacteria must
332 be cultivated in pure cultures from their growth environment (3). Although the
333 predominant bacterial species inhabiting the hair surface have been identified (20,22,23),
334 few studies have reported bacterial isolation from hair and their functionality remains
335 unclear. In particular, the predominant bacterial species isolated (*Cutibacterium acnes*
336 subsp. *defendens and Cutibacterium acnes* subsp. *acnes,* and *Micrococcus luteus*) from
337 hair in this study had different carbon metabolic capabilities such as mannitol and glucose
338 than those of the reference strains (Table 2). Mannitol is one of the components in hair
339 care products (26) while human sweat contain glucose (12). Thus, these compounds
340 would be utilized as carbon sources by hair bacteria. It is hypothesized that several
341 bacterial isolates on hair would obtain carbon metabolic capabilities for hair-specific
342 carbon sources. These results suggested the necessity of bacterial isolation from target
343 samples to understand bacterial functionality in hair. In this study, 64 bacterial species
344 belonging to 26 genera were isolated from hair samples using different culture conditions
345 and nutrient media. The isolation possibility proposed and evaluated, is discussed later.
346 A component of standard media at high levels has been reported to not only stimulate
347 the bacterial growth rate but also inhibit growth (3, 9), which would result in the inhibition
348 of colony formation in certain slow-growing bacteria. However, dilution of the standard

medium is considered effective for isolating bacteria that cannot form colonies using high-nutrient media (28,29). In this study, non-diluted and 10% diluted media were investigated to compare the bacterial species in NM, LB, TS, CASO, and GAM media (Fig. 1). Sixteen species belonging to seven genera were isolated solely using 10% diluted media, whereas 17 species belonging to 10 genera were isolated using non-diluted media (Fig. 1). These results suggest that hair bacteria would consist of nutrient-rich and nutrient-poor bacteria. Contrary to the general use of standard media to isolate skin bacteria (17), the use of both standard nutrient media and diluted media is required to efficiently isolate hair-associated bacteria.

Several species including *Cutibacterium* spp., *Staphylococcus* spp., *Brevundimonas huaxiensis*, *Janibacter* ~~limosu~~sp. (LC819243), and *Microbacterium algeriense* were isolated under only anaerobic condition (Fig. 2). These bacteria (30–34) have been reported to be facultative anaerobes except for *Janibacter limosus* (a top hit 16S similarity of *Janibacter* sp. (LC819243)) (35). In particular, *Cutibacterium* spp. with the highest relative abundance of ca. 40% on human hair (22), showed good growth under anaerobic condition (30,36). This is why these facultative anaerobes would be isolated under anaerobic condition. In addition, hair root would show more anerobic condition and higher bacterial density than hair shaft (20). From these results, it is suggested that

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367 facultative anaerobes would grow on hair root well, and survive on hair shaft.

368 Agar has been used as a general gelling agent to prepare microbial isolation media for
369 more than 100 years; however, it has been reported to contain inhibitors of several
370 microorganisms (37,38), which prevents the efficient isolation of several environmental
371 microorganisms. In this study, bacterial isolation was performed with standard agar media
372 containing unpurified substances, including an inhibitor and purified agarose (Fig. 3). In
373 total, 16 species belonging to 10 genera were isolated from agarose-only media (Fig. 3E),
374 suggesting that these isolates were sensitive to inhibitors in agar. Seventeen species
375 belonging to 11 genera were isolated using the agar medium alone (Fig. 3E). As agar is
376 not purified and is composed of several substances, including agarose and agaropectin
377 (38), these isolates were suggested to require unknown substances as growth activators
378 for the isolation of hair bacteria. Therefore, the type of gelling agent is a significant factor
379 in the isolation of hair bacteria.

380 Although a nutrient for hair bacteria has not been identified, sebum components
381 secreted from the scalp skin have been proposed as nutrients (39). Isolation with standard
382 media and the addition of pig fat (PF) as a carbon source were performed under several
383 conditions (Nos. 21–24). In total, 11 species belonging to seven genera were isolated with
384 the addition of PF (Fig. 4). Notably, *Methylobacterium brachiatum*, *Brevundimonas*

385 *huaxiensis*, *Microbacterium algeriense* and *Janibacter limosus* were not isolated from
386 hair in this study without PF addition. On the other hands, type strains of those bacterial
387 species were reported to grow on standard media including NM (*Methylobacterium*
388 *brachiatum*^{T} and *Brevundimonas huaxiensis*^{T}) and TS (*Microbacterium algeriense*^{T} and
389 *Janibacter limosus*^{T}) without PF addition (34,40–42). Contrary to the type strains, these
390 isolated bacterial strains from hair were considered to prefer sebum components as the
391 carbon source. These results suggest that the addition of sebum as a carbon source to the
392 media has the potential to improve the efficiency of isolating lipid-preferring bacteria
393 from human hair. Therefore, the lipid metabolism of these isolates should be further
394 investigated.

395 Culturability, defined as the viable count expressed as a percentage of the
396 microscopically determined total cell count for isolation sources, has conventionally been
397 used as an indicator of the presence of uncultured bacteria (43). However, we proposed a
398 novel indicator "isolation possibility" to evaluate the difficulties of hair bacterial isolates
399 at genera levels using standard media under various isolation conditions (Figs. 5 and S2).
400 The isolates were categorized into two groups based on possibilities: high isolation
401 possibilities (>25%), such as *Staphylococcus* spp., *Moraxella* spp., and *Sphingomonas*
402 spp., and low isolation possibilities (≤25%), such as *Brachybacterium* sp.,

403 *Calidifontibacter* sp., and *Gulosibacter* sp. (Fig. 5D). These results suggested the
404 existence of “easily isolated bacteria” and “difficultly isolated bacteria” on human hair.
405 Because most of studies reports on bacterial isolates under successfully isolation
406 conditions without information on unsuccessful isolation conditions, the researcher must
407 make much effort to isolate a target bacterium by a culture method, which would result
408 in time-consuming, costly, and labor works. Accumulation of the isolation possibilities
409 would not only give academically valuable knowledge but also help a researcher to
410 develop a strategy of isolation methods with or without standard media. On the other hand,
411 this parameter of isolation possibility would vary depending on isolation sources (i.e.,
412 environmental samples) and isolation conditions (i.e. medium components, component
413 concentrations, gelling agents, and atmospheric environments), therefore, generalizability
414 would be required to apply this parameter for isolation of environmental microorganisms.
415 Therefore, the development of isolation methods to improve the possibility of isolating
416 difficult-to-isolate bacteria and to understand the underlying mechanisms will be crucial
417 for future microbial isolation research.
418
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557

558 **Figure legends**

559 FIG. 1. Venn diagram of isolated hair bacteria on undiluted (1, 5, 9, 13, and 19) and 10%
560 diluted media (2, 6, 10, 14, and 20).
561 (A) Aerobic isolation with LB, (B) Aerobic isolation with NM, (C) Aerobic isolation with
562 TS, (D) Aerobic isolation with CASO, (E) Anaerobic isolation with GAM, and (F)
563 Summarized Venn diagram of (A) to (E).

564

565 FIG. 2. Venn diagram of isolated hair bacteria on standard culture medium in aerobic (1,

5, 9, 13, 17, and 21) and anaerobic (3, 7, 11, 15, 17, and 21) cultivation.

(A) Isolation with LB, (B) Isolation with NM, (C) Isolation with TS, (D) Isolation with CASO, (E) Isolation with Col, (F) Isolation with PF, and (G) Summarized Venn diagram of (A) to (F).

FIG. 3. Venn diagram of isolated hair bacteria on agar (1, 5, 9, and 13) and agarose (4, 8, 12, and 15) plates.

(A) Isolation with LB, (B) Isolation with NM, (C) Isolation with TS, (D) Isolation with CASO, and (E) Summarized Venn diagram of (A) to (D).

FIG. 4. Venn diagram of isolated hair bacteria on agar plates with lipid (21 and 22) and without lipid from pig (23 and 24).

(A) Aerobic isolation with PF and PFn, (B) Anaerobic isolation with PF and PFn, and (C) Summarized Venn diagram of (A) and (B).

FIG. 5. Bacterial genera identified in colonies grown under each culture condition.

(A) Total number of bacteria isolated from 24 culture conditions, (B) Difficulty of colonization for each bacterial genus as indicated by the number of bacterial isolates, (C)

584 Number of conditions of isolated bacteria, and (D) Isolation possibility of isolated hair
585 bacteria. Probability was defined as the number of isolation conditions for each genus
586 divided by 24 of the total isolation conditions.
587
588 FIG. 6. Summary list of isolation bacterial species from hair by 24 culture conditions and
589 heat map of their relative abundances on hair. The values of relative abundance of isolated
590 bacterial species on hair were referred to the report by Watanabe et al. (22).

1 **Title**

2 **Isolated hair bacteria reveal different isolation possibilities under various conditions**

3 Running title: Distinct isolation possibilities for hair bacteria

4

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18 Keywords: hair bacteria; isolation possibilities; isolated microorganisms; standard media;

carbon metabolic capabilities

ABSTRACT

Microorganisms are assumed to inhabit various environments and organisms, including the human body. The presence of more than 700 bacterial species on scalp hair has been reported through rRNA gene amplicon analysis. However, the biological properties of bacteria on the scalp hair (hair bacteria) and their functions are poorly understood as few hair bacteria have been isolated from hair in previous studies. This study aimed to isolate hair bacteria using standard media under 24 different conditions (including medium components, component concentrations, gelling agents, and atmospheric environments). Furthermore, we evaluated the possibility of isolating strains under these isolation conditions and examined the carbon metabolic ability of several predominantly isolated strains. A total of 64 bacterial species belonging to 26 genera were isolated from hair under 24 isolation conditions. The predominant bacterial species isolated from human hair in this study showed different carbon metabolic capabilities than those of the reference strains. In addition, “isolation possibility” was newly proposed to systematically evaluate the number of isolation conditions that could cultivate a bacterial species. Based on isolation possibility, the isolates were categorized into groups with a

high number of isolation conditions (e.g., ≥25%; such as *Staphylococcus*) and those with a low number (e.g., ≤25%; such as *Brachybacterium*). These findings indicate the existence of "easily isolated microorganisms" and "difficultly isolated microorganism" from human hair.

INTRODUCTION

Microorganisms have been considered to inhabit various environments by adapting to different conditions, such as temperature, oxygen level, pH, salinity, and growth matrices (1–3). They play a role in the biological cycle of elements in nature and affect the lifestyle of other organisms, including humans (4,5). Recently, many studies have attempted to identify the microbiota in different environments using rRNA gene amplicon analysis and metagenomics using next-generation sequencing (NGS) without isolating the microorganisms (6,7). Recently, metagenome-assembled genomes (MAGs) has attracted attention as a non-culture method to be reconstructed from metagenome data, and this method would give understanding of microbiota and their interactions under a targeted environment (8). However, it would be difficult to elucidate actual functions and roles of environmental microorganisms by this method (9). Thus, the isolation of microorganisms is essential for elucidating their ecology and function in various environments (10). In addition, the acquisition of microorganisms can be applied to establish novel biotechnologies, including the chemical, pharmaceutical, and food industries, as well as for environmental conservation (3).

Microorganisms have been isolated from the environment using solid media since the end of the 19th century (10). Solid media are classified into two types: natural media or synthetic media that mimic the natural environment (11,12) and standard media such as

61 nutrient medium (NM) and Luria-Bertani (LB) medium (10,13). Standard media have
62 been considered advantageous for the isolation of many types of microorganisms and the
63 isolation processes require extensive investigation of isolation conditions (media
64 (component concentration and composition) and culture conditions (such as pH,
65 temperature, and aerobic or anaerobic conditions)), which would result in labor- and time-
66 consuming work. Most microorganisms are unisolated or uncultured using standard
67 media, even after extensive investigation under many isolation conditions. The number
68 of microorganisms cultured or uncultured in a targeted environment has been evaluated
69 based on a quantitative parameter “culturability”, defined as the viable count expressed
70 as a percentage of the microscopically determined total count of cells for the isolation
71 sources used in that particular cultivation experiment (14). However, few studies have
72 reported the number of isolation conditions that can cultivate an isolated microorganism.
73 Here, we proposed a novel parameter; “isolation possibility”, because a systematic
74 evaluation of isolation possibility using standard media would improve the efficiency of
75 isolating useful and targeted microorganisms.

76 Understanding the resident bacterial ecosystem in the human body is crucial for
77 clarifying its impact on human health (15,16). To date, the function of skin bacteria and
78 the interactions between skin bacteria and human hosts have been elucidated using

79 microorganisms isolated from the human body (17). Notably, skin bacteria have drawn
80 attention to the skin's barrier function, immunomodulation, and their implications for
81 dermatological condition (18,19). However, we recently reported on a unique bacterial
82 community structure of human hair from root to tip（hair bacteria), consisting of
83 predominant bacterial phyla including Actinobacteria, Firmicutes, and Proteobacteria
84 by16S rRNA gene amplicon analysis using NGS (20–23). Furthermore, several types of
85 strains related to the predominant hair bacterial species indicated by 16S rRNA gene
86 amplicon analysis have been reported to influence human keratinocyte cellular activity,
87 which suggests the health of the scalp and hair, including repair of the damaged scalp and
88 hair growth by hair bacteria (24). Thus, the isolation of hair bacteria would be significant
89 not only to elucidate their characteristics and functions in human hosts but also for human
90 scalp/hair health. In addition, only a few reports are available on evaluating the growth
91 characteristics of hair bacteria; therefore, the ability of hair bacteria to metabolize
92 substrates remains unclear.
93 The objectives of this study were to isolate hair bacteria under various isolation
94 conditions using standard media, evaluate the possibility of isolating strains, and elucidate
95 the metabolic ability of the carbon sources of several isolated strains.
96

MATERIALS AND METHODS

Samples and collection

Scalp hair shaft samples were collected from 18 healthy individuals (10 females and 8 males) ranging in age from 20 to 40 years who consented to participate in the study. Scalp hair shaft samples were cut using sterile scissors with nitrile gloves. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2013. The participants provided written informed consent with the approval of the Ethics Committee of the Graduate School of Bioscience and Biotechnology at Kyushu University (Authorized No. 104). The methods were carried out in accordance with approved guidelines.

Isolation culture conditions

The isolation procedure was based on the standard operating procedure SOP-GG-02-00 (25), with hair replacing the source material in this study. The standard media used were Luria-Bertani medium (LB), nutrient broth medium (NM), trypticase soy medium (TS), casein-peptone soy meal-peptone medium (CASO), Columbia Blood Medium (Col), Gifu Anaerobic Medium (GAM), and pig fat medium (PF). The compositions of the

115 respective media were as follows: LB with 1 g/L of tryptone (Thermo Fisher Scientific,
116 Tokyo, Japan), 5 g/L of yeast extract (BD), and 10 g/L of NaCl; NM with 5.0 g/L of
117 peptone (Becton, Dickinson and Company (BD), NJ, USA) and 3 g/L of meat extract
118 (Nacalai Tesque, Kyoto, Japan); TS with 30 g/L of trypticase soy broth (BD); CASO with
119 8 g/L of tryptone (BD), 15 g/L of yeast extract (BD), 5.0 g /L of peptone from soymeal
120 (Nacalai Tesque), and 5 g/L of NaCl; Col with peptone from casein (BD) 10.0 g/L,
121 peptone from meat (BD) 5.0 g/L, heart extract (BD) 3.0 g/L, yeast extract (BD) 5.0 g/L,
122 starch 1.0 g/L, NaCl 5.0 g/L, defibrinated sheep blood (BD) 5.0 g/L and agar 13.0 g/L.;
123 GAM with 59 g/L of GAM Broth (Nissui Pharmaceutical Co., Ltd., Tokyo, BD); PF with
124 5 g/L of pig lipid extract, 0.05 g/L NH_4NO_3, 2 g/L yeast extract (BD); and non-pig fat
125 (PFn) with 0.05 g/L of NH_4NO_3, 2 g/L yeast extract (BD); and an AnaeroPack (Mitsubishi
126 Gas Chemical Co., Inc., Tokyo, Japan) was used in the anaerobic jar for anaerobic
127 cultivation. LB, NM, TS, CASO, Col, PF, and PFn were incubated at 30°C and GAM at
128 37°C. LB, NM, TS, CASO, Col, and PF were cultured under both aerobic and anaerobic
129 conditions. Gelling agents, agar, and agarose were used for LB, NA, TS, and CASO
130 assays. Low nutrient concentrations and 10% dilution medium conditions for LB, NM,
131 TS, CASO, and GAM were also used. The culture conditions are listed in Table 1.
132

Colony purification and DNA extraction

Colonies were obtained by placing hair on a plate and incubating under each culture condition. Single colonies were inoculated into a novel plate twice for purification. Single colonies were suspended in TE solution. The mixture was heated at 100°C for 10 min and centrifuged at 13,000 rpm for 20 min. The supernatant was the DNA extraction product and was stored at -20°C until use.

PCR and 16S rRNA gene sequence analysis

The region of the 16S rRNA gene in each sample was amplified using universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3′), 1492R (5'-TACGGYTACCTTGTTACGACTT-3′). PCR was performed using Premix Taq (TaKaRa, Shiga, Japan): 5 min at 94°C, 30 cycles of denaturation 94°C for 1 min, annealing 55°C for 45 s, elongation at 72°C for 2 min, and final elongation at 72°C for 10 min. To purify the PCR products, the target bands were extracted by 1.5% agarose gel electrophoresis and purified using the FastGeneTM Gel/PCR Extraction Kit (Nippon Genetics, Japan). DNA analysis was performed using a Sanger sequencing platform performed by GENEWIZ (Tokyo, Japan), and the obtained sequences were used to identify the bacterial species using EzBioCloud (https://www.ezbiocloud.net/) based on a similarity score

(≥98.7%) with those of the type strain. Phylogenetic analysis employed the neighbor joining method with 1000 bootstrap replicates, and phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (version X) software (https://www.megasoftware.net/). The sequences of 64 identified hair bacterial isolates were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC800711–LC800725, LC800727-LC800774, and LC819243.

Evaluation of metabolized carbon sources by hair bacteria

The carbon metabolism capacity was exploited by the API®50CH kit (Biomerieux Japan, Tokyo). The target bacterial strains evaluated were six species identified in previous studies as having a high prevalence in the scalp environment: *Cutibacterium acnes* subsp. *defendens, Cutibacterium acnes* subsp. *acnes, Staphylococcus epidermidis, Staphylococcus caprae* and *Micrococcus luteus.* Target bacterial isolates were incubated and purified. Colonies were suspended in the pre-culture medium for bacterial adaptation, using the API®20A medium (Biomerieux Japan, Tokyo) for *Cutibacterium acnes* subsp. *defendens* and *Cutibacterium acnes* subsp. *acnes*, and API®50CHL medium for *Staphylococcus epidermidis*, *Staphylococcus caprae*, and *Micrococcus luteus*. The cultures were inoculated into API®50CH plates and incubated at 30°C for 48 h.

169 *Cutibacterium* spp. plates were assayed with the BCP reagent. The carbon metabolism
170 capacity of bacterial type strain was obtained from the APIWEB (Biomerieux Japan Ltd,
171 https://apiweb.biomerieux.com/identIndex) and compared with the isolates.
172
173 **RESULTS**
174 **Comparison of aerobically isolated bacteria using undiluted and diluted standard**
175 **media**
176 Hair bacteria were isolated from LB, NM, TS, CASO, and GAM plates using undiluted
177 and 10% diluted standard media. Totally 41 species were isolated from human hair and
178 identified under each condition (Fig. 1). The numbers of isolated bacteria on both
179 undiluted and diluted media were eight species belonging to six genera: *Dermacoccus*
180 *nishinomiyaensis* (NM and GAM), *Bacillus subtilis* sbsp. *subtilis* (LB, NM, TS, and
181 CASO), *Staphylococcus epidermidis* (NM, CASO and GAM), *Staphylococcus warneri*
182 (LB, NM, and CASO), *Brevibacterium casei* (CASO and GAM), *Brevundimonas nasdae*
183 (NM, TS), *Moraxella osloensis* (LB, NM, TS, and GAM), and *Roseomonas mucosa* (LB,
184 NM, and CASO). The numbers detected solely on undiluted media were 16 species
185 belonging to 10 genera,: *Brachybacterium muris* (TS), *Corynebacterium aquatimens*
186 (LB), *Corynebacterium senegalense* (NM and TS), *Cutibacterium acnes* subsp. *defendens*

(GAM), *Gulosibacter faecalis* (LB), *Staphylococcus argenteus* (TS), *Staphylococcus caprae* (LB and NM), *Staphylococcus haemolyticus* (NM), *Endobacter medicaginis* (NM), *Mesobacillus boroniphilus* (CASO), *Methylobacterium longum* (NM), *Rothia kristinae* (LB), *Sphingomonas mali* (NM), *Sphingomonas palmae* (CASO), *Sphingomonas panni* (LB), and *Sphingomonas* sp. (LC800757) (CASO). The numbers detected solely on 10% diluted media were 16 species belonging to seven genera: *Calidifontibacter indicus* (LB), *Microbacterium aerolatum* (LB), *Microbacterium foliorum* (NM), *Microbacterium maritypicum* (NM), *Microbacterium endophyticus* (GAM), *Lederbergia wuyishanensis* (GAM), *Staphylococcus capitis* subsp. *capitis* (CASO), *Staphylococcus capitis* subsp. *urealyticus* (CASO), *Staphylococcus hominis* subsp. *novobiosepticus* (CASO), *Staphylococcus lugdunensis* (LB and NM), *Staphylococcus saccharolyticus* (CASO), *Brevibacterium sediminis* (NM), *Brevibacterium vesicularis* (NM), *Pseudomonas oryzihabitans* (CASO), *Sphingomonas* sp. (LC800754) (CASO), and *Sphingomonas* sp. (LC800758) (LB). These results showed that similar numbers of different bacterial species were obtained under undiluted (16 species) and diluted media (16 species) conditions and that the use of both standard nutrient media and diluted media would be efficient in isolating hair bacteria.

205 **Comparison of isolated bacteria under aerobic and anerobic conditions**

206 Hair bacteria were isolated via aerobic or anaerobic cultivation on LB, NM, TS, CASO,
207 Cal, and PF plates. Totally 38 species were isolated from human hair and identified under
208 each condition (Fig. 2). The number of bacteria isolated in both aerobic and anaerobic
209 cultures was four species belonging to three genera: *Staphylococcus caprae* (LB, NM,
210 and CASO), *Staphylococcus epidermidis* (NM and CASO), *Moraxella osloensis* (LB,
211 NM, TS, and PF), and *Roseomonas mucosa* (NM and PF). The number of isolated bacteria
212 solely in aerobic cultivation was 22 species belonging to 12 genera,: *Bacillus subtilis*
213 subsp. *subtilis* (LB, TS, CASO, and PF)*, Brachybacterium muris* (TS), *Corynebacterium*
214 *aquatimens* (LB), *Corynebacterium senegalense* (NM and TS), *Dermacoccus*
215 *nishinomiyaensis* (NM and PF), *Gulosibacter faecalis* (LB), *Rothia kristinae* (LB),
216 *Staphylococcus argenteus* (TS and PF), *Staphylococcus haemolyticus* (NM),
217 *Staphylococcus hominis* subsp. *hominis* (PF), *Staphylococcus warneri* (LB and CASO),
218 *Brevibacterium casei* (CASO), *Brevibacterium nasdae* (LB, TS), *Endobacter medicaginis*
219 (NM), *Mesobacillus boroniphilus* (CASO), *Methylobacterium brachiatum* (PF),
220 *Methylobacterium longum* (NM), *Sphingomonas mali* (NM), *Sphingomonas palmae*
221 (CASO), *Sphingomonas panni* (LB), and *Sphingomonas* sp. (LC800757) (CASO). The
222 number of bacteria isolated solely in anaerobic cultivation was 11 species belonging to

five genera: *Brevundimonas huaxiensis* (PF), *Cutibacterium acnes* subsp. *acnes* (LB, NM, TS, CASO, and Col), *Cutibacterium acnes* subsp. *defendens* (LB, NM, TS, CASO, and Col), *Cutibacterium namnetense* (CASO and Col), *Janibacter* sp. (LC819243) (PF), *Microbacterium algeriense* (PF), *Staphylococcus* sp. (LC800761) (PF), *Staphylococcus capitis* subsp. *urealyticus* (LB), *Staphylococcus lugdunensis* (Col), *Staphylococcus saccharolyticus* (LB, CASO, and PF), and *Staphylococcus saprophyticus* subsp. *saprophyticus* (LB). These results indicate that more species of hair bacteria can colonize under aerobic conditions than under anaerobic conditions.

Comparison of isolated bacteria using different gelling agents with agar and agarose

Hair bacteria were isolated on LB, NM, TS, and CASO plates using agar and agarose as gelling agents. Totally 38 species were isolated from human hair and identified under each condition (Fig. 3). The number of isolated bacteria on both agar and agarose was eight species belonging to five genera: *Dermacoccus nishinomiyaensis* (LB, NM, and CASO), *Moraxella osloensis* (LB, NM, and TS), *Roseomonas mucosa* (NM, TS, and CASO), *Sphingomonas panni* (NM and CASO), *Sphingomonas* sp. (LC800757) (LB), *Staphylococcus argenteus* (NM), *Staphylococcus caprae* (LB, NM, TS, and CASO), and *Staphylococcus epidermidis* (LB, TS, and CASO). The number of isolated bacteria on

241 solely on agar was 16 species belonging to 10 genera: *Bacillus subtilis* subsp. *subtilis*
242 (CASO, LB, and TS), *Brachybacterium muris* (TS), *Brevibacterium casei* (CASO),
243 *Brevundimonas nasdae* (LB and TS), *Corynebacterium aquatimens* (LB),
244 *Corynebacterium senegalense* (NM and TS), *Endobacter medicaginis* (NM),
245 *Gulosibacter faecalis* (LB), *Mesobacillus boroniphilus* (CASO), *Methylobacterium*
246 *longum* (NM), *Rothia kristinae* (LB), *Sphingomonas mali* (NM), *Sphingomonas palmae*
247 (CASO), *Staphylococcus haemolyticus* (NM), and *Staphylococcus warneri* (LB and
248 CASO). The number of bacteria isolated solely on agarose was 16 species belonging to
249 nine genera: *Janibacter hoylei* (NM), *Kocuria arsenatis* (TS), *Micrococcus endophyticus*
250 (TS), *Micrococcus luteus* (LB and NM), *Moraxella tetraodonis* (TS and CASO),
251 *Paracoccus panacisoli* (LB and NM), *Sphingomonas* sp. (LC800765) (CASO),
252 *Sphingomonas aquatilis* (NM), *Sphingomonas pseudosanguinis* (TS), *Staphylococcus*
253 *capitis* subsp. *capitis* (CASO), *Staphylococcus hominis* subsp. *hominis* (CASO),
254 *Staphylococcus hominis* subsp. *novobiosepticus* (TS), *Staphylococcus saccharolyticus*
255 (TS), *Stenotrophomonas bentonitica* (LB), *Stenotrophomonas geniculata* (CASO), and
256 *Tianweitania* sp. (LC800759) (TS). The results showed that the bacterial species
257 colonizing on agar and the agarose plates were different and the number of isolates was
258 obtained without bias.

Comparison of isolated bacteria using media with and without pig fat addition

Hair bacteria were isolated by adding pig fat, similar to human fat, to standard media for aerobic and anaerobic cultivation. In total, 17 species were isolated from human hair and identified under each condition (Fig. 4). The number of bacteria isolated on media with and without pig fat were three species belonging to three genera: *Dermacoccus nishinomiyaensis* (aerobic PF and PFn), *Staphylococcus* sp. (LC800761) (aerobic PFn and anaerobic PF), and *Moraxella osloensis* (aerobic PF and PFn). The number of isolated bacteria on media solely with the pig fat was 10 species belonging to 7 genera: *Bacillus subtilis* subsp. *subtilis* (aerobic PF), *Janibacter* sp. (LC819243) (anaerobic PF), *Microbacterium algeriense* (anaerobic PF), *Staphylococcus argenteus* (aerobic PF), *Staphylococcus epidermidis* (anaerobic PF), *Staphylococcus hominis* subsp. *hominis* (aerobic PF), *Staphylococcus saccharolyticus* (anaerobic PF), *Brevundimonas huaxiensis* (anaerobic PF), *Methylobacterium brachiatum* (aerobic PF), and *Roseomonas mucosa* (aerobic and anaerobic PF). The number of isolated bacteria from media without pig fat was four species belonging to four genera: *Dermacoccus* sp. (LC800771) (aerobic PFn), *Micrococcus endophyticus* (anaerobic PFn), *Rhizobium qilianshanense* (aerobic PFn), and *Sphingomonas* sp. (LC800772) (aerobic PFn). Our results showed that more hair

277 bacteria could be isolated using pig fat-supplemented media than using media without pig
278 fat.
279
280 **Assessment of the carbon metabolism of hair-isolated bacteria.**
281 We analyzed the carbon metabolism of several isolated bacteria using API 50 (Table
282 2). The predominant hair bacteria, including *Cutibacterium acnes* subsp. *defendens,*
283 *Cutibacterium acnes* subsp. *acnes, Staphylococcus epidermidis, Staphylococcus caprae,*
284 and *Micrococcus luteus,* showed differences in carbon metabolism between the type
285 strains (*Cutibacterium acnes* subsp. *defendens* JCM 6473^T, *Cutibacterium acnes* subsp.
286 *acnes* NBRC 107605^T, *Staphylococcus epidermidis* NBRC 100911^T, *Staphylococcus*
287 *caprae* DSM 20608^T and *Micrococcus luteus* NBRC 3333^T) and isolates obtained in this
288 study. The isolated strains *Cutibacterium acnes* sbsp. *defendens* and *Cutibacterium acnes*
289 sbsp. *acnes* could metabolize mannitol. *Staphylococcus caprae* did not show the ability
290 to metabolize lactose or trehalose. *Micrococcus luteus* showed the ability to metabolize
291 D-glucose, D-fructose, D-mannose, D-maltose, D-lactose, and D-sucrose. In addition, the
292 major isolated strains consistently showed the ability to metabolize D-glucose, D-fructose,
293 and D-mannose. The results showed that the predominant bacterial species inhabiting the
294 isolated hair had carbon metabolic capabilities that were different from those of the

295 reference strains.

296

297 **Evaluation of isolation possibility of hair bacteria under various conditions**

298 Hair bacteria were classified into 26 genera and 64 species were isolated from human
299 hair under 24 isolation conditions, including medium components, component
300 concentrations, gelling agents, and atmospheric environments (Table S1, Fig. S1). To
301 systematically evaluate the number of species that can be cultivated under an isolation
302 condition and the number of isolation conditions that can cultivate a bacterial species, the
303 numbers of isolated bacteria from human hair were counted, based on 24 isolation
304 conditions (Figs. 5A) and 26 genera (Figs. 5B, 5C and 5D) Four isolation conditions (Nos.
305 1, 5, 6, and 16) obtained a large number (≥9 species); while low number (≤2 species) of
306 hair bacteria were isolated by several isolation conditions (Nos. 10, 11, 15, 19, and 24)
307 (Fig. 5A). Conversely, 11 genera were isolated by only one isolation condition:
308 *Brachybacterium* sp. (No. 9), *Calidifontibacter* sp. (No. 2), *Gulosibacter* sp. (No. 1),
309 *Kocuria* sp. (No. 12), *Rothi*a sp. (No. 1), *Lederbergia* sp. (No. 20), *Endobacter* sp. (No.
310 5), *Mesobacillus* sp. (No. 13), *Pseudomonas* sp. (No. 14), *Rhizobium* sp. (No. 23), and
311 *Tianweitania* sp. (No. 12) (Fig. 5B). In total, 49 *Staphylococcus* spp., 15 *Moraxella* spp.,
312 and 12 *Sphingomonas* spp. were isolated under 20, 15, and 10 isolation conditions,

313 respectively (Figs. 5D, and 5C). In addition, the possibility of isolation, dividing the
314 number of isolation conditions for each genus by 24 of the total isolation conditions, was
315 calculated to evaluate the ease or difficulty of isolating each genus (Fig. 5D). Five genera
316 (*Dermacoccus*, *Microbacterium*, *Staphylococcus*, *Moraxella*, and *Shingomonas*) showed
317 high isolation possibilities (>25 %), whereas lower isolation possibilities (≤25%) were
318 observed in the other 21 genera. Watanabe et al. previously reported bacterial abundance
319 in hair using 16S rRNA gene amplicon analysis (22). The correlation between the number
320 of isolation conditions for each genus and its relative abundance in human hair (22) was
321 investigated (Fig. 6 and S2). The correlation coefficient was only 0.1086 (Fig. S2), which
322 suggested that it would not be possible to attribute the isolation of the target bacteria to
323 their abundance on the scalp hair. These results indicate that hair bacteria can be classified
324 into two groups: a high isolation possibility group, which is easy to isolate under various
325 conditions, and a low isolation possibility group, which is difficult to isolate and requires
326 specific conditions.
327
328 **DISCUSSION**
329 To understand the functionality of bacteria in the environment, individual bacteria must
330 be cultivated in pure cultures from their growth environment (3). Although the

predominant bacterial species inhabiting the hair surface have been identified (20,22,23), few studies have reported bacterial isolation from hair and their functionality remains unclear. In particular, the predominant bacterial species isolated (*Cutibacterium acnes* subsp. *defendens and Cutibacterium acnes* subsp. *acnes,* and *Micrococcus luteus*) from hair in this study had different carbon metabolic capabilities such as mannitol and glucose than those of the reference strains (Table 2). Mannitol is one of the components in hair care products (26) while human sweat contain glucose (12). Thus, these compounds would be utilized as carbon sources by hair bacteria. It is hypothesized that several bacterial isolates on hair would obtain carbon metabolic capabilities for hair-specific carbon sources. These results suggested the necessity of bacterial isolation from target samples to understand bacterial functionality in hair. In this study, 64 bacterial species belonging to 26 genera were isolated from hair samples using different culture conditions and nutrient media. The isolation possibility proposed and evaluated, is discussed later.

A component of standard media at high levels has been reported to not only stimulate the bacterial growth rate but also inhibit growth (3, 9), which would result in the inhibition of colony formation in certain slow-growing bacteria. However, dilution of the standard medium is considered effective for isolating bacteria that cannot form colonies using high-nutrient media (28,29). In this study, non-diluted and 10% diluted media were

investigated to compare the bacterial species in NM, LB, TS, CASO, and GAM media (Fig. 1). Sixteen species belonging to seven genera were isolated solely using 10% diluted media, whereas 17 species belonging to 10 genera were isolated using non-diluted media (Fig. 1). These results suggest that hair bacteria would consist of nutrient-rich and nutrient-poor bacteria. Contrary to the general use of standard media to isolate skin bacteria (17), the use of both standard nutrient media and diluted media is required to efficiently isolate hair-associated bacteria.

Several species including *Cutibacterium* spp., *Staphylococcus* spp., *Brevundimonas huaxiensis*, *Janibacter* sp. (LC819243), and *Microbacterium algeriense* were isolated under only anaerobic condition (Fig. 2). These bacteria (30–34) have been reported to be facultative anaerobes except for *Janibacter limosus* (a top hit 16S similarity of *Janibacter* sp. (LC819243)) (35). In particular, *Cutibacterium* spp. with the highest relative abundance of ca. 40% on human hair (22), showed good growth under anaerobic condition (30,36). This is why these facultative anaerobes would be isolated under anaerobic condition. In addition, hair root would show more anerobic condition and higher bacterial density than hair shaft (20). From these results, it is suggested that facultative anaerobes would grow on hair root well, and survive on hair shaft.

Agar has been used as a general gelling agent to prepare microbial isolation media for

more than 100 years; however, it has been reported to contain inhibitors of several microorganisms (37,38), which prevents the efficient isolation of several environmental microorganisms. In this study, bacterial isolation was performed with standard agar media containing unpurified substances, including an inhibitor and purified agarose (Fig. 3). In total, 16 species belonging to 10 genera were isolated from agarose-only media (Fig. 3E), suggesting that these isolates were sensitive to inhibitors in agar. Seventeen species belonging to 11 genera were isolated using the agar medium alone (Fig. 3E). As agar is not purified and is composed of several substances, including agarose and agaropectin (38), these isolates were suggested to require unknown substances as growth activators for the isolation of hair bacteria. Therefore, the type of gelling agent is a significant factor in the isolation of hair bacteria.

Although a nutrient for hair bacteria has not been identified, sebum components secreted from the scalp skin have been proposed as nutrients (39). Isolation with standard media and the addition of pig fat (PF) as a carbon source were performed under several conditions (Nos. 21–24). In total, 11 species belonging to seven genera were isolated with the addition of PF (Fig. 4). Notably, *Methylobacterium brachiatum*, *Brevundimonas huaxiensis*, *Microbacterium algeriense* and *Janibacter limosus* were not isolated from hair in this study without PF addition. On the other hands, type strains of those bacterial

385 species were reported to grow on standard media including NM (*Methylobacterium*
386 *brachiatum*^{T} and *Brevundimonas huaxiensis*^{T}) and TS (*Microbacterium algeriense*^{T} and
387 *Janibacter limosus*^{T}) without PF addition (34,40–42). Contrary to the type strains, these
388 isolated bacterial strains from hair were considered to prefer sebum components as the
389 carbon source. These results suggest that the addition of sebum as a carbon source to the
390 media has the potential to improve the efficiency of isolating lipid-preferring bacteria
391 from human hair. Therefore, the lipid metabolism of these isolates should be further
392 investigated.

393 Culturability, defined as the viable count expressed as a percentage of the
394 microscopically determined total cell count for isolation sources, has conventionally been
395 used as an indicator of the presence of uncultured bacteria (43). However, we proposed a
396 novel indicator "isolation possibility" to evaluate the difficulties of hair bacterial isolates
397 at genera levels using standard media under various isolation conditions (Figs. 5 and S2).
398 The isolates were categorized into two groups based on possibilities: high isolation
399 possibilities (>25%), such as *Staphylococcus* spp., *Moraxella* spp., and *Sphingomonas*
400 spp., and low isolation possibilities (≤25%), such as *Brachybacterium* sp.,
401 *Calidifontibacter* sp., and *Gulosibacter* sp. (Fig. 5D). These results suggested the
402 existence of "easily isolated bacteria" and "difficultly isolated bacteria" on human hair.

403 Because most of studies reports on bacterial isolates under successfully isolation
404 conditions without information on unsuccessful isolation conditions, the researcher must
405 make much effort to isolate a target bacterium by a culture method, which would result
406 in time-consuming, costly, and labor works. Accumulation of the isolation possibilities
407 would not only give academically valuable knowledge but also help a researcher to
408 develop a strategy of isolation methods with or without standard media. On the other hand,
409 this parameter of isolation possibility would vary depending on isolation sources (i.e.,
410 environmental samples) and isolation conditions (i.e. medium components, component
411 concentrations, gelling agents, and atmospheric environments), therefore, generalizability
412 would be required to apply this parameter for isolation of environmental microorganisms.
413 Therefore, the development of isolation methods to improve the possibility of isolating
414 difficult-to-isolate bacteria and to understand the underlying mechanisms will be crucial
415 for future microbial isolation research.
416

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Figure legends

FIG. 1. Venn diagram of isolated hair bacteria on undiluted (1, 5, 9, 13, and 19) and 10% diluted media (2, 6, 10, 14, and 20).

(A) Aerobic isolation with LB, (B) Aerobic isolation with NM, (C) Aerobic isolation with TS, (D) Aerobic isolation with CASO, (E) Anaerobic isolation with GAM, and (F) Summarized Venn diagram of (A) to (E).

FIG. 2. Venn diagram of isolated hair bacteria on standard culture medium in aerobic (1, 5, 9, 13, 17, and 21) and anaerobic (3, 7, 11, 15, 17, and 21) cultivation.

(A) Isolation with LB, (B) Isolation with NM, (C) Isolation with TS, (D) Isolation with

566 CASO, (E) Isolation with Col, (F) Isolation with PF, and (G) Summarized Venn diagram
567 of (A) to (F).
568
569 FIG. 3. Venn diagram of isolated hair bacteria on agar (1, 5, 9, and 13) and agarose (4, 8,
570 12, and 15) plates.
571 (A) Isolation with LB, (B) Isolation with NM, (C) Isolation with TS, (D) Isolation with
572 CASO, and (E) Summarized Venn diagram of (A) to (D).
573
574 FIG. 4. Venn diagram of isolated hair bacteria on agar plates with lipid (21 and 22) and
575 without lipid from pig (23 and 24).
576 (A) Aerobic isolation with PF and PFn, (B) Anaerobic isolation with PF and PFn, and (C)
577 Summarized Venn diagram of (A) and (B).
578
579 FIG. 5. Bacterial genera identified in colonies grown under each culture condition.
580 (A) Total number of bacteria isolated from 24 culture conditions, (B) Difficulty of
581 colonization for each bacterial genus as indicated by the number of bacterial isolates, (C)
582 Number of conditions of isolated bacteria, and (D) Isolation possibility of isolated hair
583 bacteria. Probability was defined as the number of isolation conditions for each genus

584 divided by 24 of the total isolation conditions.

585

586 FIG. 6. Summary list of isolation bacterial species from hair by 24 culture conditions and

587 heat map of their relative abundances on hair. The values of relative abundance of isolated

588 bacterial species on hair were referred to the report by Watanabe et al. (22).

Figure

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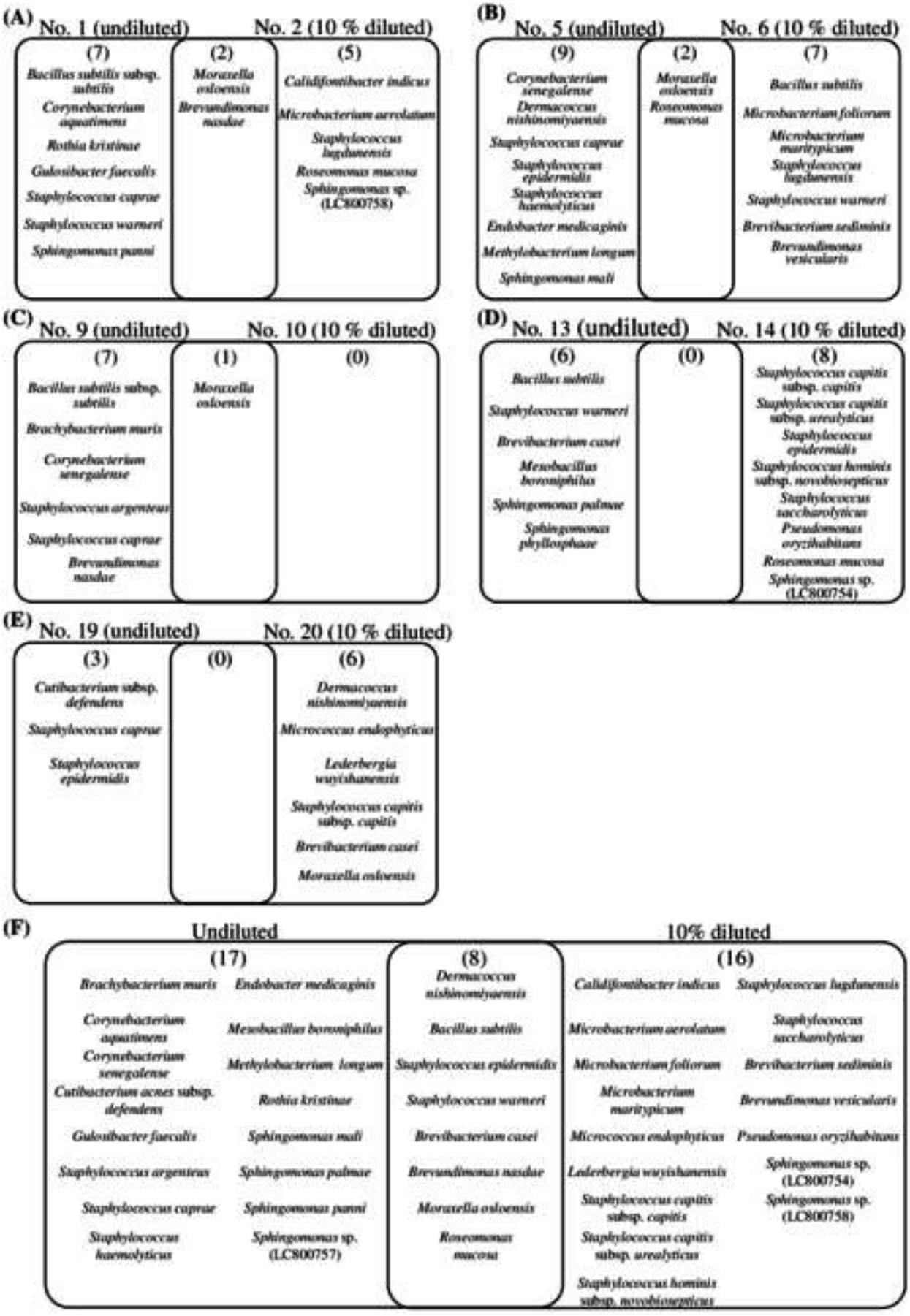

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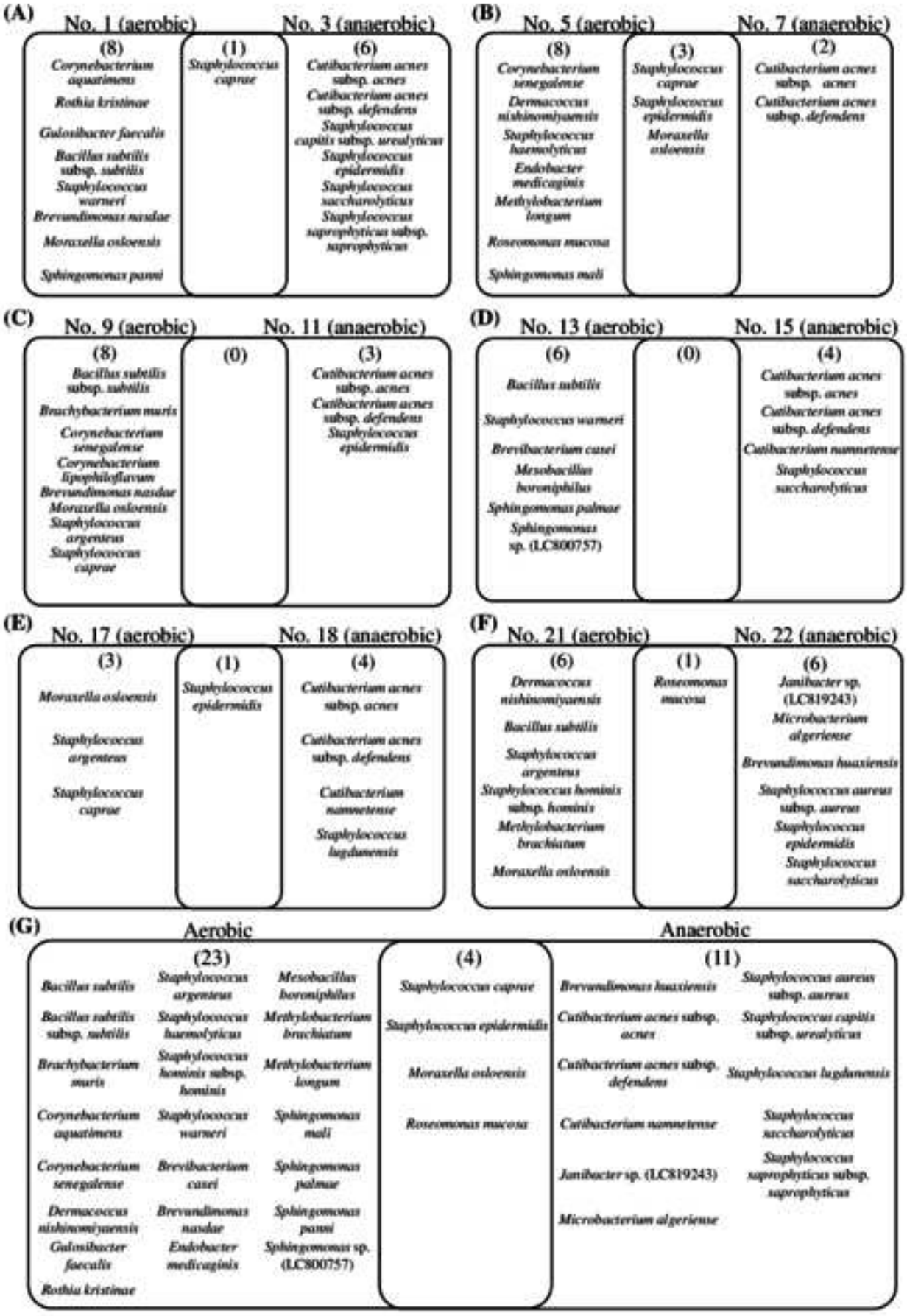

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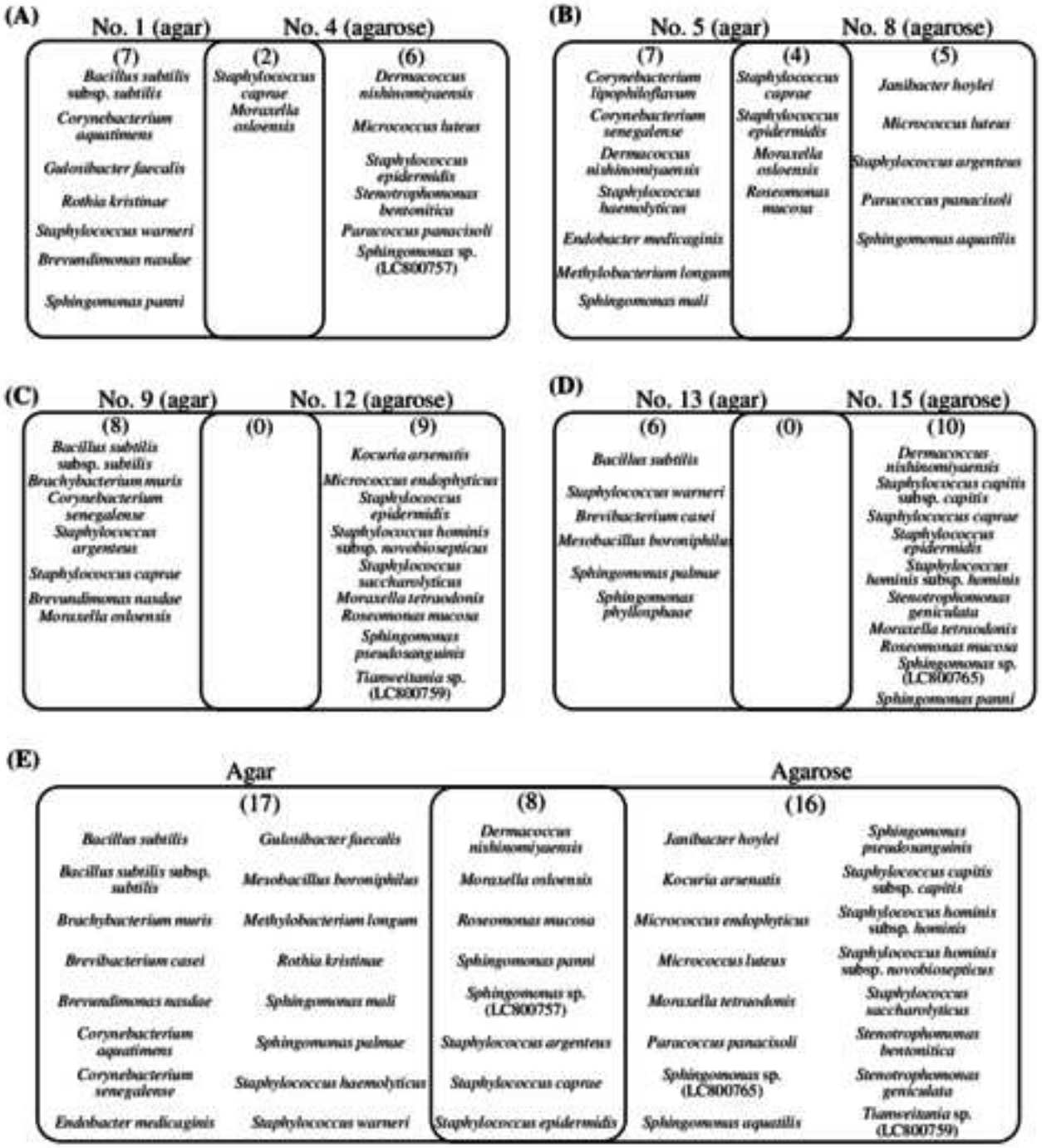

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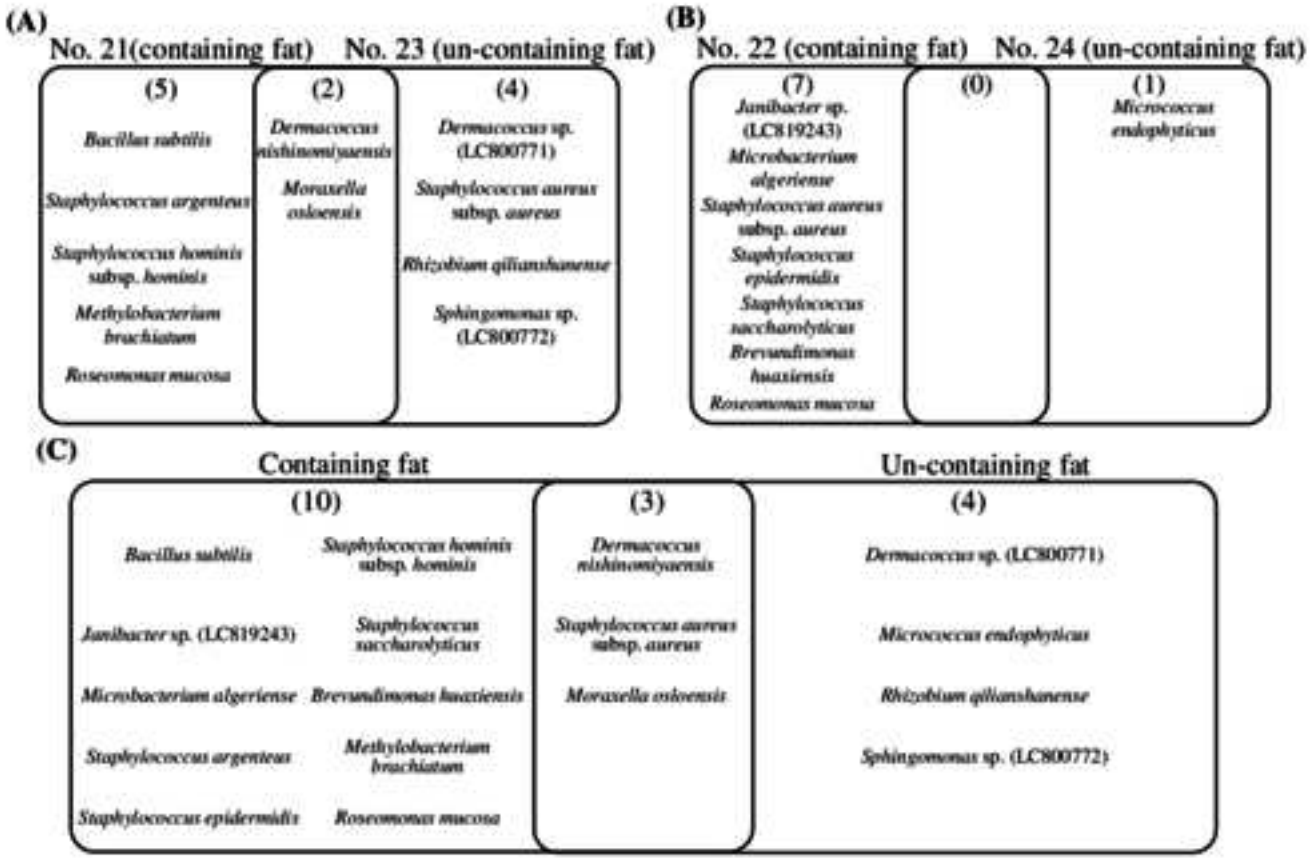

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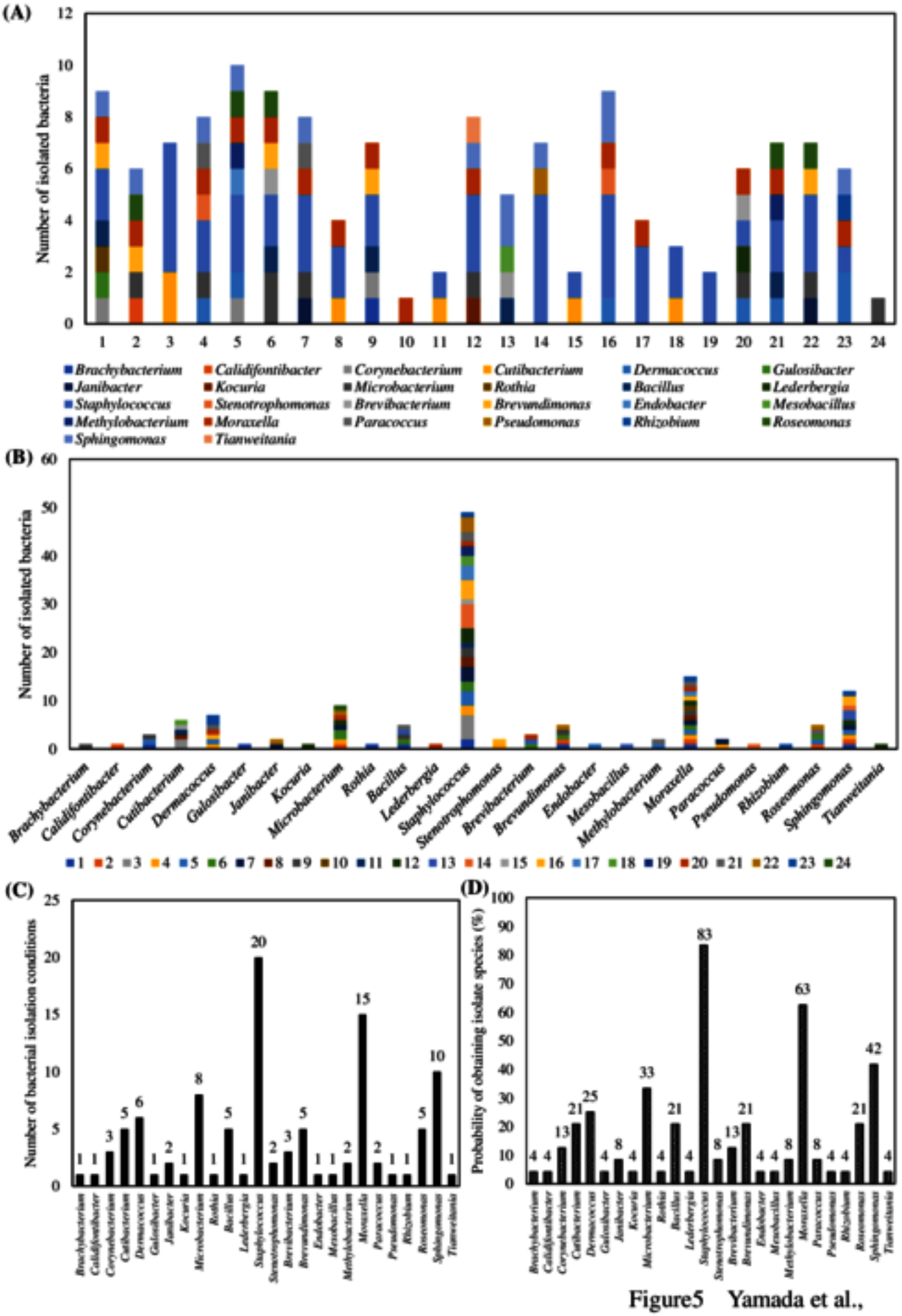

Figure

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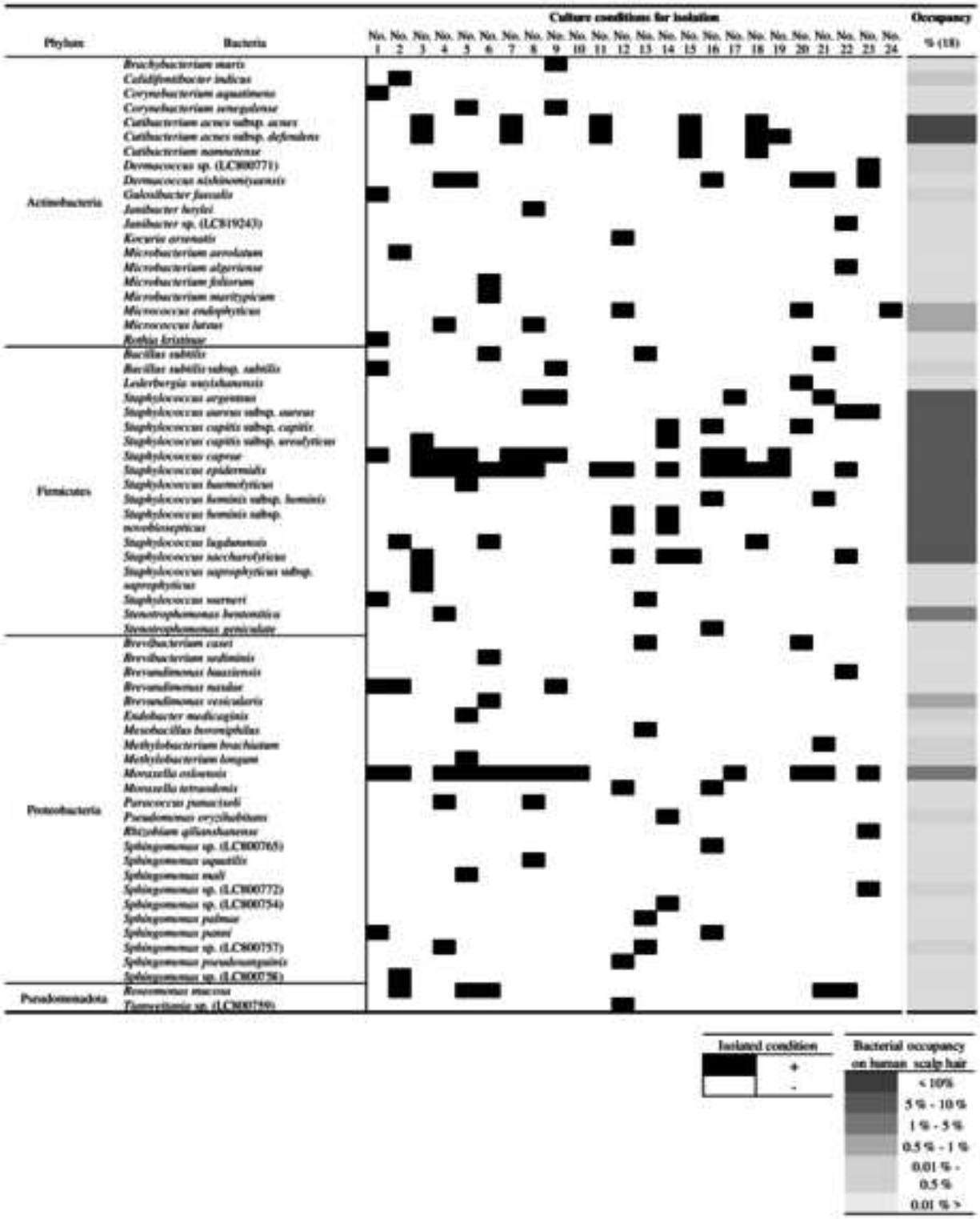

Figure 6 Yamada et al.,

Table1. List of culture conditions for isolation

Culture No.	Medium	Oxygen	Gelling agents	10% dilution
1	LB	+	agar	-
2	LB	+	agar	+
3	LB	-	agar	-
4	LB	+	agarose	-
5	NM	+	agar	-
6	NM	+	agar	+
7	NM	-	agar	-
8	NM	+	agarose	-
9	TS	+	agar	-
10	TS	+	agar	+
11	TS	-	agar	-
12	TS	+	agarose	-
13	CASO	+	agar	-
14	CASO	+	agar	+
15	CASO	-	agar	-
16	CASO	+	agarose	-
17	Col	+	agar	-
18	Col	-	agar	-
19	GAM	-	agar	-
20	GAM	-	agar	+
21	PF	+	agar	-
22	PF	-	agar	-
23	PFn	+	agar	-
24	PFn	-	agar	-

Table 2. Carbohydrate metabolism profile of isolation bacteria analyzed by the API50 CHL.

	C. d[a]	*C. a*[b]	*S. e*[c]	*S. c*[d]	*M. l*[e]
Control	-	-	-	-	-
N-Acetylglucosamine	+	+	-	-	-
Inositol	+	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	-	-	-	-	-
D-Xylose	-	-	-	-	-
L-Xylose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Ribose	+	+	-	-	-
D-Adonitol	+	+	-	-	-
D-Galactose	+	+	+	-	-
D-Glucose	+	+	+	+	+*
D-Fructose	+	+	+	+	+*
D-Mannose	+	+	+	+	+*
L-Sorbose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
Glycerol	+	+	+	-	+
Erythritol	+	+	-	-	-
D-Mannitol	+*	+*	-	-	-
D-Sorbitol	-	-	-	-	-
Dulcitol	-	-	-	-	-
Xylitol	-	-	-	-	-
D-Arabitol	+	+	-	-	-
L-Arabitol	-	-	-	-	-
Salicin	-	-	-	-	-
Esculin Ferric citrate	-	-	-	-	-
D-Cellobiose	-	-	-	-	-
D-Maltose	-	-	+	-	+*
D-Lactose	-	-	+	-*	+*
D-Saccharose	-	-	+	-*	+*
D-Melibiose	-	-	-	-	-
D-Trehalose	-	-	-	-	-
Inulin	-	-	-	-	-
D-Melezitose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
Starch	-	-	-	-	-
Glycogen	-	-	-	-	-
Gentiobiose	-	-	-	-	-
D-Turanose	-	-	+	-	-
Methyl-ß-D-xylopyranoside	-	-	-	-	-
Methyl-α-D-mannopyranoside	+	+	-	-	-
Methyl-α-D-glucopyranoside	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
Potassium gluconate	-	-	-	-	-
Potassium 2-ketogluconate	-	-	-	-	-
Potassium 5-ketogluconate	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Amygdalin	-	-	-	-	-
Arbutin	-	-	-	-	-

[a]*Cutibacterium acnes* subsp. *defendens,* [b]*Cutibacterium acnes* subsp. *acnes,* [c]*Staphylococcus epidermidis,* [d]*Staphylococcus caprae,* and [e]*Micrococcus luteus*.

*Isolated bacteria with different metabolic capacities compared to type strain. (*Cutibacterium acnes* subsp. *defendens* JCM 6473^T, *Cutibacterium acnes* subsp. *acnes* NBRC 107605^T, *Staphylococcus epidermidis* NBRC 100911^T, *Staphylococcus caprae* DSM 20608^T and *Micrococcus luteus* NBRC 3333^T

Isolated hair bacteria reveal different isolation possibilities under various conditions

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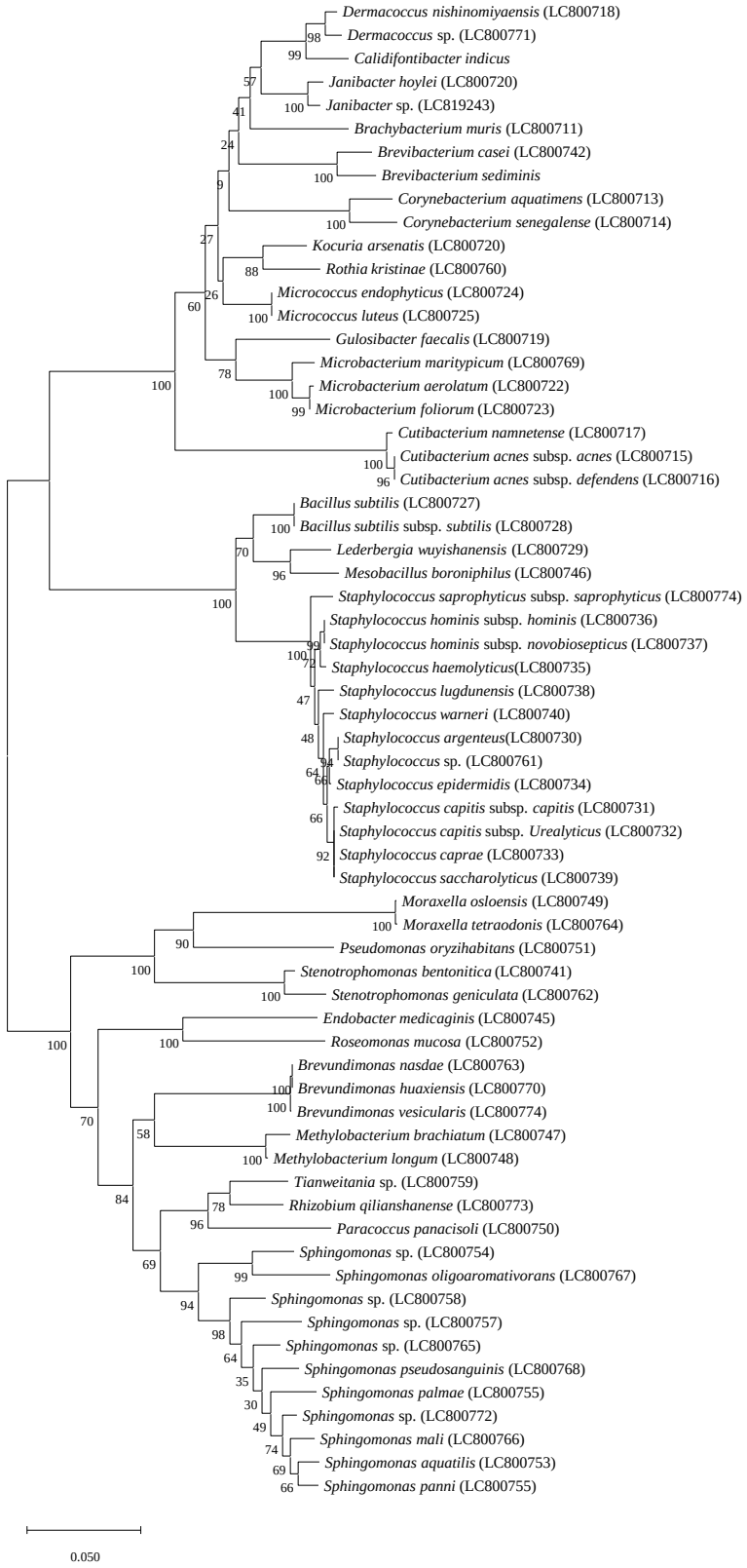

Figure S1. Phylogenetic tree of isolation bacteria from hair by 24 culture conditions. Accession number of LC800711-LC800725, LC800727-LC800774 and LC819243 were registered in the DNA Data Bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/index-e.html)

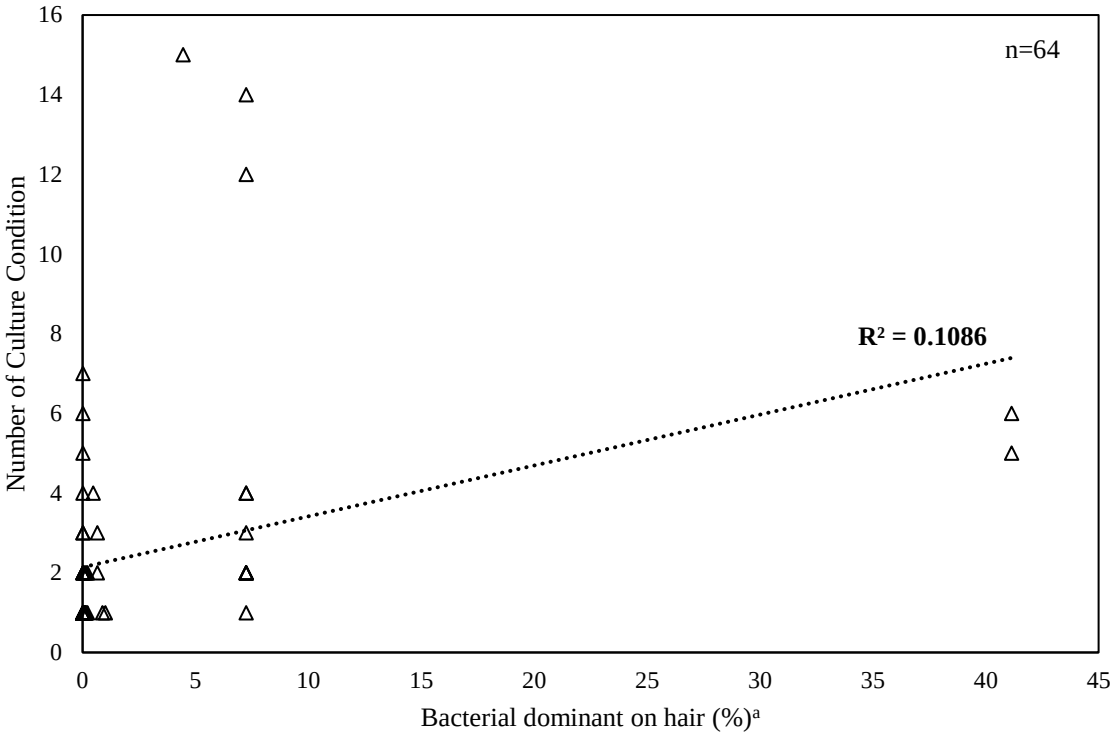

Figure S2. Correlative relationship between the level of difficulty level of isolated bacteria and their occupancy rate on scalp hair. Totally 26 genera from 64 samples were plotted. The correlation coefficient was calculated between the prevalence rates of bacterial colonization on human hair obtained from [a]Watanabe et al. 2021 (1) and the number of bacterial isolation conditions for each genus obtained in this research.

References

1. **Watanabe, K., Yamada, A., Nishi, Y., Tashiro, Y., and Sakai, K.:** Host factors that shape the bacterial community structure on scalp hair shaft. Sci Rep., **11**, 1-11 (2021).