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Novel volatiles of skin-borne bacteria inhibit the growth of Gram-positive bacteria and affect quorum-sensing controlled phenotypes of Gram-negative bacteria

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ABSTRACT

The skin microbiota is import for body protection. Here we present the first comprehensive analysis of the volatile organic compound (VOC) profiles of typical skin-resident corynebacterial and staphylococcal species. The VOC profile of *Staphylococcus schleiferi* DSMZ 4807 was of particular interest as it is dominated by two compounds, 3-(phenylamino)butan-2-one and 3-(phenylimino)butan-2-one (schleiferon A and B, respectively). Neither of these has previously been reported from natural sources. Schleiferon A and B inhibited the growth of various Gram-positive species and affected two quorum-sensing-dependent phenotypes – prodigiosin accumulation and bioluminescence – of Gram-negative bacteria. Both compounds were found to inhibit the expression of prodigiosin biosynthetic genes and stimulate the expression of prodigiosin regulatory genes *pigP* and *pigS*. This study demonstrates that the volatile schleiferons A and B emitted by the skin bacterium *S. schleiferi* modulate differentially and specifically its interactions with members of diverse bacterial communities. A network of VOC-mediated interspecies interactions and communications must be considered in the establishment of the (skin) microbiome and both compounds are interesting candidates for further investigations to better understand how VOCs emitted by skin bacteria influence and modulate the local microbiota and determine whether they are relevant to antibiotic and anti-virulence therapies.

Introduction

The skin is the body's most extensive organ and serves as a barrier between the internal and external environments. It protects the organism from harmful agents, excessive loss of water and microbial assault [37,49,59]. Since the skin is in permanent contact with the external environment, it is heavily colonized by diverse species of microorganisms, collectively known as the skin microbiota, which plays a key role in health and disease [55]. Thus, over the past decade, analysis of the function of the skin microbiome has become a topic of considerable interest. It is well known that the skin microbiota includes fungi, viruses and mites, but bacteria are generally dominant. Based on the analysis of 16S rRNA, it has been shown that approximately 1000 species of bacteria can be found on the human skin [21,22]. They belong to 19 phyla, of which *Actinobacteria*, *Firmicutes* and *Bacteriodetes* predominate. Although it was recently shown that the host genome has an impact on the skin microbiota, little is known about how its composition is controlled [62]. Nevertheless, most skin-resident bacteria are non-pathogenic commensals, and it has become apparent that some species are beneficial to their host. The microbiota is made up of complex dynamic communities of microorganisms, which, for example, interact with immune cells to modulate the skin immune system by priming T-cells to recognize non-self antigens for appropriate immune responses [6,40,53]. By colonizing the skin, the normal bacterial population can also compete with and eliminate pathogens through surface occupation. They can inter-

Abbreviations: mVOC, microbial volatile organic compound; QS, quorum sensing.

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act with other microorganisms by secreting various metabolites, including toxins and antibiotics, such as the anti-microbial peptides bacteriocins [5]. For example, strains of *Staphylococcus warneri* can produce warnerin, which inhibits the growth of a large number of Gram-positive and Gram-negative bacteria [48]. In the same way, *Staphylococcus epidermidis* and *Staphylococcus gallinarum* secrete the lantibiotics epidermin and gallidermin, respectively, which belong to a class of bacteriocins that inhibit other Gram-positive bacteria [18,30,57]. Bacteria on the skin can also produce compounds of low molecular weight, which are often volatile due to their high vapor pressure and low boiling point. These are collectively termed microbial volatile organic compounds (mVOCs) [51,58,60,68]. The potential effects of mVOCs on the skin microbiota have largely been overlooked. Nevertheless, it is known that the secretions produced by the skin glands are usually odorless, bacterial metabolism can transform these substances into odoriferous volatile compounds [25,26,60,67,77]. Thus, species of *Corynebacterium* degrade various precursor compounds found in sweat into short branched-chain fatty acids, such as (*E*)-3-methyl-2-hexenoic acid, which is the primary contributor to the typical axillary odor and is a key scented volatile [41,80]. In addition, *S. epidermidis* degrades leucine present in the sweat to produce 3-methylbutanoic acid, which is the major component of foot odor [1]. Other studies have shown that volatile aliphatic carboxylic acids and dimethyl disulfide produced by the skin microbiota are the principal cues that mosquitoes use to locate humans [50,68,70].

The spectrum and diversity of known mVOCs were recently summarized in the mVOC database [34], which lists more than 1200 mVOCs that have been described so far. Volatile profiles of individual microorganisms often reveal compounds that are completely new to nature. One such species is the rhizobacterium *Serratia plymuthica* 4Rx13, which releases more than 100 volatiles, including the novel compound sodorifen that has a unique and unusual structure [71,74]. Moreover, some bacteria produce compounds of particular interest, which possess antibiotic, antifungal, nematicidal or plant-growth-promoting properties and/or potentially function as signal molecules in communication within microbial communities [2,9,14,15,16,24,28,52,56,64].

It is likely that the skin represents a habitat characterized by strong interactions between its normal microbial residents and/or with other environmental microorganisms. However, while little is known about how these communities maintain their stability on the skin, it is tempting to speculate that mVOCs might play important roles in microbial interactions and defenses. In order to gain a better understanding of the role of these mVOCs, the VOC profiles of different bacterial species naturally found on the skin were first analyzed. The effects of bacterial volatiles on other bacteria were then studied, and it was found that volatiles produced by *Staphylococcus schleiferi* DSMZ 4807 inhibited the growth of Gram-positive bacteria and affected the phenotypes of Gram-negative bacteria that are controlled by quorum sensing. Among the volatiles released by *S. schleiferi* isolates, amino/imino ketones were identified that had never been reported from any other organism. Therefore, the structures, biological effects and modes of action of these substances, which were designated schleiferons A and B, on the growth of Gram-positive and Gram-negative bacteria are described.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study, and their origins, are summarized in Table S3. Brain heart infusion medium (BHI) (Roth, Germany) was used as the culture medium for all strains except *Serratia marcescens* V11649 and *S. plymuthica* AS9, which were grown in peptone glycerol broth (5 g peptone, 2 g K_2HPO_4, 10 mL glycerol per L of medium), and *Vibrio harveyi* DSMZ 6904, which was grown in Basic medium (10 g tryptone, 5 g yeast extract, 1.5 mL of 50% glycerol, 20 g NaCl, 1 g $MgSO_4$, 6 g Tris-HCl, pH 7.5 with HCl per L of medium). Bacterial stocks were prepared by adding glycerol (final concentration 25%) to an overnight culture, and stored at −70 °C.

Collection and analysis of mVOCs

A single colony of each strain was transferred from a Petri dish to 8 mL of BHI and incubated at 30 °C under agitation (170 rpm) for 24 h in order to obtain a fresh, pure pre-culture. The cell density of each pre-culture was measured at OD_{600} (0.05–1) and an aliquot was transferred into a modified 250-mL conical flask containing 100 mL of culture medium (final OD_{600} of 0.005). The culture was set up in a closed-airflow VOC collection system ([27], modified) connected to a pump (Gardner Denver Thomas GmbH, Memmingen, Germany) and incubated at 30 °C under agitation (Fig. S8). Charcoal-purified air, sterilized by passage through a wad of cotton wool, was introduced into the conical flask containing the bacterial culture at a constant flow rate (500 mL min^{-1}). After passing over the bacterial culture, the volatile-enriched air was further funneled into a trap containing 30 mg of adsorbent matrix (Porapak™, Waters, Eschborn, Germany). After a defined incubation period (see specific figure or figure legend), the volatiles were eluted from the matrix with 300 μL of dichloromethane. Nonyl acetate (10 μL; equivalent to a final concentration of 5 ng μL^{-1} in the eluate) was added as an internal standard. Samples were analyzed using a Shimadzu GC/MS QP 5000 (equipped with a 60 m × 0.25 mm × 0.25 μm DB5-MS column). Using a CTC autosampler, 1 μL of the eluate was injected directly (without flow splitting) at 200 °C with a sampling time of 2 min. Helium was used as the carrier gas. Mass spectra were obtained using the scan mode. Compounds were identified by comparing their retention times and mass spectra with those of the authentic compounds or with those available in the National Institute of Standards and Technology (NIST) 107 library (version 1998). As a control experiment, the volatiles emitted from the media were determined at respective time intervals and were always subtracted from the bacterial volatile profiles and did not appear in the analyses. The media volatile profiles never showed schleiferon A or B. Furthermore, the volatiles of the media continuously decreased from interval to interval, indicating that at later stages during bacterial growth when schleiferon became dominant the volatiles of the media became minor compounds. To ensure that the volatiles analyzed were derived from the bacteria, two control experiments were performed: (i) the supernatant of the overnight culture was sterile filtered and incubated, and (ii) the supernatant was heated to inactivate enzymes, sterile filtered and incubated. Schleiferons A and B were not detected in either experiment, and only volatiles from the media were present (data not shown). The quantities of schleiferons A and B produced were calculated based on the internal standard. Schleiferons A and B were synthesized from 2-phenylethylamine and acetoin (Schulz et al., in preparation).

Effects of S. schleiferi *VOCs on other microorganisms*

Dual culture experiments were performed using 96-well microtiter plates (Fig. S3) or bipartite Petri dishes. A total of 40 wells of each microtiter plate were filled with 200 μL of an *S. schleiferi* DSMZ 4807 culture (OD_{600} 0.005) and another 40 wells were inoculated with 200 μL of the test bacterium culture (*Staphylococcus sciuri* V405, *Staphylococcus saccharolyticus* B5709, *S. epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729, *Enterococcus faecalis* ATCC 51299, *Escherichia coli* DH5α, *Pseudomonas fluorescens* V12141, *S. marcescens* V11649 and *Salmonella enterica* RV4) of