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Proteobacteria from the human skin microbiota: Species-level diversity and hypotheses

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ABSTRACT

The human skin microbiota is quantitatively dominated by Gram-positive bacteria, detected by both culture and metagenomics. However, metagenomics revealed a huge variety of Gram-negative taxa generally considered from environmental origin. For species affiliation of bacteria in skin microbiota, clones of 16S rRNA gene and colonies growing on diverse culture media were analyzed. Species-level identification was achieved for 81% of both clones and colonies. Fifty species distributed in 26 genera were identified by culture, mostly belonging to *Actinobacteria* and *Firmicutes*, while 45 species-level operational taxonomic units distributed in 30 genera were detected by sequencing, with a high diversity of *Proteobacteria*. This mixed approach allowed the detection of 100% of the genera forming the known core skin Gram-negative microbiota and 43% of the known diversity of Gram-negative genera in human skin. The orphan genera represented 50% of the current skin pan-microbiota. Improved culture conditions allowed the isolation of *Roseomonas mucosa*, *Aurantimonas altamirensis* and *Agrobacterium tumefaciens* strains from healthy skin. For proteobacterial species previously described in the environment, we proposed the existence of skin-specific ecotypes, which might play a role in the fine-tuning of skin homeostasis and opportunistic infections but also act as a shuttle between environmental and human microbial communities. Therefore, skin-associated proteobacteria deserve to be considered in the One-Health concept connecting human health to the health of animals and the environment.

Introduction

Various human microbiota are now deciphered in depth thanks to metagenomics and new generation DNA sequencing (NGS) [1,2]. These methods allowed comparative microbial ecology by examining the influence of environmental factors, body sites and pathology on the diversity of microbiome [3–12]. Compared to culture-based approaches, NGS has generally extended the range of microbial diversity of complex communities and detected yet-to-be-described bacterial taxa. However, culture-based approaches detect living microorganisms and may be more sensitive for the detection of minority bacteria able to grow on artificial media in monoculture [13,14]. Polyphasic studies associating molecular and culture-based analyses remained scarce [15,16] despite the development of high-throughput methods named culturomics [13].

Human skin is colonized by a complex microbial community, considered for a long time as dominated by Gram-positive bacteria such as staphylococci, micrococci, corynebacteria, *Propionibacterium* spp., *Brevibacterium* spp., and members of the genus *Acinetobacter* being the most frequently encountered Gram-negative bacteria in human skin microbiota. These bacteria belong to the long-term resident microbiota, based on the frequency with which they have been detected [2,4,15,17–21]. Beside these well-described bacteria, culture-independent approaches demonstrate that Gram-negative bacteria, particularly *Proteobacteria*, represent an important component of the skin microbiota [2,12,19–22]. Despite their detection in numerous metagenomic studies and diverse physio-pathological conditions, cutaneous *Proteobacteria* remained poorly described, mainly because isolates were not available and sequences generated by NGS were generally too short to obtain an accurate species affiliation.

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This study aimed to precise the phylogenetic relationships and taxonomy of *Proteobacteria* from healthy human skin microbiota by analysis of 16S rRNA gene sequences of more than 800 bp and by strain cultivation. Phylotypes and isolates will be described to the species and/or genotype level in order to compare skin-associated *Proteobacteria* with related environmental ecotypes.

Materials and Methods

Cutaneous samples, isolates and clones

The present study was an ancillary study proposed beside clinical study on atopy (Institut de Recherche Pierre Fabre, unpublished data). Briefly, in the main study, donors are tertiary workers (no healthcare workers) in urban areas without particular exposure to animals and soil. They took a shower using mild soap between 4 and 6 h before sampling. For each donor, one sample was taken from the inner forearm protected by clean personal garments until sampling. Sampling was performed according to the method described by Fleurette using a transfer fluid able to maintain the viability and to avoid proliferation of the microbiota [23]. Briefly, the open end of a sterile glass cylinder, with an area of 3.14 cm^2 was manually placed on the skin. Two milliliters of RTF medium [23] sterilized by 0.22 μm filtration was used to collect skin microbiota. Four successive spots were realized each during 1 min with the same liquid or a total forearm skin area of 12.56 cm^2. One tenth of the liquid was used for microbial culture, the remaining was stored at −20 °C for molecular analysis.

A total of 311 isolates and 278 16S rRNA gene clones obtained from two healthy-donors included in the clinical study were analyzed herein. Isolates were obtained by culture on Columbia agar supplemented with 5% sheep blood (Biomérieux) incubated under aerobic and anaerobic (Anoxomat) conditions at 37 °C for 5 days. One colony of each morphotype observed was harvested, sub-cultured and stored at −20 °C in cryopreservative medium (Eugon broth + 10% glycerol). A selective isolation of Gram-negative bacilli was performed at 30 °C for 5 days using culture media implemented by vancomycin (7.5 mg/L): R2A agar (Pronadisa), Schaedler agar (Difco) and Chocolat agar (Difco) for 8 samples from 2 other healthy donors.

The 16S rRNA clones library had been obtained after total DNA extraction directly from samples (MagNA Lyser Green beads, Roche Molecular Biochemicals), and purification (QIAamp DNA micro purification kit, Qiagen, Germany). The amplification of a 863 bp-sequence of 16SrRNA gene was performed using the universal primers, Universel1 (5′ AGCAGCCGCGGTRATWC 3′) and Universel2 (5′ ACGGGCGGTGTG TAC 3′) [24,25]. The purified amplicons (QIAquick PCR Purification kit, Qiagen) were ligated into the plasmid vector pGEM®-T Easy, then transformed into JM109 High Efficiency competent cells using the pGEM®-T Easy Vector Systems kit (Promega). JM109 transformed cells were streaked onto Luria–Bertani agar plates containing 100 μg/mL ampicillin, 40 μg/mL X-gal, 0.5 mM IPTG for blue/white screening as previously described [15]. The insert of each selected white clone was amplified and sequenced (ABI PRISM 3130 Genetic Analyzer, Applied Biosystems, USA) using T7 and SP6 primers as previously described [26].

Bacterial identification

Each colonial morphotype was submitted to identification by molecular methods based on 16S rRNA gene sequencing [15]. For members of the genus *Staphylococcus*, ITS 16S–23S and *tuf* gene sequencing were used for species affiliation [27,28].

Phylogenetic analysis and taxon affiliation

Sequences used for further sequence analysis corresponded to high-quality sequences, i.e. presenting less than 0.5% undetermined positions. Nucleotide sequences were analyzed using the Blast program in NCBI and Greengenes database. Chimera was detected using Bellorophon software in the Greengenes website (greengenes.lbl.gov). For database comparison, we retained the stringent threshold value of 98.7% of similarity with a fully defined cultured strain (type or non-type) as recommended for bacterial species delineation [29], for the affiliation of a clone to a species- or a genus-level operational taxonomic unit (OTU). When a similarity level of more than 98.7% was obtained for an uncultured bacterial clone only, the sequence was classified as 16S rRNA gene clone and affiliated to a genus according Greengenes database. Beside sequences identified herein, the sequences used to reconstruct phylogenies were chosen by Blast analysis as follows. For each clone sequence, we included the most related deposited sequence and the most related sequences corresponding to (1) validated species and (2) human skin clone. The dataset of sequences was aligned using ClustalW software [30]. The most appropriate substitution model determined according to Akaike information criterion calculated with Modeltest (v.3.7) was GTR plus gamma distribution, plus invariant sites [31]. ML phylogenetic analysis was performed using PHYML v2.4.6, gamma shape parameter being estimated from the dataset [32]. ML bootstrap support was computed using PhyML after 100 reiterations.

The Shannon-Wiener (H′) and Simpson (D) diversity indexes were calculated for each phylum and according to the type of method (culture or uncultured approaches) [33,34].

Results

Taxonomic diversity in the skin microbiota

Table 1 shows species-level identification of the bacterial isolates and clones. Fig. 1 summarizes the qualitative and quantitative diversity repartition of the skin microbiota according to phylum and type of cell wall structure. Our approach allowed species-level identification for 81% of both clones and colonies. The culture detected 50 species-level OTUs including 39 taxonomic species distributed in 26 genera among 311 colonies tested. The sequencing of 278 clones allowed the identification of 45 species-level OTUs in 30 genera, including 26 taxonomic species, 6 pairs of undifferentiated taxonomic species, 7 groups of related species and 5 unaffiliated OTUs (Table 1).

Gram-positive bacteria belonging to *Firmicutes* and *Actinobacteria* represented 90.3% of the colonial morphotypes studied. Genera belonging to *Firmicutes* (175 isolates) were *Bacillus*, *Streptococcus*, *Enterococcus*, *Gemella*, *Eubacterium* and *Staphylococcus*. The latter was the most diverse genus of the cultivable skin microbiota, with 15 different species identified. *Actinobacteria* appeared more diverse to the genus level since the 106 isolates affiliated to this phylum belonged to 10 different genera (Table 1; Fig. 1). Uncultured clones affiliated to *Firmicutes* and *Actinobacteria* were minority (Table 1; Fig. 1) compared to clones belonged to Gram-negative bacterial phyla (Table 1): *Proteobacteria* of the alpha (27.0%), beta (23.9%) and gamma (47.2%) subdivisions and *Bacteroidetes* (1.9%). Most of the Gram-negative genera identified by sequencing were not detected in culture, except for *Acinetobacter*, *Pseudomonas* and *Sphingomonas*. Indeed, only 30 of the total of 311 bacterial colonies tested (9.7%) corresponded to Gram-negative bacteria. Fourteen isolates belonged to *Proteobacteria* (*Enterobacteriaceae* and non-fermentative bacilli) (Table 1), and 16 isolates of Gram-negative anaerobes were represented by one unique species, *Prevotella buccae* in the phylum *Bacteroidetes*.

Fig. 1 revealed that the distribution of clones and colonies by phylum and type of cell wall structure differed markedly. Cultivating Gram-positive bacteria were quantitatively the most represented (175 colonies, Fig. 1) but by contrast their taxonomic diversity was low (21 species, H′ and D indexes of 0.3236 and 0.0057 respectively) (Fig. 1; Table 2). The ratio Gram-positive versus Gram-negative varied clearly according to the method used: the Gram-positive bacteria were mostly detected by culture (ratio of 9.37) but only partially by the molecular