Characterization of the Axillary Microbiota of Japanese Male Subjects with Spicy and Milky Odor Types by Pyrosequencing

HIROYA OKAMOTO¹, SHOKO KOIZUMI¹, HIRONORI SHIMIZU², OTOMI CHO³, AND TAKASHI SUGITA³

¹Product Assurance Division, Mandom Corporation, 5-12 Juniken-cho, Chuo-ku, Osaka 540-8530, Japan
²Technical Development Center, Mandom Corporation, 5-12 Juniken-cho, Chuo-ku, Osaka 540-8530, Japan
³Department of Microbiology, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan

Received 22 March, 2017/Accepted 19 September, 2017

Malodorants in the human axilla are produced from human biogenic precursors by axillary bacterial enzymes. In the present study, we used pyrosequencing analysis to identify the axillary bacterial microbiota of 13 Japanese male subjects with cumin-like, spicy body odor (C type), and 9 with milky, skin-based body odor (M type). Anaerococcus, Corynebacterium, and Staphylococcus predominated in both C- and M-type subjects, followed by Moraxella and Peptoniphilus. These genera accounted for 96.2-99.9% of the total bacterial population, except in the microbiota of one C-type subject. However, the axillary bacteria in C-type subjects were more abundant than that in M-type subjects. These results suggest that the level of colonization by axillary bacteria is important for the production of malodorants.

Key words : Axillary odor / Corynebacterium / 16S rRNA.

INTRODUCTION

Human body odor is generated by bacteria that colonize the initially odorless secretions of sweat and sebaceous glands (Taylor et al., 2003; James et al., 2013). The distribution and sizes of these glands vary markedly among individuals and thus body odor is unique to an individual. Moreover, different body parts, such as the axillae, feet, and scalp, have distinct odors (Fredrich et al., 2013; Troccaz et al., 2015). Hence, there are many different types and intensities of body odor. Body odor can have a significant negative impact on one’s social life, making the prevention or elimination of body odor important for the quality of life.

Cosmetic companies invest considerable effort in creating technologically superior products for minimizing body odor. Deodorant manufacturers typically claim that their products are specifically designed to prevent body odor and stress-induced sweating for as long as possible. Investigation of the mechanism of odor generation is critical to the development of such products.

Axillary odor can be quite strong and shows considerable interindividual variation. Hara et al. (2015) evaluated the quality and intensity of axillary odor in healthy Japanese males. They found that most Japanese males (57%) have a weak, milk-like odor, while some males (18%) have a strong spicy odor. The major constituents of human axillary odor are volatile fatty acids, such as 3-methyl-2-hexenoic acid, and short sulfanylalkanols, such as 3-methyl-3-sulfanyl-hexan-1-ol (Zeng et al., 1991; Natsch et al., 2003; Hasegawa et al., 2004; James et al., 2004a; James et al., 2004b; Natsch et al., 2004; Troccaz et al., 2004; Starkenmann et al., 2005; Natsch et al., 2006). Fatty acids are secreted as glutamine conjugates, and are released upon cleavage by corynebacterial enzymes. Sulfanylalkanols are secreted as cysteine or cysteine-glycine conjugates, and are released in a similar way. Bacteria play an important role in generating odor from odorless secretions (Fredrich et al., 2013; Leyden et al., 1981).

Axillary microbiota have been investigated using culture-dependent methods, which have identified species across many genera, including Corynebacterium, Micrococcus, Staphylococcus, Propionibacterium, and...
Brevibacterium (Leyden et al., 1981). More recently, molecular-based approaches to characterizing skin bacteria have resulted in the detection of a much greater diversity of microorganisms (Fredrich et al., 2013; Troccaz et al., 2015). The lipophilic genus Corynebacterium is associated with strong axillary odor, and that Staphylococcus is associated with weak axillary odor (Taylor et al., 2003).

In the present study, we characterized the axillary bacterial microbiota of Japanese subjects with two types of axillary odor (strong, spicy odor and weak, milk-like odor) using culture-independent methods via pyrosequencing.

**MATERIALS AND METHODS**

**Enrollment of subjects**

Twenty-two healthy male subjects (36.9±12.9 years) participated in this study after they provided written informed consent. Thirteen of these subjects had type C (cumin-like, spicy) and nine had type M (milky, skin-based) axillary odor, based on independent direct sensory evaluation by four experts. Those with M-type odor were considered to have minimal body odor. Use of deodorants was prohibited for three days prior to the start of the study; bathing, eating spicy foods, and drinking alcoholic beverages were prohibited for 24 h prior to the study; and smoking was prohibited on the day of the study.

**Sampling of axillary microorganisms**

A washing solution (0.1 M phosphate buffer, pH 7.9, containing 0.1% Tween 80) was prepared by adding 1 mL Tween 80 to a mixture of 80 mL 75 mM Na2HPO4 and 920 mL 75 mM NaH2PO4, followed by sterilization in an autoclave. The sampling area was washed with a fragrance-free soap, and the subjects were instructed to wear a deodorized shirt. After 24 h, a sterilized glass tube (3.2 cm interior diameter) was pressed against each test region, 1.5 mL washing solution was added, the skin surface was lightly rubbed with a sterilized pellet mixer, and the washing solution was retrieved with a pipette. This procedure was repeated twice and the liquid thus obtained was used as the bacterial suspension.

The study protocol has been reviewed and approved by the Institutional Review Board.

**DNA extraction**

The bacterial cells collected by centrifugation were suspended in 300 µL of lysing solution (100 mM Tris-HCl [pH 8.0], 30 mM EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) and the cell suspension was frozen by liquid nitrogen. Freeze-thaw process was performed three times. The solution was incubated for 15 min at 100°C, and then was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was precipitated with ethanol using Ethatimate (Nippon Gene, Toyama, Japan) as a precipitation activator. The DNA pellet was resuspended in 50 µL of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]).

**Sequence processing and data analysis**

Primer and barcode sequences were removed from the data and possible chimeras were excluded from the analysis. Sequences ≥ 400 bp in length were analyzed. V1-V3 16S sequences were classified to the genus level using the RDP classifier. The R package vegan (http://CRAN.R-project.org/package = vegan) was used to construct a Shannon diversity index boxplot. Significance was tested using a one-tailed t-test and two-sample variance. A three-dimensional principal coordinate analysis (PCoA) plot was normalized using weighted values (http://www.quilme.org).

**Colonization by Corynebacterium and Staphylococcus**

Levels of the colonization of Corynebacterium and Staphylococcus were measured by quantitative PCR using genus-specific primers and a TaqMan probe based on the bacterial 16S rRNA gene according to the method of Gao et al. (2010). The total level of bacterial colonization of the skin was also determined.

**RESULTS**

**Characterization of the skin bacterial microbiota**

The skin bacterial microbiota of 22 Japanese male subjects with different body odor types was characterized by the pyrosequencing of the 16S rRNA gene. The dataset included 206,396 high-quality sequences, including 9,224±2,245 (mean ± standard deviation) sequences for C-type and 9,609±2,404 for M-type subjects. The predominant genera of the microbiota were similar in the C- and M-type odor groups. Anaerococcus, Corynebacterium, and Staphylococcus were the most abundant genera, followed by Moraxella and Peptonophilus.

**Axillary bacterial microbiota of subjects with C- and M-type odor**

Anaerococcus, Corynebacterium, Staphylococcus, Moraxella, and Peptonophilus were abundant in the samples of both C- and M-type odor subjects (Fig.1). The proportions of these five predominant genera did not differ between the two odor types (Fig.2). They accounted for 96.2-99.9% of the total bacterial population in all subjects, with the exception of subject ID 100.
difference between the C and M types (Fig.3). A PCoA to evaluate sample diversity and analyze the relationships among samples indicated that the microbiota of C- and M-type subjects were not distinct (Fig.4).

Levels of colonization by abundant genera

Colonization levels of Corynebacterium, Staphylococcus, and total bacteria were determined by (C type). Anaerococcus, Corynebacterium, Moraxella, and Peptoniphilus each comprised only 1.0% of the total population in subject ID 100, while Staphylococcus was predominant (29.6%). Stenotrophomonas (22.8%) and Microbacterium (18.7%) were also more abundant in this subject.

Shannon’s diversity index was calculated to determine the diversity of the samples, and indicated no significant
qPCR using specific primers and TaqMan probes. The total bacterial population was larger in C-type subjects (1.4±1.3×10⁴ copies/µL) than in M-type subjects (4.8±4.2×10³ copies/µL) (p<0.05) (Fig. 5). In addition, the level of Corynebacterium was greater in C-type subjects (7.0±8.9×10³ copies/µL) than in M-type subjects (1.3±1.6×10³ copies/µL). The Staphylococcus populations in C-type subjects (4.4±4.4×10³ copies/µL) and M-type subjects (1.7±2.6×10⁴ copies/µL) were not significantly different (p>0.05).

DISCUSSION

Using pyrosequencing analysis, this study revealed that Anaerococcus, Corynebacterium, Staphylococcus, Moraxella, and Peptoniphilus were the predominant genera in axillary samples from Japanese male subjects with C- and M-type odors. Axillary bacterial enzymes convert human biogenic substances such as proteins, fatty acids, steroids, skin lipids, amino acids, glycerol, and lactic acid into axillary malodorants (James et al., 2013). For example, the malodorant E-3-methyl-2-hexenoic acid is metabolized from N-E-3-methyl-2-hexenoyl-L-glutamine by N-acetylglutamine aminocyclase, which is produced by Corynebacterium spp. This enzyme also metabolizes N-3-hydroxy-3-methylhexanoyl-L-glutamine into the malodorant N-3-hydroxyl-3-methylhexanoic acid.

To the best of our knowledge, although no comprehensive analysis of the axillary bacterial microbiota of Japanese subjects has been reported, pivotal studies of axillary microbiota using culture-independent methods have been conducted in Germany (Egert et al., 2011), Belgium (Callewaert et al., 2013), and Switzerland (James et al., 2013). The German study revealed that Anaerococcus, Corynebacterium, Peptoniphilus, Propionibacterium, and Staphylococcus were the most abundant genera, and accounted for >90% of the total bacterial population in 10 male subjects, with Staphylococcus predominating (>50%). That study used 16S rRNA gene-based terminal-restriction fragment length polymorphism fingerprinting in combination with clone-library analysis. The Swiss study found the bacterial populations of axillary samples from 24 Caucasian male and female subjects to be 84.7-100% Staphylococcus, Corynebacterium, and Propionibacterium. Anaerococcus and Peptoniphilus were the fourth and fifth most abundant genera, respectively. Finally, the Belgian study, which made use of pyrosequencing analysis, found that the axillary samples of 53 subjects contained mainly Corynebacterium and Staphylococcus.

Our study of Japanese subjects showed results similar to these three previous reports. However, one major difference was that Propionibacterium was not abundant in our samples (0.4±0.4% in C type, 0.6±0.5% in M type) whereas the German study reported the levels of Propionibacterium to be >10%. Our study found marked inter-individual variation in axillary microbiota, but Corynebacterium and Staphylococcus were the predominant genera in all but one subject. The axillary bacterial microbiota of subject ID 100 (C-type) differed from the other subjects. Subject ID 100 was the youngest subject (18 years of age). The Belgian and German studies reported that the axillary bacterial microbiota differed considerably among individuals. Our results also indicated interpersonal diversity in the axillary microbiome, and additionally suggest that the diversity of the axillary microbiome may be affected by age.

In our study, proportions of Corynebacterium and Staphylococcus were inversely correlated: Corynebacterium was present in high abundance when Staphylococcus was at low abundance, with a high correlation coefficient (r²=0.80 in C-type subjects, r²=0.73 in M-type subjects). Although the DNA sequence information obtained from pyrosequencing in this study did not permit identification to the species level, a BLAST search (http://blast.ncbi.nlm.nih.gov/) suggested that the predominant Corynebacterium and Staphylococcus species were C. urei, C. hominis or phylogenetically closely related species. Our results, taken with those of previous studies, imply that there is little or no ethnic variation in axillary microbiota.

Studies using the culture method have found that the overall abundance of bacteria is correlated to the strength of the axillary odor (Taylor et al., 2003). It is surprising that the composition of the axillary microbiota of both C- and M-type subjects were similar in this study. However, quantitative analysis (qPCR) yielded

![FIG. 5. Levels of colonization of abundant axillary bacteria, as determined by qPCR. C (cumin-like, spicy odor); and M (milky, skin-based odor).](image-url)
interesting results. The total level of bacterial colonization of C-type subjects was 3-fold higher than that of M-type subjects. The levels of colonization with Corynebacterium (5.4-fold, p<0.05) and Staphylococcus (2.7-fold, p>0.05) in C-type subjects were also higher than those in M-type subjects. These findings suggest that the degree of colonization of axillary bacteria, rather than the composition of the axillary microbiota, is important for the generation of malodorants. The levels of Anaerococcus, Moraxella, and Peptoniphilus colonization were not determined because specific primers and TaqMan probes were not available for these genera.

In conclusion, we found that the total level of bacterial colonization of C-type subjects was higher than that of M-type subjects in Japanese males. However, further investigation is required to clarify the specific character of human axillary odor, and explore the impact of factors such as gender or race.

REFERENCES

Callewaert, C., Kerckhof, F. M., Granitsiotis, M. S., Gele, M. Van., Wiele, T. Van de., and Boon, N. (2013) Characterization of Staphylococcus and Corynebacterium clusters in the human axillary region, PLoS One, 8,e70538.

Egert, M., Schmidt, I., Höhne, H. M., Lachnit, T., Schmitz, R. A., and Breves, R. (2011) rRNA-based profiling of bacteria in the axilla of healthy males suggests right-left asymmetry in bacterial activity, FEMS Microbiol. Ecol., 77, 146-153.

Friedrich, E. I., Barzantny, H., Brune, I., and Tauch, A. (2013) Daily battle against body odor: towards the activity of the axillary microbiota, Trends Microbiol., 21, 305-312.

Gao, Z., Perez-Perez, G. I., Chen, Y., and Blaser, M. J. (2010) Quantitation of major human cutaneous bacterial and fungal populations, J. Clin. Microbiol., 48, 3575-3581.

Hara, T., Kyuka, A., and Shimizu, H. (2015) Butane-2,3-dione: the key contributor to axillary and foot odor associated with an acidic note, Chem. Biodivers., 12, 248-258.

Hasegawa, Y., Yabuki, M., and Matsukena, M. (2004) Identification of new odoriferous compounds in human axillary sweat, Chem. Biodivers., 1, 2042-2050.

James, A. G., Casey, J., Hyliands, D., and Mycock, G. (2004a) Fatty acid metabolism by cutaneous bacteria and its role in axillary malodour, World J. Microbiol. Biotechnol., 20, 787-793.

James, A. J., Hyliands, D., and Johnston, H. (2004b) Generation of volatile fatty acids by axillary bacteria, Int. J. Cosmet. Sci., 26, 149-156.

James, A. G., Austin, C. J., Cox, D. S., Taylor, D., and Calvert, R. (2013) Microbiological and biochemical origins of human axillary odour, FEMS Microbiol. Ecol., 83, 527-540.

Leyden, J. J., Mc Ginley, K. J., Holde, E., Labows, J. N., and Kligman, A. M. (1981) The microbiology of the human axilla and its relationship to axillary odor, J. Invest. Dermatol., 77, 413-416.

Natsch, A., Gfeller, H., Gygax, P., Schmid, J., and Acuna, G. (2003) A specific bacterial aminoacylase cleaves odorant precursors secreted in the human axilla, J. Biol. Chem., 278, 5718-5727.

Natsch, A., Schmid, J., and Flachsmann, F. (2004) Identification of odoriferous sulfanylalkanols in human axilla secretions and their formation through cleavage of cysteine precursors by a C-S lyase isolated from axilla bacteria, Chem. Biodivers., 1, 1058-1072.

Natsch, A., Derrer, S., Flachsmann, F., and Schmid, J. (2006) A broad diversity of volatile carboxylic acids, released by a bacterial aminoacylase from axilla secretions, as candidate molecules for the determination of human-body odor type, Chem. Biodivers., 3, 1-20.

Starkenmann, C., Niclass, Y., Troccaz, M., and Clark, A. J. (2005) Identification of the precursor of (S)-3-methyl-3-sulfanylhexan-1-ol, the sulfury malodour of human axilla sweat, Chem. Biodivers., 2, 705-716.

Taylor, D. I., Daulby, A., Grinshaw, S., James, G., Mercer, J., and Vaziri, S. (2003) Characterization of the microflora of the human axilla, Int. J. Cosmet. Sci., 25, 137-145.

Troccaz, M., Starkenmann, C., Niclass, Y., Waal, M. van de., and Clark, A. J. (2004) 3-Methyl-3-sulfanylhexan-1-ol as a major descriptor for the human axilla-sweat odour profile, Chem. Biodivers., 1, 1022-1035.

Troccaz, M., Gaia, N., Baccucci, S., Schrenzel, J., Cayeux, I., Starkenmann, C., and Lazarevic, V. (2015) Mapping axillary microbiota responsible for body odours using a culture-independent approach, Microbiome, 3, 3.

Zeng, X. N., Leyden, J. J., Lawley, H. J., Sawano, K., Nohara, I., and Preti, G. (1991) Analysis of characteristic odors from human male axillas, J. Chem. Ecol., 17, 1469-1492.