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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

OD_{600} (0.005). The microtiter plate was incubated at 30 °C in a microtiter plate reader (SpectraMax M2, MWG-Biotech, Ebersberg, Germany) for 72 or 96 h. Every 30 min the plate was shaken for 15 s and the cell density was measured. In the control experiment, the plate was filled with 200 μL of BHI instead of the *S. schleiferi* DSMZ 4807 culture.

For dual culture in bipartite Petri dishes, one compartment of the plate was filled with 10 mL of a 96 h culture of *S. schleiferi* or *S. warneri* (control) and the other was inoculated with 10 mL of the test bacterium. In a second control experiment, 10 mL of BHI instead of the *S. schleiferi*/*S. warneri* culture were added to one compartment of the Petri dish. The plate was incubated for 24 h at 30 °C, and the growth of the test bacteria was monitored by determining living cell numbers (CFU) and cell densities (OD_{600}).

For the liquid dual culture system (Fig. S9), *S. schleiferi* DSMZ 4807 and *S. warneri* CCM 2730 cultures (OD_{600} 0.005) were grown at 30 °C under agitation (170 rpm). After 48 h or 96 h incubation, 100 mL of each culture was transferred into a 250-mL modified conical flask (Fig. S9). In this set-up, charcoal-purified and sterilized air was pumped as described above into a bacterial culture flask (*S. schleiferi* or *S. warneri* as a control) at a constant rate of 700 mL min^{-1}, and the volatile-enriched air was allowed to pass through an outlet of the first flask into a second flask containing *S. marcescens* V11649 or *V. harveyi* DSMZ 6904 (150 mL culture OD_{600} 0.005). After 24 h, the growth of *S. marcescens* or *V. harveyi* was monitored by determining living cell numbers (CFU) and cell densities (OD_{600}).

Effects of synthetic schleiferons A and B on quorum-sensing-dependent phenotypes of Gram-negative bacteria

Overnight cultures of *S. marcescens* V11649, *S. plymuthica* AS9 or *V. harveyi* DSMZ 6904 were incubated at 30 °C under agitation (170 rpm) (20 °C for *S. plymuthica*). An aliquot of this culture (OD_{600} 0.5–1) was then transferred into a 25-mL Erlenmeyer flask or to one side of a bipartite Petri dish, both containing 4 mL of culture medium (final OD of 0.005). Various concentrations of schleiferon A or B solved in DMSO were added either directly into the bacterial culture or onto a 6 mm Whatman filter paper disk, which was placed in the other side of the Petri dish. The bacterial culture was incubated under agitation in the Erlenmeyer flask, and the Petri dish or Erlenmeyer flask were incubated at 30 °C (20 °C for *S. plymuthica*). After 20 h the cell density was determined. Prodigiosin was extracted from *S. marcescens* and *S. plymuthica* cultures and quantified as described below. *V. harveyi* bioluminescence was determined using the Stella Image Reader (Raytest, Straubenhardt, Germany) and quantified with Aida Image Analyser software. In control experiments, DMSO (dimethyl sulfoxide) was used instead of schleiferons A and B.

Extraction of prodigiosin

S. marcescens or *S. plymuthica* cultures were centrifuged at 10,000 × *g* for 5 min, the supernatant was discarded and the pellet resuspended in 96% ethanol acidified with 5% HCl. After centrifugation at 10,000 × *g* for 5 min, prodigiosin was quantified by measuring its absorption in the supernatant at 534 nm.

Determination of minimum inhibitory concentrations (MICs) for schleiferons A and B

MIC determinations were carried out on various Gram-positive and Gram-negative test species in 25-mL Erlenmeyer flasks (each containing 4 mL of culture medium), which had been inoculated (to OD_{600} = 0.005) with aliquots of cultures grown to OD_{600} 0.5–1 overnight at 30 °C and 170 rpm. Different concentrations of schleiferon A or B diluted with DMSO were added to the test cultures and incubated under agitation at 37 °C. After 20 h, the CFU and cell densities (OD_{600}) were determined. DMSO was used instead of schleiferon A or B in control experiments.

Preparation of RNA probes

Dioxygenin-labelled RNA probes for the corresponding genes were generated by PCR, using genomic DNA as template isolated from *S. plymuthica* AS9 with the NucleoSpin® Tissue kit (Macherey-Nagel). Each reaction contained 0.2 μg DNA, 3 μL reaction buffer, 1 μL *Taq* polymerase (both from Thermo Scientific), 1 μL each of dATP, dCTP and dGTP, 6.5 μL dTTP (from 10 mM stock solutions), 3.5 μL of 1 mM DIG-11_dUTP (Roche, Mannheim, Germany), 2 μL of each primer (Table S4) and 24 μL of ddH_2O.

RNA isolation and Northern blot analysis

Total RNA was isolated using the hot phenol-SDS method [45] from *S. plymuthica* AS9 cultures (OD 0.005) grown in the presence or absence of (340.5 μg mL^{-1}) schleiferon A or B, as described above. Control experiments were treated with DMSO. RNA integrity was confirmed by agarose gel electrophoresis and quantified by spectrophotometry (Smart Spec 3000, BioRad), using 20 μg of the total RNA of each sample on a 1% gel. RNA was separated under denaturing conditions (72.5 mL running buffer (20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA × $2H_2O$, pH 7.0) and 2.5 mL formaldehyde). The RNA was transferred onto a positively charged nylon membrane (Roche, Mannheim, Germany) by capillary blotting using 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 16 h. Following the transfer, the RNA was cross-linked to the membrane by UV radiation (0.120 J/cm^2) for 1 min (Techne, Thermo-DUX, Wertheim, Germany). 16S rRNA bands on the membrane indicated the efficiency of the blotting and served as a control for the quantity of RNA in each lane. DIG-labelled probes were used to detect specific RNA molecules by hybridization [54].

Preparation of inverted membrane vesicles

E. coli TKR2000 was transformed with plasmid pNKN or pNKQ encoding wild-type LuxN or LuxQ, respectively. For overproduction of CqsS, *E. coli* Rosetta (DE3) pLysS was transformed with pKK-CqsS-6His encoding wild-type CqsS. Inside-out membrane vesicles were prepared as described previously [66].

Heterologous production of LuxP and LuxU

E. coli MDAI-2 was transformed with the plasmid pGEX_LuxP and purified as described elsewhere [42]. LuxU was overproduced using *E. coli* JM109 transformed with pQE30LuxU-6His, and purified as described by Timmen et al. [66]. All proteins were stored at −80 °C.

Phosphorylation assay

Phosphorylation reactions were performed in phosphorylation buffer (50 mM Tris/HCl pH 8.0, 10% (v/v) glycerol, 500 mM KCl, 2 mM DTT) at room temperature (69). The hybrid histidine kinases LuxQ, CqsS and LuxN were used as full-length membrane integrated proteins in inverted membrane vesicles at final concentrations of 5.5 mg mL^{-1} for LuxQ and CqsS and 2 mg mL^{-1} for LuxN. The reaction mixture contained 0.36 mg mL^{-1} LuxU and 0.48 mg mL^{-1} LuxP, unless otherwise indicated. To incorporate LuxP into LuxQ-containing membrane vesicles, three cycles of freezing and thawing were performed. Unless otherwise indicated, schleiferon A or B was

added at a final concentration of 340.5 μg mL^{-1} in DMSO (the corresponding volume of DMSO alone was used in the controls). The phosphorylation reaction was started by adding radiolabeled Mg^{2+}-ATP, typically 100 μM [γ-^{32}P]ATP (0.94 Ci mmol^{-1}; PerkinElmer, Rodgau-Jügesheim, Germany) and 110 μM $MgCl_2$, and stopped at various time points by the addition of SDS loading buffer, followed by fractionation of the reaction on SDS-polyacrylamide gels. Gels were dried at 80 °C on filter paper, exposed to a phosphoscreen for at least 24 h and scanned using a Typhoon Trio™ variable mode imager (GE Healthcare, München, Germany).

Bioluminescence assay

To compare the bioluminescence yields of wild-type *V. harveyi* ATCC BAA-1116 (BB120) (recently reclassified as *Vibrio campbellii* ATCC BAA-1116 [36]) with those of a *luxO*-deletion mutant (Δ*luxO*), cells from a culture grown overnight in LM medium (20 g L^{-1} NaCl, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract) were diluted to an OD_{600} of 0.05 in AB medium [20] and cultivated aerobically at 30 °C. Schleiferon A or B (at 340.5 μg mL^{-1}) was added to the exponential growth phase at the indicated time point. As a control, the corresponding volume of DMSO was added to the control culture. Cultivation as well as measurement of OD_{600} and luminescence (every 20 min) was performed in microtiter plates using a Tecan Infinite® F500 system (Tecan, Crailsheim). Data are reported as relative light units (RLU) in counts per second per milliliter per OD_{600}.

Statistical analysis

Comparisons between means were carried out according to the Student's t-test. Differences were considered significant at a p value of <0.05. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed using the web-based software tool MetaboAnalyst 3.0 [78].

Results

Volatile profiles of skin bacteria

The emission of VOCs into headspaces was comprehensively investigated for the two dominant families of the skin microbiota, Staphylococcaceae and Corynebacteriaceae. When grown in BHI broth, whose composition resembles that of human tissue, members of these families produced a variety of volatile compounds. The VOC profiles of *Corynebacterium* consisted of 11 different compounds (Table S1). Of these, 2-nonanone (#9), 2-phenylethanol (#12), 2-undecanone (#20), 8-pentadecanone (#46) and 2-pentadecanone (#47) were produced by all *Corynebacterium* isolates, and very large amounts of 2-phenylethanol were released by *Corynebacterium striatum* isolates (Fig. S1 (I–VII)). *Staphylococcus* strains emitted more than 50 compounds in all, among which ketones were dominant (Table S1). After a multivariate analysis, principal component analysis confirmed that Staphylococcaceae and Corynebacteriaceae could be partitioned into two distinct groups on the basis of their specific VOC profiles (Fig. 1A). Hierarchical clustering analysis also distinguished two clearly separated clusters corresponding to the two bacterial families. Moreover, each cluster was further divided into several subclusters according to the respective VOC profile of the bacterial isolate (Fig. 1B). Based on their VOC profiles, individual *C. striatum* and *Corynebacterium jeikeium* isolates, respectively, were more closely related to each other than to the other *Corynebacterium* species, while in the Staphylococcaceae family, representatives of the same bacterial species (e.g., *S. epidermidis*, *S. sciuri* or *S. schleiferi* isolates) did not always cluster together, and *S. sciuri* isolates together with *Staphylococcus intermedius* were distinctly separated from the other *Staphylococcus* species. Among the *Staphylococcus* strains, the VOC profiles of *S. schleiferi* isolates were particularly intriguing. Both strains produced more than 30 VOCs, among which were 2- and 3-methylbutanoic acids (#4 and #5), 2-phenylethylamine (#10), farnesol (#49) and several ketones (Table S2). The ketones #31, #35, #43 and #44 appeared in relatively high quantities and were only emitted by *S. schleiferi* isolates (Table S1). The structures of compounds #31 and #35 were elucidated as reported elsewhere (Schulz et al., in preparation). The two substances were identified as 3-(2-phenylethylamino)butan-2-one and (*E*)-3-(2-phenylethylimino)butan-2-one, and were named schleiferons A and B, respectively (Fig. 2A). Neither has previously been reported from natural sources. Schleiferons A and B were emitted in different amounts by the three *S. schleiferi* isolates DSMZ 4807, V431 and H34 (Figs. S2A and S2B, respectively), with DSMZ 4807 releasing approximately 30- to 100-fold higher levels of both compounds than V431 and H34, respectively, although all three strains had similar growth curves. During bacterial growth, schleiferons A and B first appeared in the stationary phase after 48 h incubation, and reached their maximum levels by 96 h. At 48 h, schleiferons A and B represented 34% and 2.4%, respectively, of the total VOC spectrum (Fig. 2B), which increased to 70% and 10% by 72 h, and 73% and 14% at 96 h.

Effects of S. schleiferi *volatiles on various microorganisms*

The effects of the bouquet of volatiles produced by a 24 h-old culture of *S. schleiferi* were evaluated on various Gram-positive and Gram-negative bacteria in co-culture experiments in which 96-well microtiter plates were inoculated with *S. schleiferi* and the test bacterium at the same time. After co-cultivation, the growth rates of the test species incubated with and without *S. schleiferi* did not differ significantly (Fig. S3A and B). Conversely, volatiles emitted by the tested species had no effect on the growth of *S. schleiferi* (Fig. S3C). Nevertheless, since schleiferons A and B were part of the volatile cocktail produced by *S. schleiferi* and were emitted in large quantities in the late stationary phase (Fig. 2B), co-culture experiments (in bipartite Petri dishes) were performed by inoculating a 96 h-old culture of *S. schleiferi* with *S. epidermidis* RP62A, *S. haemolyticus* CCM 2729 (Gram-positives), *S. enterica* RV4 or *S. marcescens* V11649 (Gram-negatives). After 24 h co-cultivation, *S. schleiferi* volatiles inhibited the growth of the Gram-positive bacteria significantly, but did not affect that of the Gram-negative species (Fig. 3A).

Effects of synthetic schleiferons A and B on Gram-positive bacteria

As documented in Fig. 3A, co-cultivation of *S. schleiferi* with other *Staphylococcus* strains significantly inhibited the growth of the latter. We therefore determined whether schleiferons A and B, when added to a liquid culture, were toxic for *Staphylococcus* isolates or for Gram-positive bacteria in general by testing their effects at different concentrations and determining their minimum inhibitory concentrations (MICs). Growth of all tested Gram-positive bacteria was indeed significantly inhibited by schleiferon A or B in a concentration-dependent manner (Fig. 3B, Table 1). Moreover, schleiferon A was approximately 10 times more effective than schleiferon B and both compounds were more toxic to corynebacteria and *Micrococcus luteus* than to staphylococci. The respective MICs ranged from 35 to 70 μM for schleiferon A, while *Staphylococcus*, *Enterococcus* and *Bacillus* strains were 2- to 16-fold less susceptible (Table 1). Interestingly, schleiferon A was less active on *S. schleiferi* DSMZ 4807 and *S. schleiferi* H34 than on *S. schleiferi* V431 and most other *Staphylococcus* strains. Among the *Staphylococcus* strains tested, schleiferon A was most effective against *S.*

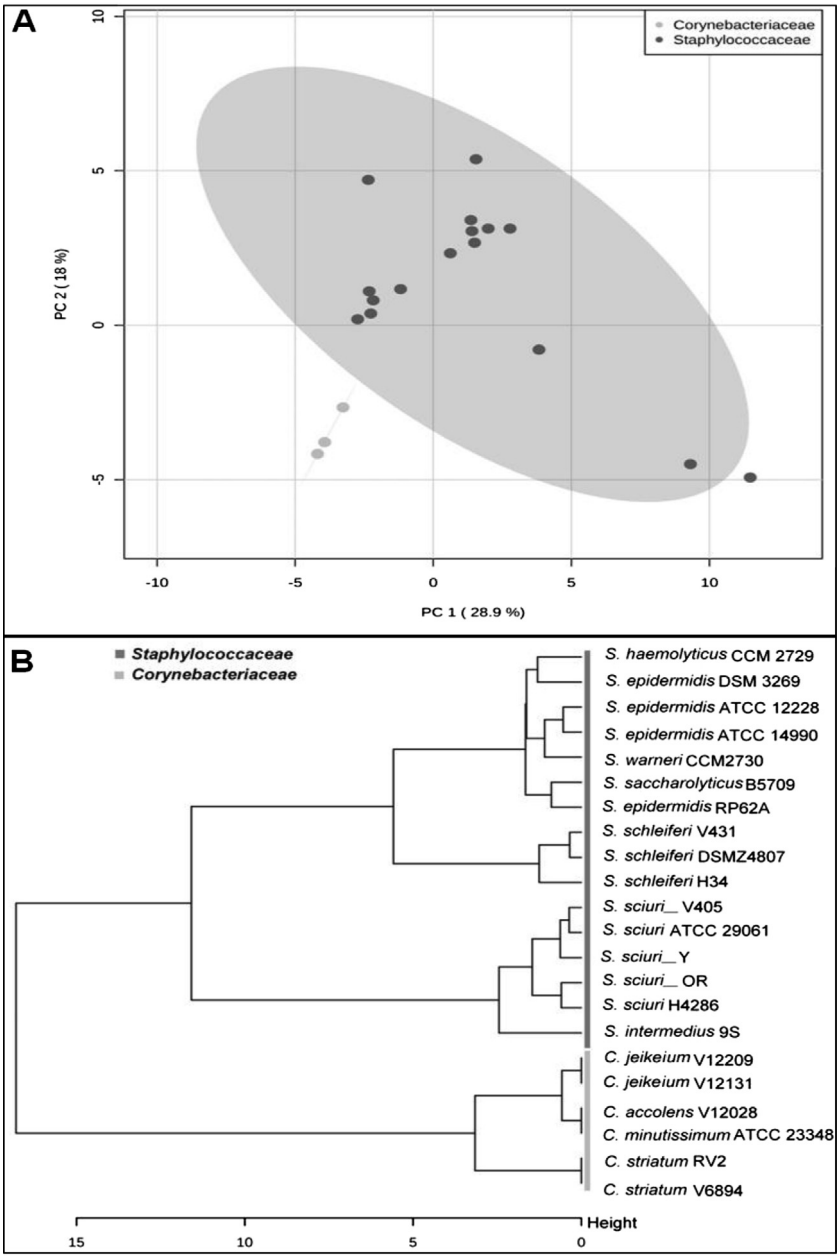

Fig. 1. Multivariate analysis of the volatile profiles of the Staphylococcaceae and Corynebacteriaceae families.
Multivariate analyses were performed using MetaboAnalist 3.0 [78], a comprehensive online tool for metabolomic data analysis. (A) Principal component analysis distinguished bacterial families on the basis of their VOC profiles. The shaded areas represent 95% of the confidence interval. (B) Dendrogram of bacterial species based on hierarchical clustering analysis of all VOCs emitted by the bacterial taxa investigated (original data in Table S1).

warneri, *S. epidermidis* and *S. haemolyticus*. In contrast, in a bipartite Petri-dish experiment, schleiferons A and B did not inhibit the growth of *S. epidermidis* and *S. haemolyticus* (Fig. S3D). Strikingly, when equivalent concentrations of schleiferon A or B were tested on Gram-negative bacteria (e.g., *S. enterica*), little or no growth inhibition was observed (Fig. 3B, Table 1).

S. schleiferi *volatiles repress quorum-sensing-dependent phenotypes of Gram-negative bacteria*

As shown in Fig. S4A, *S. schleiferi* VOCs had little effect on the growth of Gram-negative bacteria, while they significantly reduced the levels of prodigiosin produced by *S. marcescens* V11649. Prodigiosin is a red pigment (with a broad range of biological activities) produced by several bacteria, including *Serratia* strains, in which prodigiosin biosynthesis is known to be controlled by the quorum sensing system [61,75,76]. Therefore, the impact of VOCs was studied not only on prodigiosin synthesis in *S. marcescens* V11649 but also on bioluminescence in *V. harveyi* DSMZ 6904. When the former was co-cultivated with a 48 h-old culture of *S. schleiferi* for 24 h (Fig. S9), prodigiosin production was inhibited by approximately 40% (Fig. 4A and C) and repressed by more than 60% in a 96 h-old culture (Fig. 4B and D), indicating that the inhibitory effect depended on the concentration of VOCs present. Similarly, *V. harveyi* cultures showed an almost 60% reduction in bioluminescence when fumigated with *S. schleiferi* volatiles (Fig. 4E and F). It is important to note here that the bacterial cell density was not affected, and that exposure to volatiles from *S. warneri* had no impact on either prodigiosin synthesis or bioluminescence (Fig. 4).

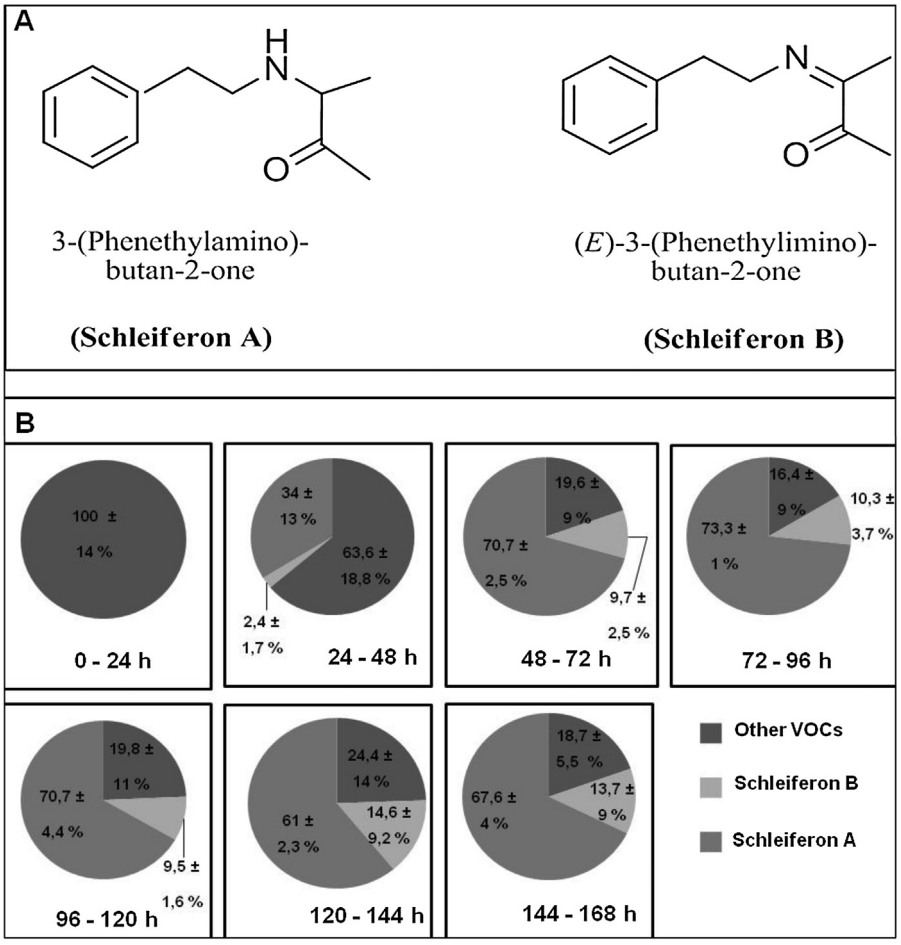

Fig. 2. Structure of schleiferons (A) and VOC spectrum of *Staphylococcus schleiferi* DSMZ 4807 (B).
VOCs were quantified and the relative contributions (%) of schleiferons A and B of the VOC spectrum of *S. schleiferi* cultures were determined at intervals of 24 h over the course of 1 week (based on data in Fig. S2) (100% (0–24 h) = 16.6 ng; 100% (24–48 h) = 34.2 ng; 100% (48–72 h) = 104.5 ng; 100% (72–96 h) = 130.3 ng; 100% (96–120 h) = 136.8 ng; 100% (120–144 h) = 109.3 ng; 100% (144–168 h) = 108.1 ng).

Table 1
Minimum inhibitory concentrations (MIC) of schleiferons A and B on different Gram-positive bacteria.

Bacteria		MIC (μM) of schleiferon	
Families	Species	A	B
Corynebacteriaceae	*Corynebacterium jeikeium* V12209	34.8	281
Corynebacteriaceae	*Corynebacterium striatum* RV2	34.8	281
Corynebacteriaceae	*Corynebacterium minutissimum* ATCC 23348	69.6	563
Staphylococcaceae	*Staphylococcus schleiferi* DSMZ 4807	278	1130
Staphylococcaceae	*Staphylococcus schleiferi* H34	278	2250
Staphylococcaceae	*Staphylococcus schleiferi* V431	139	2250
Staphylococcaceae	*Staphylococcus intermedius* 9S	139	563
Staphylococcaceae	*Staphylococcus saccharolyticus* B5709	139	1130
Staphylococcaceae	*Staphylococcus sciuri* V405	139	1130
Staphylococcaceae	*Staphylococcus warneri* CCM 2730	96.7	1130
Staphylococcaceae	*Staphylococcus epidermidis* RP62A	69.6	281
Staphylococcaceae	*Staphylococcus haemolyticus* CCM 2729	69.6	563
Micrococcaceae	*Micrococcus luteus* V515	34.7	141
Enterococcaceae	*Enterococcus faecium* ATCC 51559	278	2250
Enterococcaceae	*Enterococcus faecalis* ATCC 51299	557	3380
Bacillaceae	*Bacillus subtilis* B2g	278	1130
Enterobacteriaceae	*Salmonella enterica* RV4	4450	>10,000

Effects of synthetic schleiferons A and B on quorum-dependent phenotypes

To verify the hypothesis that schleiferons A and B emitted by *S. schleiferi* were responsible for the quorum-quenching effect of total VOCs on prodigiosin synthesis and bioluminescence emission by the test bacteria, cultures of *S. plymuthica* AS9, *S. marcescens* V11649 and *V. harveyi* DSMZ 6904 were incubated with different concentrations of pure (synthetic) schleiferons A and B. With increasing concentrations of the ketones, production of prodigiosin (Fig. S4B, 5A and B, S5A) and emission of bioluminescence (Fig. 5C, S5D), respectively, were eventually repressed to virtually undetectable levels. Inhibition of prodigiosin production by both *S. marcescens* (Fig. 5A) and *S. plymuthica* (Fig. 5B), as well as repres-

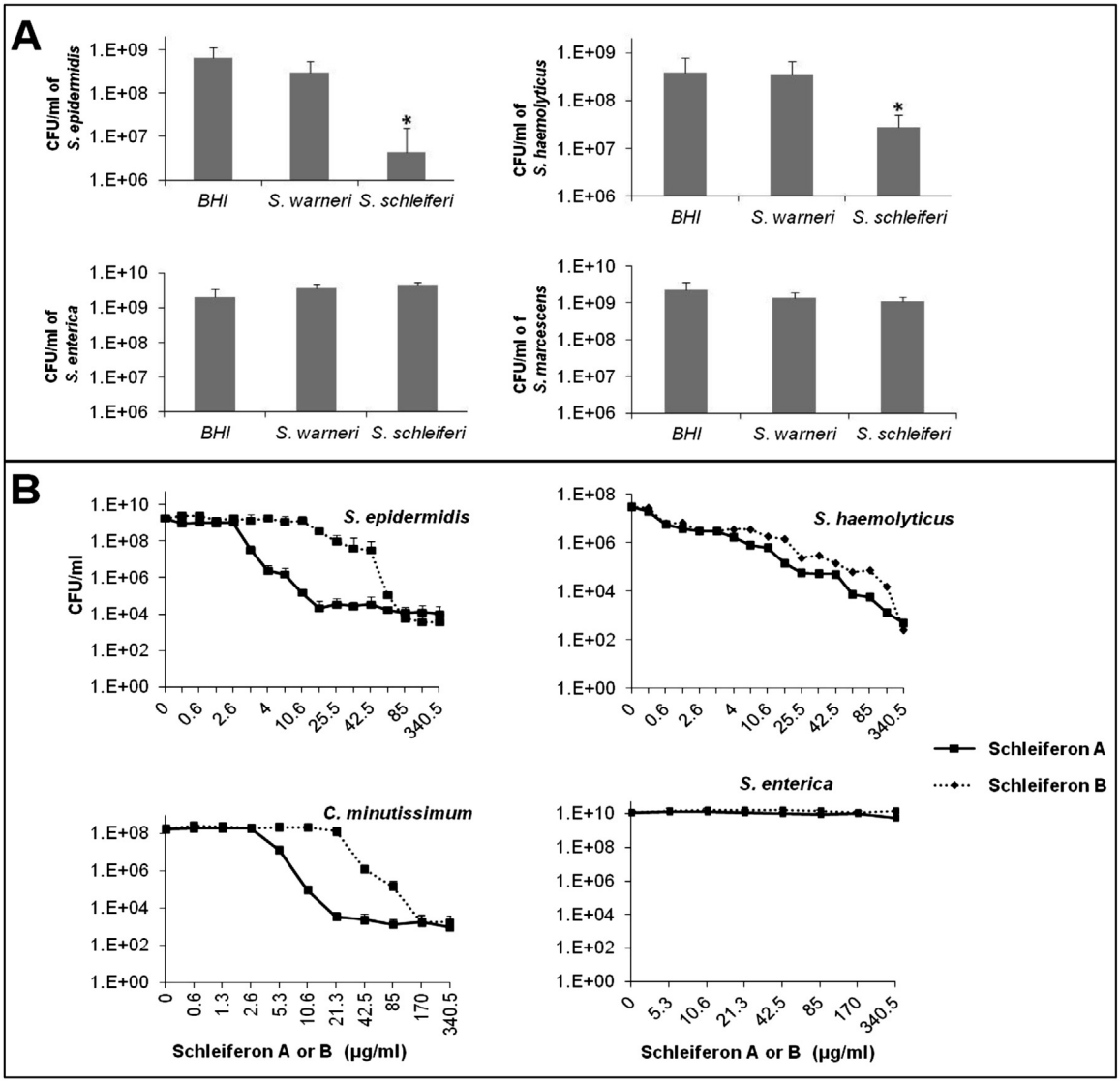

Fig. 3. Effects of *Staphylococcus schleiferi* and *S. warneri* volatiles (A) and synthetic schleiferons (B) on the growth of different Gram-positive and Gram-negative bacteria. (A) Gram-positive (*Staphylococcus epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729) and Gram-negative (*Serratia marcescens* V11649, *Salmonella enterica* RV4) bacteria were co-cultivated with a 96-h old culture of *S. schleiferi* or *S. warneri* in bipartite Petri dishes for 24 h. Growth of the bacteria was monitored by determining viable cell numbers (CFU). Uninoculated growth medium (BHI) was used as a control. $^{*}p < 0.05$.
(B) *Staphylococcus epidermidis* RP62A, *Staphylococcus haemolyticus* CCM 2729, *Corynebacterium minutissimum* ATCC 23348 and *Salmonella enterica* RV4 were grown at 37 °C in BHI medium in the presence of the indicated final concentrations of schleiferon A or B. After 20 h, viable cell numbers (CFU) were determined. Data are the means of three independent experiments and bars indicate the mean standard deviation. When error bars are not visible, they were smaller than the symbols.

sion of bioluminescence in *V. harveyi* (Fig. 5C), was already obvious at a low concentration (5.3 µg mL^{-1}) of the test substance and increased gradually with an increasing concentration of added schleiferon A or B. At 42.5 µg mL^{-1}, both phenotypes were suppressed by approximately 50% and at the maximum concentration (340.5 µg mL^{-1}) almost no prodigiosin or bioluminescence was detected, while the cell density was only slightly affected by schleiferon A or B. Moreover, determination of the numbers of viable cells in *S. marcescens* (Fig. S5B) and *S. plymuthica* (Fig. S5C) cultures treated with schleiferon A or B (CFU mL^{-1}) revealed no significant differences compared to the control (bacteria treated with DMSO).

Schleiferons A and B act downstream of the quorum-sensing phosphorelay of V. harveyi

Induction of bioluminescence in *V. harveyi* depends on a complex quorum-sensing (QS) signaling cascade. At low cell density, in the absence of autoinducers, the three hybrid histidine kinases LuxN, LuxQ (in interplay with LuxP) and CqsS autophosphorylate the transfer of the phosphoryl group via a phosphorelay to the histidine phosphotransferase protein (HPr) LuxU, and subsequently to the response regulator LuxO (69). Phosphorylated LuxO activates transcription of five regulatory sRNAs which, together with the RNA chaperone Hfq, destabilize the transcript coding for the master regulator LuxR. When the concentration of LuxR in cells is low, induction of bioluminescence is impossible. At high cell density, in the presence of high concentrations of autoinducers, autophosphorylation is inhibited, LuxR is synthesized, and the bioluminescence phenotype is expressed [72]. To gain insight into the molecular mechanism of schleiferon A- and B-mediated inhibition of bioluminescence in *V. harveyi*, in vitro phosphorylation assays of the three hybrid histidine kinases were performed. LuxQ, CqsS and LuxN, respectively, were heterologously expressed in *E. coli*, and inverted membrane vesicles bearing the full-length proteins were

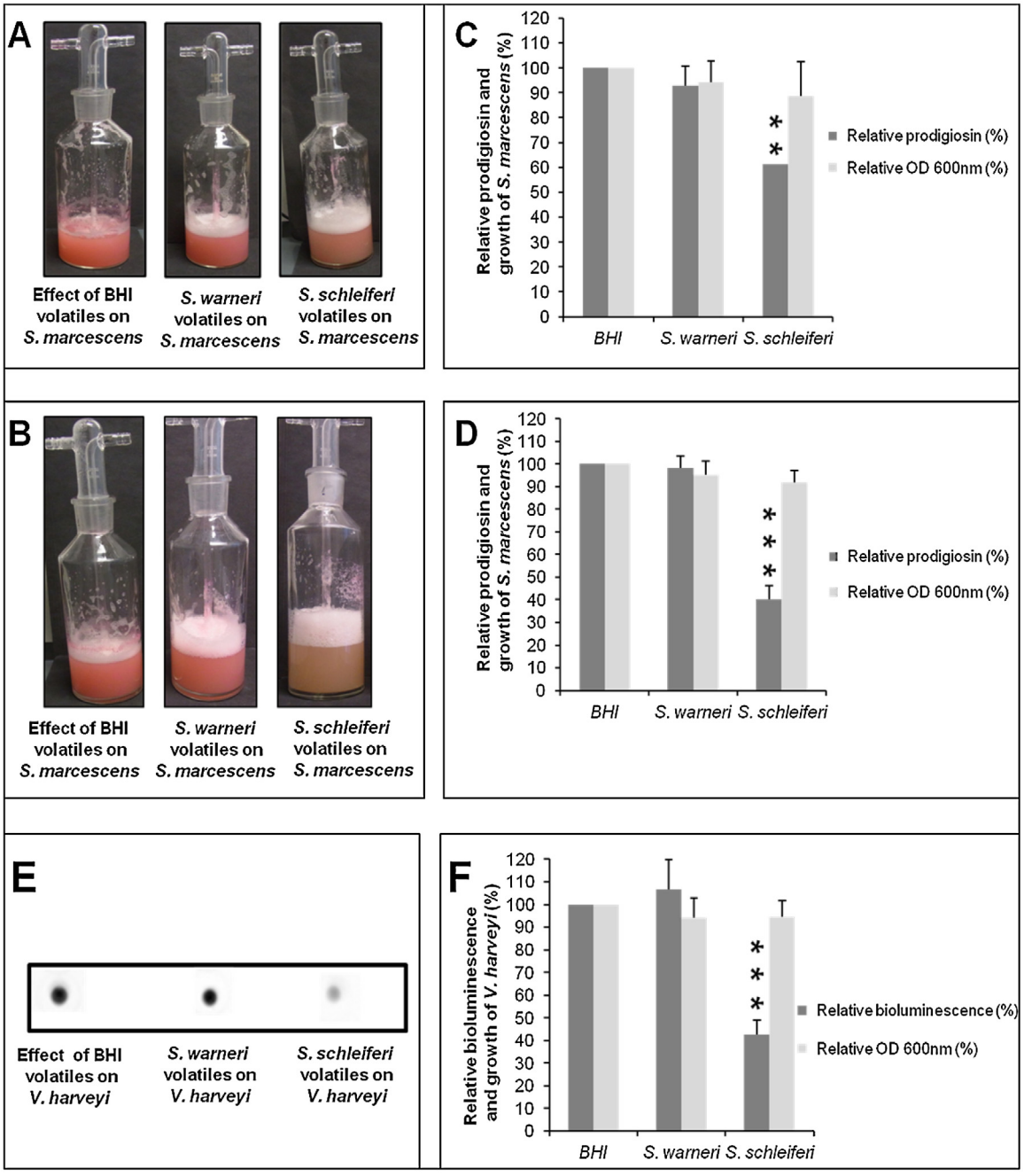

Fig. 4. Volatiles of *Staphylococcus schleiferi* inhibit prodigiosin production in *Serratia marcescens* and bioluminescence in *Vibrio harveyi*.
S. marcescens was cultivated in a dual culture system (Fig. S9) for 24 h with a 48-h (A) or a 96-h (B) culture of *S. schleiferi* or *S. warneri* (BHI: brain heart infusion medium). Cell density (OD_{600} of 100% = 4) and relative levels of prodigiosin in the culture medium (%) (OD_{534} of 100% = 1.4) for *S. marcescens* were measured after 24 h dual cultivation of *S. marcescens* with a 48 h (C) or 96 h (D) culture of *S. schleiferi*/*S. warneri*. (E) shows the light image of *V. harveyi* bioluminescence cultivated in a dual culture system (Fig. S9) for 24 h with a 96-h old culture of *S. schleiferi*/*S. warneri*, and (F) shows the relative growth (% cell density) (OD_{600} of 100% = 5) and bioluminescence emission (%) (100% = 7.9E + 05) for *V. harveyi*. The culture medium was used as a control. Data are the means of 3–5 independent experiments and bars indicate the mean standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$.

directly used for phosphorylation assays. The effect of schleiferons A and B (340.5 μg mL^{-1} final concentration) was investigated on the autophosphorylations catalyzed by the three kinases and on the LuxU phosphotransfer reaction. However, no significant increase was detected in phosphorylated LuxU compared to the controls, which were treated with DMSO alone (Fig. S6A–C).

Moreover, in an independent in vivo assay, it was found that bioluminescence of the *luxO* deletion mutant, which exhibits autoinducer-independent (i.e., constitutive) bioluminescence, was still inhibited by application of either schleiferon (Fig. S7). These results demonstrated that the decrease in bioluminescence caused by schleiferons A and B was not related to any perturbation of the QS phosphorelay by these compounds.

Synthetic schleiferons A and B stimulate the regulators of prodigiosin biosynthesis in S. plymuthica *AS9*

Prodigiosin biosynthesis has been well characterized in *Serratia* sp. strain ATCC 39006 [61,75,76]. Homologous genes involved in the biosynthesis of this red pigment are found in *S. plymuthica* AS9 [29]. The *pig* cluster (*pigA-O*) encodes the enzymes that catalyze the synthesis of the pigment from proline and octenal to prodigiosin,

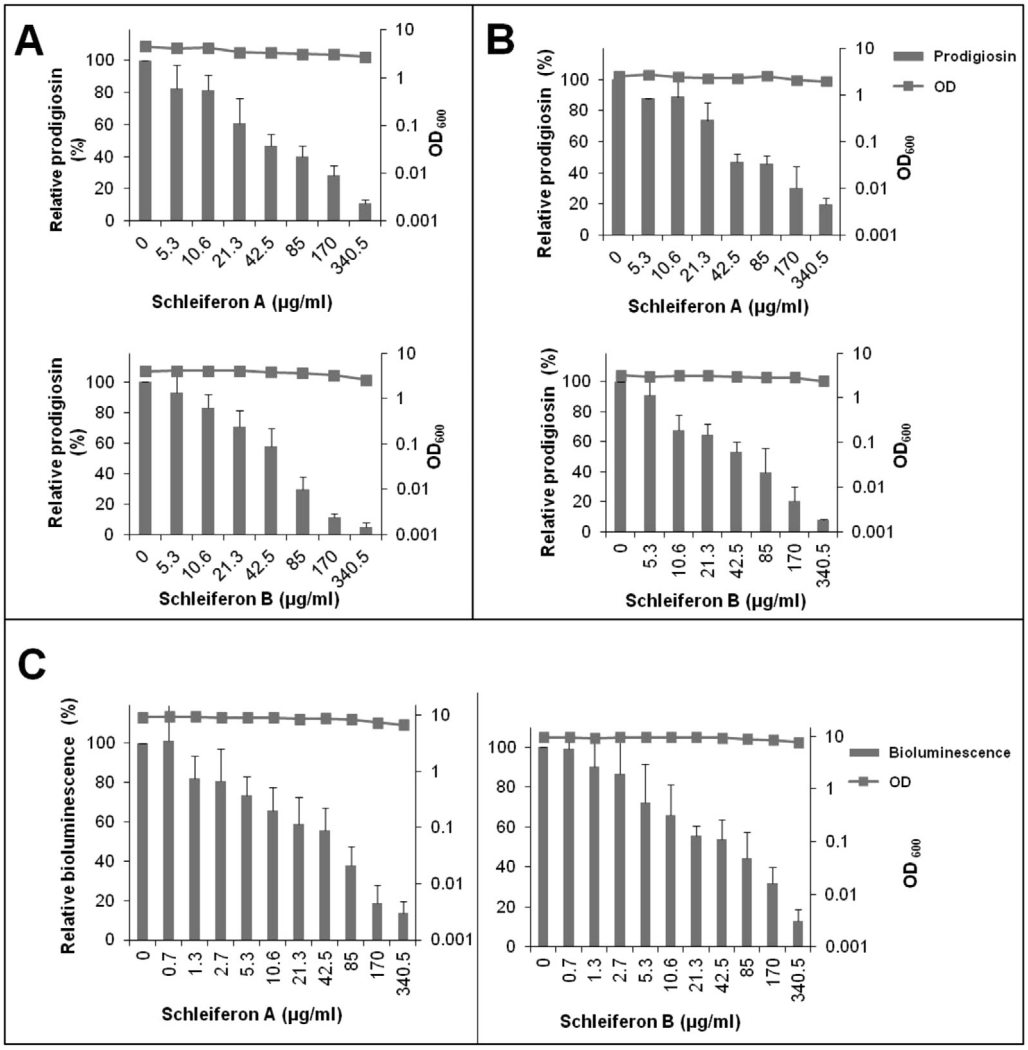

Fig. 5. Inhibition of prodigiosin production in *Serratia marcescens* and *S. plymuthica* (A and B) and bioluminescence emission by *Vibrio harveyi* (C) by synthetic schleiferons A and B.

Bacteria were incubated with increasing concentrations of schleiferon A or B (final concentrations are shown). Bacterial growth was monitored by measuring the cell density at OD_{600}, prodigiosin levels were determined from the OD_{534} value and relative prodigiosin production was calculated as the ratio between prodigiosin and cell density (100% = 2 for *S. marcescens* (A) and 0.98 for *S. plymuthica* AS9 (B)). Relative bioluminescence emission (%) (100% = 8.7E + 05) was calculated as the ratio between bioluminescence intensity and cell density (C). Controls: bacteria were incubated with DMSO and their prodigiosin or bioluminescence emission levels were set as 100%. Data are means of at least five independent experiments and bars indicate the mean standard deviation.

while *pigP*, *pigS* and *luxR* code for transcriptional regulators of the cluster and *luxS* encodes the synthase responsible for production of the quorum-sensing auto-inducer AI-2 [23,61]. It was hypothesized that schleiferons A and B might repress prodigiosin synthesis by acting on the transcription factors. To test this idea, Northern blot analysis was used (Fig. 6) to determine the levels of the *pigP*, *pigS* and *luxR* transcripts. As a result, increased *pigP*, *pigS* and *luxS* mRNA levels were observed after application of schleiferon B, while the *luxR* level was unaffected (Fig. 6A). Furthermore, the expression levels of the prodigiosin gene cluster of *S. plymuthica* AS9 were investigated. Since the biosynthetic genes are co-transcribed [61], the transcription level of one of the genes (*pigG*) was analyzed using Northern blotting and a decrease was found in the *pigG* transcript (Fig. 6B), which consequently indicated a decrease in transcription of the *pig* gene cluster.

Discussion

In this study, it was shown that skin bacteria were a rich source of natural volatile organic compounds (VOCs) and novel compounds were identified that selectively inhibited the growth of Gram-positive bacteria and influenced specific phenotypes (prodigiosin synthesis, bioluminescence) in Gram-negative bacteria. It was demonstrated that schleiferons A and B, volatiles emitted by *S. schleiferi*, had detectable effects on other bacterial species that colonize the skin. Similar results have been reported by Chernin et al. [11] for rhizobacterial volatiles acting on plants, or have been reviewed by Audrain et al. [3]. These results underlined the need to take volatile-based modes of interaction and communication into account when considering microbiome establishment and dynamics.

While 2-pentadecanone was emitted by all skin bacteria investigated in this study, some VOCs were found to be strain and/or species specific. In particular, fatty carboxylic acids, such as 3-methylbutanoic and 2-methylbutanoic acid, were produced by all *Staphylococcus* strains examined. This confirmed the finding of Ara et al. [1], who showed that *S. epidermidis* was able to metabolize the amino acid leucine present in sweat secretions to 3-methylbutanoic acid, which is also the main component of foot odor. In contrast to staphylococci, not all corynebacteria generated and emitted

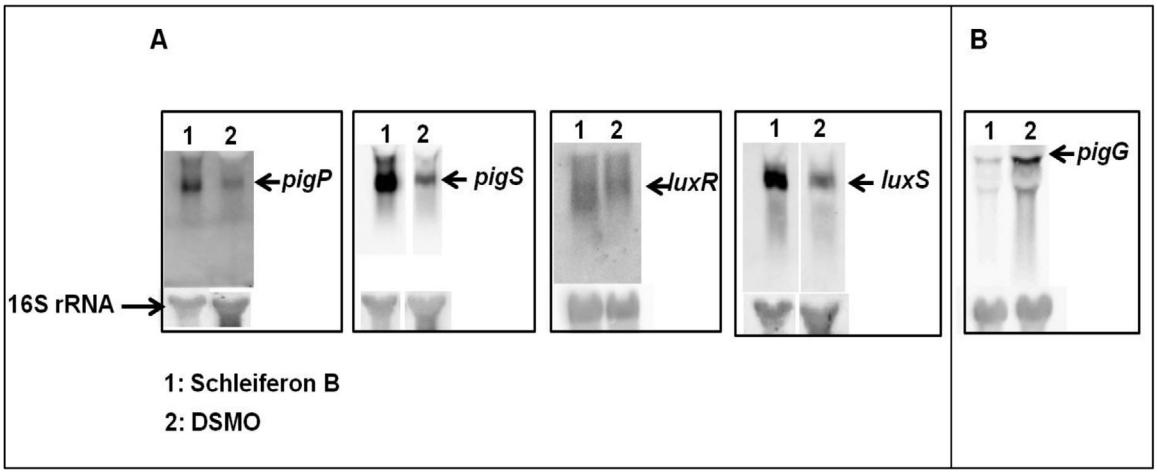

Fig. 6. Expression levels of the prodigiosin regulatory genes (A) and biosynthetic genes (B) in *Serratia plymuthica* AS9.
The bacterium was incubated at 20 °C with schleiferon A or B (340 μg mL^{-1} final concentration). As a control, the corresponding volume of DMSO was added to the cells. After 24 h, total RNA was extracted and Northern blot analysis was performed to quantify the expression of the genes (c) involved in the regulation (A) and biosynthesis (B) of prodigiosin. (A) *pigP*, *pigS*, *luxR*, and *luxS* correspond to the genes SerAS9, SerAS9_1501, SerAS9_0078 and SerAS9_0772, respectively. (B) *pigG* corresponds to the gene *SerAS9*_1747 in the *pig* biosynthesis cluster. The bands of 16S rRNA served as an indication of the blotting efficiency and demonstrated the loading of RNA in each lane.

these VOCs. This finding highlights the important role of staphylococci in foot odor formation. However, 2-phenylethanol was emitted by all *Corynebacterium* strains analyzed here and was produced in particularly large amounts by *C. striatum* isolates, while among staphylococci only *S. schleiferi* and *S. sciuri* isolates were able to produce this compound. Early studies had shown that 2-phenylethanol is in general a common bacterial volatile [58] and that it is present in the headspace of the skin microbiome [68]. In light of the results presented here, it is likely that 2-phenylethanol belongs to the cocktail of volatiles generated by bacterial residents of the skin, and is mainly produced by corynebacteria. This volatile has a characteristic rose-like odor and has been shown to act as a mosquito repellent [69]. Moreover, this compound has been reported to possess antimicrobial, antiseptic and disinfectant properties [19,39], which has prompted speculation that it may be emitted by corynebacteria during interspecies competition for niches on the skin in order to eliminate other competitors and/or inhibit the growth of pathogens on the skin.

Another interesting observation was that the VOC profiles of the two dominant families of skin bacteria could be easily distinguished. This difference can probably be explained by metabolic differences between the two genera, even though they share the same environment and the same growth conditions. Moreover, this is in good agreement with previous studies, which showed that the spectrum of volatiles emitted by bacterial communities depends not only on growth conditions and growth medium but also on the metabolic activities of their constituent species [9,10,65,73]. Furthermore, the work of Kwaszewska et al. [31] demonstrated that corynebacterial and staphylococcal communities co-habiting on human skin do not employ the same sets of enzymatic activities to metabolize substrates such as carbohydrates, lipids and proteins, and that *Corynebacterium* species express less proteinase, phospholipase and saccharolytic activity. These observations could also explain why the number of VOCs emitted by the *Staphylococcus* strains studied here was fivefold higher than that of the corynebacterial strains, allowing members of the two genera to be clearly differentiated by PCA on the basis of their VOC spectra alone (Fig. 1).

In the course of the analysis of the VOCs of these species, two new volatiles were identified – schleiferons A and B – which have never previously been associated with any biological source. In the sample of taxa, these substances were only produced by *S. schleiferi* isolates. On the basis of other work, Schulz et al. (unpublished) have postulated a biosynthetic route for these compounds via acetoin and 2-phenylethylamine, both of which are produced by *S. schleiferi*. Acetoin is an essential physiological metabolite produced by many bacteria and serves as a precursor in the biosynthesis of branched-chain amino acids. Although it is primarily formed by decarboxylation of alpha-acetolactate, it can also be secreted as a by-product of pyruvate oxidation or decarboxylation reactions [reviewed in Ref. [79]]. On the other hand, instances of bacterial production of 2-phenylethylamine are far less prominent in the literature, and the capacity to synthesize it is not widely distributed even among staphylococci [7,32,63]. Nevertheless, some enterococci, as well as lactic acid bacteria isolated from food products, have been shown to synthesize this compound via decarboxylation of L-phenylalanine by tyrosine decarboxylase [4,33,38,47]. Thus, we speculate that 2-phenylethylamine is synthesized by *S. schleiferi* using the same mechanism or via an as yet undescribed specific L-phenylalanine decarboxylase. Acetoin appears early in the VOC profile of *S. schleiferi* DSMZ 4807, after 24 h growth. In bacteria, this compound is usually produced during exponential growth in order to prevent over-acidification of the cytoplasm and the surrounding medium before the major carbon source has been used up [79]. However, 2-phenylethylamine was only detected after 48 h, which could explain why schleiferons A and B only attained their maximum levels in the late stationary phase of bacterial growth. Since schleiferon A already comprised up to 30% of volatiles at 48 h (Fig. 2B), sufficient 2-phenylethylamine must have been available prior to that time.

Microbe-microbe interactions are known to be mediated via secondary metabolites [56]. To gain insight into the biological role(s) of schleiferons A and B, *S. schleiferi* DSMZ 4807 was co-cultivated with bacteria that are naturally found on the skin, and with other microorganisms from different microbiota. The results revealed that *S. schleiferi* volatiles selectively inhibited the growth of Gram-positive bacteria. The growth inhibition was only significant when the bacteria were co-cultivated with *S. schleiferi* cells that were in the late stationary phase, the period in which the concentrations of schleiferons A and B reached their maxima. This suggested that these compounds might contribute to the reduced growth rate of Gram-positive species. This notion was further supported by the finding that *S. warneri*, which does not synthesize either schleiferon, did not affect any of the bacteria tested. Moreover, when chemically synthesized schleiferons A

and B were tested separately, they both specifically inhibited the growth of Gram-positive bacteria in a concentration-dependent manner, with schleiferon A being significantly more active than schleiferon B.

During competition for nutrients or territory, skin bacteria release different antimicrobial compounds to prevent adherence of pathogens and/or competitors [8,13]. Schleiferons were significantly more active (3- to 16-fold) against *Corynebacterium* strains and *M. luteus* (another skin bacterium) and less deleterious to *Bacillus subtilis* (rhizobacterium) and *Enterococcus* strains (gut bacteria). Moreover, they were also active against other skin *Staphylococcus* strains, although the latter were considerably less affected than corynebacteria or *M. luteus*. Therefore, the production of schleiferons can be an advantage for staphylococci during bacterial interactions and might help them retain their balance in the skin flora. In addition, among these skin bacteria, the prominent schleiferon producer *S. schleiferi* DSMZ 4807 was 2- to 8-fold more resistant to these VOCs and its growth was noticeably affected at very high concentrations, which was only be reached in the late stationary phase. Altogether, these results highlighted the role(s) that schleiferons may have in skin bacterial interactions, suggesting that mVOCs might contribute to maintain species diversity and to shape the evolution of the community composition or structure the microbiome. However, the mode of action by which these compounds inhibit the growth of Gram-positive bacteria is not yet known and remains to be investigated in the future.

In contrast, the growth of Gram-negative bacteria was unaffected by either schleiferon A or B, although both compounds inhibited quorum-dependent phenotypes, specifically prodigiosin production in *Serratia* strains and bioluminescence by *V. harveyi*. Incubation of cell extracts with schleiferon A or B did not alter their prodigiosin content (results not shown), implying that the two agents disrupted its synthesis at the transcriptional or post-transcriptional level. Homologues of the prodigiosin biosynthesis genes (*pigA-N*) of *Serratia* sp. ATCC 39006 are present in *S. marcescens* and *S. plymuthica* [29,35,76] and the genes in these clusters are co-transcribed and directed by a promoter upstream of *pigA* [61]. The Northern blot analysis revealed that the transcription level of *pigG* (representing the co-transcribed *pig* cluster) was significantly inhibited in the presence of schleiferon B (Fig. 6B), which ultimately led to the reduction of prodigiosin synthesis and accumulation. A complex hierarchical network of regulatory proteins control the biosynthesis of prodigiosin [17,23,75] and, since the production of this pigment is regulated by both the quorum-dependent and -independent mechanisms, the transcription levels of both master transcriptional regulator genes *pigP* and *luxR* were analyzed. Fineran et al. [17] previously identified the PigP protein as a master transcriptional regulator of secondary metabolism in some Enterobacteriaceae. They found that this protein could control prodigiosin production in *Serratia* sp. ATCC 39006 either by directly regulating the expression of the *pig* biosynthesis cluster or indirectly via transcriptional control of genes for six other regulators (*pigQ*, *pigR*, *rap*, which overlap the quorum-sensing circuit, and *pigV*, *pigS* or *pigX*). When schleiferon was added to a *S. plymuthica* AS9 culture, the transcriptional level of *pigP* was significantly increased in comparison to the control, suggesting control via this *pig* regulator. Moreover, when the transcription levels of *pigS* were analyzed, a significant increase was also found in its transcription level. *pigS* encodes for an ArsR family regulator that is able to repress the expression of other proteins (BlhA; OrfY; PmpA, B and C) involved in prodigiosin biosynthesis [23]. Therefore, the transcription level of *pigS* correlated with the activation of the expression of the master regulator PigP, and both up-regulations might favor a reduction of prodigiosin in *S. plymuthica* AS9. Similarly to *pigP*, the expression of *lux*S (encoding for the auto-inducer 2 synthase) was also up-regulated. In contrast to PigP, SmaR in *Serratia* sp. ATCC 39006 (*LuxR* homolog in *S. plymuthica* AS9) is a quorum-sensing master transcriptional repressor [17,23,61]. LuxR represses expression of the *pig* biosynthesis gene cluster directly or indirectly via the repression of other transcriptional regulators, such as PigR, PigQ or Rap [17]. When the transcription levels of *luxR* were analyzed in *S. plymuthica* AS9 after application of schleiferon, it was found surprisingly that they were almost unaffected. Altogether, these results support the notion that in Gram-negative bacteria exposed to schleiferon A or B, the expression of the master regulator PigP was stimulated and led to the inhibition of prodigiosin production. Nevertheless, the transcriptional regulators that overlap the quorum-dependent circuit that are under the control of PigP still have to be investigated.

The discussion has so far concerned molecular circuits in the context of how schleiferons A and B induced the repression of prodigiosin biosynthesis. Nevertheless, it can also be postulated whether or not schleiferon could act on other targets of quorum sensing (e.g., as an auto-inducer antagonist) by interfering with auto-inducer receptors to influence prodigiosin or bioluminescence production through stimulation of autophosphorylation [12,72]. Surprisingly, no increase in phosphorylation of the quorum sensing receptors was found after schleiferon A or B addition, suggesting a target(s) downstream of the hybrid histidine kinase. These results were corroborated by the schleiferon-mediated inhibition of bioluminescence in a *luxO* deletion mutant, which constitutively produces bioluminescence independently of auto-inducers and the QS receptors.

In summary, it has been shown that a large number of VOCs were present in the headspace of skin bacteria, including bio-organic compounds that have never been recognized before in natural settings. Schleiferons A and B selectively inhibited bacterial growth or affected bacterial metabolism and gene expression. Considering the problem of continued emerging antibiotic-resistant pathogens, it would be very interesting to test these new natural compounds on pathogenic strains. As these mVOCs interfere in bacterial communication by affecting gene expression, their impact on the expression of virulence factor genes needs to be determined in order to evaluate their efficacy in anti-virulence therapy. The local microbiota plays an important role in buttressing the skin's role as a barrier against colonization by different microorganisms and invasion by pathogens by engaging in competitive interactions that control access to nutrients and/or space. Future experiments will need to elucidate the relevance of these microbial volatiles to such interactions on the skin, and will contribute to a better understanding of the implications of the microbiota in health and disease conditions.

Contributions

MK and MCL performed the PCA and the hierarchical clustering analysis (Fig. 1), SRR and SS elucidated the structure (Fig. 2A) and helped to analyze the VOCs of the headspaces (Tables S1 and S2), whereas NL and KJ performed the phosphorylation experiments and the in vitro bioluminescence assay (Figs. S6 and S7, respectively). All other experiments were performed by MCL. MCL and BP planned the experiments and wrote the manuscript. All authors interpreted the results and approved the submitted version of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.08.008.

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