Combination of high-throughput microfluidics and FACS technologies to leverage the numbers game in natural product discovery

Markus Oberpaul,1,† Stephan Brinkmann,1,† Michael Marner,1 Sanja Mihajlovic,1 Benedikt Leis,1 Maria A. Patras,1 Christoph Hartwig,1 Andreas Vilcinskas,1,2 Peter E. Hammann,3 Till F. Schäberle1,2,4,† Marius Spohn1,3,*** and Jens Glaeser1,3,***

1 Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Branch for Bioresources, Giessen, 35392, Germany.
2 Institute for Insect Biotechnology, Justus-Liebig-University-Giessen, Giessen, 35392, Germany.
3 Evotec International GmbH, Göttingen, 37079, Germany.
4 German Center for Infection Research (DZIF), Partner Site Giessen-Marburg-Langen, Giessen, 35392, Germany.

Summary

High-throughput platforms facilitating screening campaigns of environmental samples are needed to discover new products of natural origin counteracting the spreading of antimicrobial resistances constantly threatening human and agricultural health. We applied a combination of droplet microfluidics and fluorescence-activated cell sorting (FACS)-based technologies to access and assess a microbial environmental sample. The cultivation performance of our microfluidics workflow was evaluated in respect to the utilized cultivation media by Illumina amplicon sequencing of a pool of millions of droplets, respectively. This enabled the rational selection of a growth medium supporting the isolation of microbial diversity from soil (five phyla affiliated to 57 genera) including a member of the acidobacterial subgroup 1 (genus Edaphobacter). In a second phase, the entire diversity covered by 1071 cultures was used for an arrayed bioprospecting campaign, resulting in > 6000 extracts tested against human pathogens and agricultural pests. After redundancy curation by using a combinatorial chemical and genomic fingerprinting approach, we assigned the causative agents present in the extracts. Utilizing UHPLC-QTOF-MS/MS-guided fractionation and microplate-based screening assays in combination with molecular networking the production of bioactive ionophorous macrotetrolides, phospholipids, the cyclic lipopeptides massetolides E, F, H and serratamolide A and many derivatives thereof was shown.

Introduction

Continuously increasing levels of drug resistance and the consequent loss of existing drug and control agents for the treatment of infections poses an enormous threat to human health care systems and plant disease management strategies (Lewis, 2013; Lakemeyer et al., 2018). This crisis has to be counteracted by continued innovation in discovery campaigns to increase the probability of finding new anti-infective lead structures (Schäberle and Hack, 2014; Tacconelli et al., 2018; Theuretzbacher et al., 2020).

A rich source for new chemical entities are microbial-derived natural products (NPs), which always has been a major inspiration for development of drugs and control agents (Newman and Cragg, 2020). NP evolution brought forth a vast diversity of unique molecules that are optimized for interactions with their respective molecular targets; the latter a plethora of biological macromolecules themselves (Firm and Jones, 2003; Bon and Waldmann, 2010; Hong, 2011). Fortunately, the predicted potential for chemical variety encoded within the microbial diversity is yet only scarcely exploited. This is...
mainly due to the fact that major NP discovery efforts of past decades had been focusing on rather easily cultivable microorganisms and consequently on a limited phylogenetic diversity (Monciardini et al., 2014). Along with the likely also not yet-exhausted potential of classical NP producers, for example *Streptomyces* spp. (Adamek et al., 2019; Belknap et al., 2020), additional phylogenetic branches of the bacterial kingdom represent a promising source for discovery of novelty (Gross and Loper, 2009; Panthee et al., 2016; Tracanna et al., 2017). Thus, driven by the dogma that phylogenetic and genomic divergence translates directly into chemical diversity (Medema et al., 2014; Monciardini et al., 2014), greater effort is demanded to bring a broader diversity of genera and families in culture (Hoffmann et al., 2018; Nicault et al., 2020) while directly exploiting their metabolic capabilities. The still valid functionality of the methodology to valorize bacterial derived NPs was recently shown by the development and market introduction of fenpicoxamid, derived from the antifungal compound UK-2A produced by *Streptomyces* sp. 517-02. This NP provides a new target site for the control of *Septoria tritici* Blotch (STB) (Butler and Paterson, 2020) since the extremely small scale enables even single cells to adapt their environment by, for example accumulating surfactants as stabilizing agents. Pressurized in microchannels with a diameter ranging from 30 to 500 µm, this ultimately results in aqueous droplets in nl to pl scale (Leman et al., 2015). The production of millions of droplets per hour was improved over years, and simplified systems are nowadays on the market (Nge et al., 2013; Volpatti and Yetisen, 2014). Their combination with further technologies, such as fluorescence-activated cell sorting (FACS), enables the direct identification of desired events and their arrayed sorting (Zinchenko et al., 2014). An advantageous value of these technologies is the downscaling and compartmentalization which allows single cells to be physically separated into distinct vessels while maintaining the overall microbial complexity (Zengler et al., 2002; Theberge et al., 2010). This separation is mandatory to avoid growth competition between different species while also allowing single cells to utilize and shape their microenvironment according to their specific needs and at their own pace (Keller and Zengler, 2004; Boitard et al., 2015). The pico-litre scaled droplets display an environmental mimicry themselves, since the extremely small scale enables even single cells to adapt their environment by, for example accumulating self-mediating growth factors eventually breaking microbial dormancy (Boedicker et al., 2009; Ishii et al., 2010; Steward, 2012). In principal, high-throughput microfluidic-based platforms increase the microbial cultures in amount but also diversity and consequently the probability to cultivate and identify also underexplored microorganisms (Akselband et al., 2006; Baret et al., 2009).

In this study, we succeeded in isolating an extended taxonomic diversity and accessing microorganisms considered as under- or even unexploited for NP discovery. We implemented a biphasic workflow consisting of (i) an efficient cultivation of microbial diversity using microfluidics and FACS-based technologies, followed by (ii) a miniaturized bioactivity-guided NP discovery process on the obtained isolates focused on the two devastating pathogens, that is *Mycobacterium tuberculosis* (MTB) and SEPTTR. Our here described cultivation platform is based on long-term stable agarose-solified micro-droplets (~ 40 µm in size, volume of ~ 33 pl). Droplets were generated up to rates of ~ 1.3 kHz; thereby, exceeding the dimensions for an application to be affiliated as ultra-high throughput (Payne et al., 2020). At a cell distribution of λ0.1, this set up allowed the parallel encapsulation of ~ 500 000 cells h⁻¹ with a statistical probability below 0.5% to obtain co-cultures. In total, we brought 1071 microorganisms into culture, from which...
~ 74% could be identified by 16S rRNA gene sequencing. The isolated bacteria belong to five different phyla affiliated to 57 different genera. Besides classical NP producing taxa a representative of fastidious Acidobacteria and certain proteobacterial genera which are under-represented in public strain libraries (e.g. Luteibacter and Variovorax) were brought into culture. The entire obtained microbial diversity was eventually integrated into a bioactivity-guided NP discovery process. After redundancy curation of initially active extracts, the organic extracts of Erwinia, Pseudomonas and Streptomyces which most strongly inhibited the growth of MTB and/or SEPTTR were followed up by bioassay- and UHPLC-QTOF-MS/MS-guided fractionation. Complemented by extensive metabolomic analysis via molecular networking, this led to the identification of serratamolides, massetolides, phospholipids and macrotetrolides as the bioactivity causing agents.

Results and discussion

Cultivation of microbial diversity using microfluidics and FACS

In this study (platform scheme see Fig. S2), we aimed to bring microbial diversity into culture to expand our strain collection (Fox, 2014) and to exploit the bacterial proportion in a bioactivity-guided NP discovery programme. Known for their highly diverse bacterial communities (Delgado-Baquerizo et al., 2016), we selected a combined soil sample as starting material for our study. Upfront the main droplet cultivation and screening campaign, we examined the general cultivation success using our microfluidics platform and explicitly the specific impact of the chosen cultivation media on the bacterial community. Specifically adapted towards soil and previously shown to be suitable to transfer a high diversity of soil microorganisms into culture, we selected ISEM (Nguyen et al., 2018) as benchmark medium for this study. Considering the importance of using buffered media as well as mimicking neutral and acidic soils for the cultivation success (Overmann et al., 2017), we buffered the media to pH 7.2 and pH 5.5 (Tovar et al., 2020). In order to identify an additional media suitable to access a broad microbial diversity of the bioresource used in this study, we encapsulated the retrieved microorganisms (λ0.1) in eight different media (VL55-xyl, VL55-cello, 1:20 CY, 1:20 NB, M13b, 1:20 TSB, 1:10 R2A and M9). Then, we comparatively determined the cultivation success by Illumina amplicon sequencing of the sorted droplet populations among themselves and towards the starting material. The sequencing was conducted on DNA directly isolated from the eight different droplet populations. Illumina amplicon sequencing of isolated DNA yielded in total 1 947 118 classified sequences, of which 0.04% could not be classified and were defined as No Relative. The data revealed that cultivation in VL55-xyl resulted in the overall highest diversity on genus level resolution (Shannon index [SI]: 4.1), followed by M9 (SI: 3.5) and 1:20 CY (SI: 3.5) (Fig. 1). Compared to the starting material, the microbial diversity covered by VL55-xyl was not significantly reduced (P > 0.5). In contrast, the exchange of the sole C-source xylan by cellobiose led to a significant reduction in diversity (P < 0.01) and a lower SI (SI: 2.3). While showing an
Overall lower diversity, the media (1:20 CY, 1:20 NB, M13b, 1:20 TSB and 1:10 R2A), which contained complex components such as yeast or peptone showed an increased proportion of Proteobacteria (up to 77.7%) in comparison with the VL55-based media (up to 39.2%) and the starting material (36.7%). A similar diversity and an increased proportion of Proteobacteria were observed by using M9. While 1:10 R2A appears to be suitable to enrich Firmicutes (20.8% vs 0.06% in starting material), all other media buffered at pH 7.2 do not show a remarkable difference within their cultivated microbial community ($P > 0.7$; Fig. 1).

Overall, VL55-xyl with its complex C-source and its pH favourable for acidophilic and acid-tolerant bacteria was shown to be the most suitable medium for our soil material and selected it to be included in all subsequent experiments. Considering the observed effects of the Illumina analysis, particularly the impact of the provided C-source and the altered pH profile, we supplemented ISEM with a variation of six complex C-sources including xylan (mISEM$^1$) and buffered it to pH 5.5 and pH 7.2.

In this study, we applied a micron-scaled cultivation platform to receive axenic cultures from a complex bioresource, adapted for the purpose to also cultivate slow-growing microorganisms. This necessitates the analysis of droplets in high-throughput fashioned manner and the reliable discrimination between droplets harbouring grown microorganisms with manifold morphologies and the empty droplet background. Therefore, we used a FACS approach utilizing the carbocyanine dye DiOC$_2$(3) to stain microorganisms with a membrane potential grown inside the agarose-solidified droplets. DiOC$_2$(3) is a potentiometric probe that exhibited green fluorescence in all bacterial cells; however, the fluorescence shifts towards red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials (Shapiro, 2000; Biener et al., 2017). To show the feasibility of using DiOC$_2$(3) for this process step, a mixed droplet population containing 50% droplets harbouring grown microcolonies of E. coli ($\times 10$) incubated for 4 h at 37°C and 50% empty droplets was stained and subsequently measured using FACS while analysing the ratio of green (FL1-H) and red (FL3-H) fluorescence. The FL1-H to FL3-H scatter exposed 49.7% of all analysed events in the defined gate, congruent with the adjusted 50.0% of the droplet population containing grown E. coli microcolonies (Fig. 2A). This verified the applicability of DiOC$_2$(3) as a fluorescent dye for staining microorganisms grown in agarose-solidified droplets. Based on these distinguishable populations, the FACS settings (e.g. sorting gates for events of interest) were defined and later on applied for the cultivation campaign.

The cell suspension from the used soil sample retrieved using nycodenz gradient centrifugation had an estimated living cell count of $\sim 4.2 \times 10^6$ cells ml$^{-1}$ determined via FACS [considering live/dead ratio ($\sim 70:30$; Fig. S3)].

Fig. 2. Sorting of microcolonies grown in agarose-solidified droplets stained using DiOC$_2$(3).
A. Differentiation of a 50:50 mixture of empty droplets and those containing grown microcolonies of E. coli ATCC 25922 ($\times 10$). 15 000 events were analysed by FACS considering granularity (SSC-H) to size (FSC-H) and green (FL1-H) to red (FL3-H) after staining.
B. Sorting of droplets containing grown microcolonies of environmental soil microorganisms ($\times 0.1$) in VL55-xyl pH 5.5, mISEM$^1$ pH 5.5 and mISEM$^1$ pH 7.2 (10 000 events displayed). The background noise was 0.06% (empty = control without cells encapsulated). After seven days of incubation at 28°C, grown microcolonies as determined by membrane potential dye were sorted and recovered. Red dots = droplets containing fluorescently labelled cells; grey dots = empty droplets.

© 2021 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.
This was taken into consideration, while cells were encapsulated in different media (k0.1 each) and subsequently incubated at 28°C, respectively. The microbial growth within droplets was frequently monitored over time by microscopy, exemplarily showing the processing of microorganisms with various morphologies (Fig. 3A). After seven days of incubation, the samples were sorted by using DiOC2(3) staining. We could affiliate 2.30% (VL55-xyl pH 5.5), 2.89% (mISEM2 pH 5.5) and 4.02% (mISEM2 pH 7.2) of all evaluated events to droplets containing grown microcolonies (Fig. 2B). These events were sorted and distributed into 384-well MTPs, and microbial growth was again monitored by turbidimetry and microscopy, respectively (Fig. 3B). After automatic transfer of grown cultures into 96-deep well MTPs and further incubation for up to 14 days, this up-scaling procedure provided 1071 cultures in total. Sample aliquots of each culture were taken to generate cryo-conserved cultures and cell lysates for taxonomic identification by 16S rRNA gene sequencing (partial sequencing using primer 1492R). This revealed the processing of 57 different genera affiliated to five different phyla (Fig. 3C and Table S1). Comparing the cultivation, success of mISEM2 at pH 5.5 and pH 7.2 clearly shows the worth of modulating this parameter to increase the cultivated diversity. Together, 55 genera were isolated using mISEM2, while only 15 of them could be found under both pH conditions. Medium mISEM2 pH 7.2 led to the successful cultivation and affiliation of 46 genera (462 strains, SI: 2.4) while 31 genera were unique to this condition. In contrast, at pH 5.5 a total of 24 genera (108 strains, SI: 2.7) and 9 unique ones were identified. While use of acidified media led to an overall decreased amount of genera, this condition showed a higher diversity and expanded the cultivation success towards a fifth phyla (Acidobacteria) not present at the neutral pH set-up. Within both media comprising pH 5.5, we found eight representatives of the Acidobacteria, all belonging to the genus Edaphobacter. A representative thereof was recovered on plate and incorporated into our strain collection. As judged on nearly full-length 16S rRNA sequence comparison, this particular strain, namely FHG110552, is phylogenetically affiliated to the subgroup 1 of Acidobacteria and most closely related to Edaphobacter modestus Jbg-1T (~98.8% similarity) isolated from alpine and forest soils (Koch et al., 2008; Koch et al., 2008; Koch et al., 2008).
The phylum Acidobacteria is postulated to be a promising bioresource for the field of NP discovery based on its predicted biosynthetic potential encoded in their genomes (Kielak et al., 2016; Crites-Christoph et al., 2018). Though, the description of Acidobacteria NPs is yet limited to hopanoids (Damsté et al., 2017).

On phylum level, a major difference between the neutral and acidified media is the ratio between Actinobacteria and Proteobacteria, which is at pH 7.2 shifted towards the Actinobacteria, while towards Proteobacteria at pH 5.5. The same is seen in VL55-xyl covering in total 23 genera (223 strains, SI: 1.9) including Edaphobacter but also unique genera such as Labrys and Rothia, not covered by mISEM². In total, 17 Actinobacteria genera were brought into culture using mISEM² pH 7.2, whereas 8 genera in mISEM² pH 5.5 and 6 genera in VL55-xyl. This is particularly biased by the genus Agromyces, which was strongly enriched in mISEM² pH 7.2 (151 strains) while present in low abundance at pH 5.5 (7 strains).

Within the Proteobacteria, such an opposite pH-dependent shift occurs within the class of Alphaproteobacteria. At pH 7.2, the genus Ancylobacter represents almost half of all Proteobacteria (112 strains) while being significantly less abundant at pH 5.5. In contrast, the genus Phyllobacterium represents >30% of the Proteobacteria at acidic condition while <1% at neutral pH. In total, the isolated Proteobacteria proportion comprises member of 33 different genera belonging to the classes of Alpha-, Beta- and Gammaproteobacteria and include also representatives of nine scarcely cultured genera according to our definition (namely Ancylobacter, Buttiauxella, Inquilinus, Kaistia, Labrys, Luteibacter, Polaromonas, Rayranella and Varionoverax).

Bioactivity-guided bioprospecting process

To prospect the here cultured microbial diversity for NPs with desired bioactivity, we integrated all arrayed microbes in a downscaled bioactivity-guided NP discovery process. Each culture was grown in its respective isolation medium and in addition in ISP2 and BSM for four and seven days. This process aimed to more sophisticatedly provoke expression of the microbial secondary metabolite production potential. Using 96-deep well Duetz systems and 1 ml culture volumes, 6426 organic extracts were generated and used for bioactivity screenings and metabolome analysis.

In first line, the extracts were screened for their antmycobacterial activity against M. smegmatis, the opportunistic microbial pathogen S. aureus and for their antifungal activity against Z. tritici, C. albicans and A. flavus. Extracts that showed growth inhibitory properties underwent a subsequent diversity assessment by cosine similarity comparison of features detected within each corresponding UHPLC-UHR-MS chromatogram. This quality control step was included to rule out extensive analysis of redundant samples, unavoidably appearing in every microbial isolation project. After screening the methanolic extracts, 64 cultures were assigned to bioactivity by either inhibiting the growth of M. smegmatis and/or the fungal indicator strains. The cosine similarity of their associated UHPLC-UHR-MS chromatograms were determined. After excluding samples with less than 50 features, the remaining 60 samples clustered into eight distinct groups applying the cosine similarity threshold 0.9 (Fig. 4A, Table S2). From the most dominant group, we recovered Penicillium sp. (FHG110518). With no focus on fungal metabolites in this project, we decided to discontinue the work on this abundant strain causing the major fractions of observed bioactivities (~70%). In order to now identify the bacterial strains in this dataset, we complemented the redundancy curation process by genotyping the cultures using BOX-PCR (Fig. 4B). By superimposing the patterns of both grouping technologies, we identified and subsequently recovered strains of the genera Pseudomonas [FHG110502, FHG110523 and FHG110524 (each unique)], Xanthomonas (FHG110521), Rhizobium (FHG110501), Sphingomonas (FHG110503), Phyllobacterium (FHG110504), Ancylobacter [FHG110512, FHG110513, FHG110514 (equal)], Agromyces [FHG110505, FHG110506, FHG110507 (equal)], Erwinia (FHG110488) and Streptomyces (FHG110508).

In order to identify the causative NP beyond the observed crude extract activities, the cultivation of the unique strains was scaled-up to 50 ml volumes. The resulting culture broths were extracted by methanol and reconstituted in DMSO for bioactivity screenings. The extracts from the three strains Pseudomonas sp. (FHG110502), Erwinia sp. (FHG110488) and Streptomyces sp. (FHG110508) exhibited the severest growth inhibitory potency and were selected for further analysis. Their crude extract complexity was strongly reduced by their separation into 159 fractions. After rescreening all fractions, the compounds present in the active fractions were identified by their exact masses, characteristic UV absorption spectra and fragmentation signature.

Erwinia sp. FHG110488. Based on the primary activity of the crude extract against M. smegmatis and Z. tritici, the corresponding extract was fractionated and rescreened. Compound 1 with a m/z of 515.3329 [M + H]⁺, corresponding to the molecular formula C_{26}H_{46}N_{2}O_{6}, was dereplicated within an active fraction (Fig. S5). On the basis of the molecular formula and the observed MS/MS fragmentation pattern, the bioactive compound was dereplicated as the known serratamolide A (Wasserman et al., 1961; Dwivedi et al., 2008). Using MS/MS networking, six additional derivatives were
Streptomyces sp. FHG110508. A classical NP producer organism that was isolated during our process is Streptomyces FHG110508. Its organic extracts showed pronounced activity against S. aureus and the fungal indicator strains. Dereplication of active fractions revealed the presence of the macrotetrolides nonactin, monactin, dinactin and macrotetrolide G (Fig. S9, 14–17) (Phillies, 1975; Rezanka et al., 2010). Additionally, the presence of macrotetrolide D (18) was shown by molecular networking (Fig. S10). Ionophore antibiotics of the macrotetrolide family are among the most commonly observed bioactive metabolites from actinomycetes passing in various screening disciplines having antibacterial, antifungal, position AA9, the known antimycobacterial massetolide family is known to be prone to natural variations, with indications in a general correlation between greater lipophilicity and increased potency (Gerard et al., 1997).

Furthermore, three phospholipids dereplicated as lysopalmitoyl-phosphoethanolamine (11), palmitoleoyl-palmitoyl-phosphoethanolamine (12) and palmitoleoyl-oleoyl-phosphoethanolamine (13) (measured 11 m/z 454.2931 [M + H]⁺, 12 m/z 690.5073 [M + H]⁺, 13 m/z 716.5237 [M + H]⁺, respectively, were assigned the other active fractions (Fig. S8). The bioactivity of phospholipids against soral indicator strains has been reported, for example bacilysocin as an antifungal active phospholipid isolated from Bacillus subtilis (Tamehiro et al., 2002).

Pseudomonas sp. FHG110502. The antimycobacterial activity caused by Pseudomonas strain FHG110502 was associated to three cyclo-lipo-1-nonadepsipeptide 8–10 (8 m/z 1112.6811 [M + H]⁺, 9 m/z 1126.6966 [M + H]⁺, measured 11 m/z 1126.6966 [M + H]⁺, 10 m/z 1154.7282 [M + H]⁺, 11 m/z 1154.7282 [M + H]⁺, respectively. Massetolide E and F differ in AA9 by incorporation of valine and leucine, respectively. Massetolide H differs in the fatty acid chain. The hydroxydecanoic acid residue of E and F is replaced by a hydroxylaeric acid residue (Fig. S7). At

identified (measured 2 m/z 541.3480 [M + H]⁺, 3 m/z 543.3635 [M + H]⁺, 4 m/z 533.3430 [M + H]⁺, 5 m/z 547.3588 [M + H]⁺, 6 m/z 575.3896 [M + H]⁺, 7 m/z 573.3745 [M + H]⁺, 8 m/z 573.3745 [M + H]⁺, 9 m/z 690.5073 [M + H]⁺, 10 m/z 716.5237 [M + H]⁺, 11 m/z 716.5224), and 12 m/z 690.5068; and 13 m/z 716.5237 [M + H]⁺, C₂₉H₅₄N₂O₉P₁, theoretical m/z 716.5224, respectively, were assigned the other active fractions (Fig. S8). The bioactivity of phospholipids against soral indicator strains has been reported, for example bacilysocin as an antifungal active phospholipid isolated from Bacillus subtilis (Tamehiro et al., 2002).

Pseudomonas sp. FHG110502. The antimycobacterial activity caused by Pseudomonas strain FHG110502 was associated to three cyclo-lipo-1-nonadepsipeptide 8–10 (8 m/z 1112.6811 [M + H]⁺, 9 m/z 1126.6966 [M + H]⁺, 10 m/z 1154.7288 [M + H]⁺, C₂₉H₅₄N₂O₁₆, theoretical m/z 1154.7282) perfectly matching the MS/MS fragmentation signature of massetolide E, F and H, respectively. Massetolide E and F differ in AA9 by incorporation of valine and leucine, respectively. Massetolide H differs in the fatty acid chain. The hydroxydecanoic acid residue of E and F is replaced by a hydroxylaeric acid residue (Fig. S7). At

© 2021 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.
antiprotozoan, antiparasitic, insecticidal and acaricidal activity (Zizika, 1998).

**Conclusive remarks.** Our study represents a combination of applied microfluidics and FACS technologies for the cultivation of microorganisms in agarose-solidified droplets. The combination of streamlined high-throughput technologies facilitated the generation and analysis of thousands of droplets within seconds. This paved the way to a fast isolation and characterization of diverse axenic cultures, followed by downstream identification of bioactive natural products. There are no limits on a particular bioresource, organism types or cultivation conditions (media, nutrient limitation, temperature, incubation method, ±oxygen supply, etc.), since especially all parameters towards cultivation in solidified droplets are highly customizable. Microfluidic platforms are already today a core element to access and assess microbial diversity from environmental samples. Their efficient application on a diversity of bioresources will be of steadily increasing importance for various research disciplines, with particular (but not exclusive) regard to the field of environmental microbiology.

**Experimental procedures**

**Sampling procedure of forest soil**

Forrest soil samples (soil) were taken from the ‘Nature Conservation Area’ Bergwerkswald at 50.564032 N 8.672555 E (Hasenkoeppel, Giessen, Germany). Five samples within a radius of ten metres (max. 10 cm in depth) including rhizosphere, lichen, humus soil and sandy loam were pooled. Samples were taken in December 2018 (Illumina approach) and January 2019 (cultivation campaign). Microorganisms were retrieved by Nycodenz density gradient centrifugation (Barra Caracciolo et al., 2005; Oberpaul et al., 2020). All samples were directly chilled and stored at 4°C until processing for the cultivation or at –50°C for Illumina amplicon sequencing.

**Encapsulation of microorganisms using microfluidic devices**

The cell concentration of retrieved microorganisms was determined using the Bacteria Counting Kit (B7277, Invitrogen, Carlsbad, CA, USA) on a FACSCalibur (BD Bioscience, San Jose, CA, USA) according to the manufacturer’s protocol. The live/dead ratio was estimated using the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (L7007, Invitrogen).

The commercially available μEncapsulator microfluidics set-up, described in detail elsewhere (Caballero-Aguilera et al., 2021), consisting of three pulse-free pressure pumps (Mitos P-Pump), a temperature control unit (TCU-100), diverse microfluidic chips, polytetrafluoroethylene (PTFE) tubing (1/16” OD, 0.25 mm ID and 0.5 mm ID) and connectors were purchased from Dolomite Microfluidics, a brand of Blacktrace Holdings Ltd (Royston, UK). A high-speed CMOS camera PL-D721CU (Navitar, Rochester, NY, USA) on a stereomicroscope SteMi SV 11 (Carl Zeiss, Oberkochen, Germany) equipped with a halogen light source KL 2500 LCD (Schott AG, Mainz, Germany) was used to image microfluidic operations. Samples for encapsulation were loaded onto the μEncapsulator Sample Reservoir Chip. Droplet generation was realized on a fluorophilic 50 μm μEncapsulator 2 Reagent Droplet Chip with an applied water to water to oil ratio of 1:1:20 at 30°C for ~ 30 min (Video S1). Pico-Surf 1 (2% (w/w) in FC-40) (Sphere Fluidics, Cambridge, UK) was used as the continuous oil phase. Both aqueous phases consisted of cultivation media with adjusted environmental or E.coli cell concentrations to obtain target cell distributions following Poisson (Fig. S1; Collins et al., 2015). All liquids except cell suspensions were filtered through a 0.2 μm CA syringe filter (Corning, Comin, NY, USA) to prevent blocking of the chip. Prior chip loading, media were mixed 50:50 with liquid, pre-warmed 3% (w/v) SeaPlaque agarose (Lonza, Basel, Switzerland) in water. The droplets were collected in 1.5 ml reaction tubes and cooled at 4°C for 10 min to facilitate gelling of the agarose. Thereafter, droplets were incubated at 28°C in a humidity chamber for seven days for the Illumina amplicon sequencing and for the cultivation experiments. Neubauer chambers (0.1 mm depth, Paul Marienfeld GmbH KG, Lauda-Königshofen, Germany) were used for imaging of stationary droplets on a fluorescence microscope DM2000 LED equipped with a DFC450 C camera and LAS V4.7 software (Leica Microsystems, Wetzlar, Germany) for picture analysis.

**Cell staining and droplet sorting via FACS**

Analysis and sorting of droplets were performed using a FACSCalibur. Surrounding oil was de-emulsified upfront fluorescence staining and FACS analysis using Pico-Break 1 (Dolomite Microfluidics) added in a ratio of 1:200 to a sample diluted with 1× PBS to adjust towards an analysis of 2000 events s⁻¹. Fluorescence labelling of microorganisms was achieved by adding 30 μM DiOC₂(3) (B34950, Invitrogen) to the samples and an incubation time of 5 min. Sorting via FACS (laser ex: 488 nm, em: 530 ± 30 nm [FL1-H] and 670 ± 30 nm [FL3-H]) was carried out using the single-cell mode and 1× PBS as sheath fluid.

**Cultivation media**

Media used for the growth of microorganisms in droplets, and the subsequent Illumina 16S V3-V4 gene amplicon
 sequencing were as follows: VL55 supplemented with 0.05% (w/v) xylan (VL55-xyl) or 0.05% (w/v) cellobiose (VL55-cel), including selenite–tungstate solution and trace elements SL-10 (Oberpaul et al., 2020) and buffered to pH 5.5. In addition, 1:10 diluted Reaerson’s 2A medium (1:10 R2A, DSMZ medium 830), Minimal medium M9 (DSMZ medium 382), 1:20 diluted Casitone-Yeast medium (1:20 CY, DSMZ medium 67), 1:20 diluted Nutrient Broth (1:20 NB, DSMZ medium 1), M13 with 200 mg l⁻¹ ampicillin (M13b; Wiegand et al., 2019)) and 1:20 diluted Tryptic Soy Broth medium (1:20 TSB, Sigma-Aldrich, St. Louis, MO, USA) were used.

For the cultivation campaign of microbial diversity using microfluidics and FACS, either VL55-xyl or modified intensive soil extract medium (ISEM) (Nguyen et al., 2018) were used. ISEM was modified by addition of 1% (w/v) C-source solution consisting of arabinogalactan, (+)-cellobiose, β-(+)-melezitose, xylan, galacto-O-mannan from *Ceratonia siliqua* and N-acetylglucosamine (25 mg l⁻¹ of each in deionized water) (mISEM). For the cultivation of sorted and arrayed events in 384-well microplates (MTPs), we exchanged the soil extract with 0.1 g l⁻¹ yeast extract (Oxoid) and 0.1 g l⁻¹ casamino acids (Difco), 0.1 g l⁻¹ proteose peptone (Roth, Karlruhe, Germany) (mISEM²). Trace elements SL-10 and selenite-tungstate solution (each 2 ml l⁻¹) were added to all media used for the cultivation experiments.

For the bioprospecting campaign, cultures were fermented in small-scaled 96-well Duetz system (Duetz et al., 2000) (Adolf Kühner AG, Birrsfelden, Switzerland) using VL55-xyl, ISP2 (DSMZ medium 987), basal salt medium (BSM) supplemented with glycerol (Marner et al., 2020) and mISEM², adjusted to pH 5.5 and 7.2.

**Illumina amplicon sequencing and statistical analysis**

In order to evaluate the relative cultivation success, we encapsulated the retrieved environmental microorganisms (≤0.1) considering the live/dead ratio in eight different media and incubated the droplets for seven days at 28°C. Environmental DNA was extracted using the NucleoSpin® Soil DNA purification kit (Macherey Nagel, Düren, Germany) according to manufacturer’s protocol. The bacterial community composition of samples was assessed by Illumina 300-bp paired-end 16S V3-V4 amplicon next-generation sequencing using the degenerate primer pair 341F (3′-CCTACGGGNGGCWGCAG-5′) and 785R (3′-GACTACHVGGGTATCTAAKCC-5′). Sequencing was performed by LGC Genomics GmbH (Berlin, Germany) on a MiSeq (Illumina, San Diego, CA, USA), and data evaluation was supported by the SILVA pipeline (SILVA SSU Ref dataset; release 132; http://www.arb-silva.de; SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN [revision 21008] Ribocon GmbH, Bremen, Germany)) and BLASTN v2.2.30⁺ as previously described (Oberpaul et al., 2020). Phylogenetic groups with a relative abundance < 0.001% of total were excluded. Statistical calculations were done by using PAST v4.03 (Hammer et al., 2001)) including the several-sample one-way ANOVA test on ranks (Daniel, 1990) followed by a Dunn’s post hoc test (Dunn, 1964) to judge on significances among samples.

**Microfluidics-FACS bioprospecting platform**

Sorted droplets were concentrated using a 0.5 μm CA membrane filter (Whatman plc, Little Chalfont, UK) and a laboratory vacuum filtration system (Sartorius AG, Göttingen, Germany). Concentrated droplets were recovered in the respective growth medium (VL55-xyl, mISEM² pH 5.5 or pH 7.2) and arrayed into 384-well MTPs with a distribution probability of approx. 0.25 droplets per well using a Matrix Wellmate microplate dispenser (Thermo Fisher Scientific, Waltham, MA, USA). After incubation as static cultures at 28°C in a humidity chamber for up to seven days, growth detection was assessed via turbidimetry (OD) at 600 nm using a Wallac 1420 Victor2 Microplate Reader (Perkin Elmer, Waltham, MA, USA) and by microscopy using a Zeiss Axiovert 200M (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with an SPOT RT Monochrome 2.1.1 camera (Diagnostic Instruments, Sterling Heights, MI, USA). All cultures exceeding our defined turbidity threshold (determined based on media control blanks present on each processed microplate) were automatically transferred into 96-deep well MTPs pre-filled with media (Corning, New York, NY, USA) using a Precision XS liquid handling system (BioTek Instruments GmbH, Bad Friedrichshall, Germany). These plates were incubated using the Duetz system at 28°C for seven days, shaking at 220 rpm, and 2.5 cm deflection. Sample aliquots were taken for DNA preparation (for 16S rRNA gene sequencing) and to generate a cryo-conserved culture for long-term conservation at −80°C.

For preparation of cryo-conserved samples, 70% (v/v) glycerol and 5% (v/v) DMSO were filled into 96-deep well MTPs and mixed with grown culture broths (ratio 2:3) using a VIAFLO 384 (Integra Biosciences, Biebertal, Germany).

Cryo-conserved strains were recovered by agar plating and integrated into our strain collection. Bioprospecting campaigns were conducted either in 1 ml culture volume using 96-well Duetz systems or in 50 ml culture volume using 300 ml Erlenmeyer flasks, respectively. Incubation of Erlenmeyer flasks occurred at 28°C using an RC-406 orbital shaker (Infors, Bottmingen, Switzerland) with
5 cm deflection at 180 rpm for four and seven days. Fermentations were stopped by cooling microbial cultures and medium controls to −50°C. Frozen samples were lyophilized using a delta 2-24 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and an equal volume of methanol (related to the culture volume) was added. Extracts were further concentrated in vacuo (50-fold in relation to the culture volume) for UHPLC-QTOF-MS/MS analysis. For screening purposes, extract aliquots were reconstituted in DMSO (100-fold in relation to the culture broth).

**Phylogenetic classification and genotyping of isolated strains**

DNA extraction was carried out by transferring 200 μl of each grown culture supernatant into Collection Microtubes (Qiagen, Hilden, Germany) containing three zirconia beads (2.3 mm, Carl Roth, Karlsruhe, Germany). Cells were disrupted (twice, 30 Hz for 1 min) by using a Tissuelyser II (Qiagen). Plates were centrifuged at 4000 × g for 5 min, incubated at 70°C for 45 min and again centrifuged. The supernatants were transferred into fresh 96-V-bottom plates and used as template for 16S rRNA gene amplification following the PCR protocol described by (Kämpfer et al., 2014) using the primer pair E8F (5’-GAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-AGATTTGATCCTGCTGCTAG-3’) and for 18S rRNA gene amplification using the primer pair NS1 (5’-GTAGTGATCCTGGCTCAG-3’) and FR1 (5’-AICCATTCAATCGTGT-3’) following the protocol described by (Panzer et al., 2015). Genera belonging to Proteobacteria were defined as rarely cultured if less than ten representatives are recorded in the List of Prokaryotic names with Standing in Nomenclature (Parte, 2018, 2018; https://ipsn.dsmz.de/ access date: 01/2021).

All cultures affiliated to Acidobacteria were propagated on buffered R2A pH 5.5 agar to receive higher culture densities. All recovered nearly full-length 16S and rRNA sequences were affiliated to the most similar sequences of type strains using BLASTN (version: BLAST+ 2.11.0; http://blast.ncbi.nlm.nih.gov/blast.cgi) with the NCBI Reference Sequence Database (version: RefSeq Release 202; https://www.ncbi.nlm.nih.gov/refseq/). All recovered nearly full-length 16S rRNA sequences were affiliated to the most similar sequences of type strains using the pairwise alignment tool from MycoBank (https://www.mycobank.org/page/Pairwise_alignment). Representatives of different subgroups of Acidobacteria were included to visualize the phylogenetic relationship towards isolated Acidobacteria of this study. Therefore, multiple sequence alignment was done by CLUSTALW. Using the maximum-likelihood method, a phylogenetic tree was calculated in MEGA v7.0.26 (https://www.megasoftware.net) under the Tamura–Nei model (Kumar et al., 2016) performing 1000 bootstrap replications. Graphical modifications and annotations were made with iTOL v5.6.3 (https://itol.embl.de/; Letunic and Bork, 2019).

BOX-A1R-based repetitive extragenic palindromic PCR (5’-CTACGGCAAGGCGACGCTGACG-3’) was used to amplify repetitive elements to perform molecular genotyping (Koeuth et al., 1995). Genomic fingerprinting patterns (BOX-pattern) were analysed by LabChip GX Touch HT using DNA 5K Assay (Cat. No. CLS760675, PerkinElmer, Waltham, MA, USA) and GelCompar II software version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) for data interpretation. A hierarchical cluster of the obtained data was calculated based on Dice similarity matrix data by applying Ward’s method (Ward, 1963) to discriminate genotypic redundancies within pure cultures.

**Bioassays**

Crude extract screening was done by microplate broth dilution assays in final assay conc. 0.25-fold, 0.5-fold and onefold (in relation to the culture volume) against pathogenic bacteria and fungi (Staphylococcus aureus ATCC25923, Mycobacterium smegmatis ATCC 607, Aspergillus flavus ATCC 9170, Zymoseptoria campaigni Roberge in Desmazières MUCL 45407 and Candida albicans FH 2173).

To evaluate the growth inhibitory effect of microbial extracts, a seeding cell suspension of the indicator strains was prepared from pre-cultures or previously prepared spore solutions: for S. aureus, an overnight culture (37°C, 18 h, 180 rpm) was diluted to 2 × 10^4 cells ml⁻¹ in cation adjusted Mueller Hinton II medium (MHII). M. smegmatis was cultured in brain-heart infusion medium (BD) supplemented with 1% (w/v) Tween-80 (Sigma) for 48 h, before the assay cell concentration was adjusted (1 × 10^5 cells ml⁻¹). The pre-culture of C. albicans was incubated for 48 h at 28°C before a 1 × 10^5 cells ml⁻¹ suspension was prepared in MHII. Spore solutions of A. flavus and Z. tritici were diluted to 1 × 10^5 spores ml⁻¹. A dilution series of gentamycin, isoniazid or nystatin was used as positive control and cell suspensions without extract or antibiotic as negative control. Test plates were incubated in the dark (37°C, 180 rpm, 85% rH) for 18 h (A. flavus, S. aureus), 48 h (C. albicans, M. smegmatis) and 72 h at 25°C (Z. tritici). End-point detection towards optical density was determined using a LUMIstar® Omega (BMG Labtech GmbH, Ortenberg, Germany) by measuring the turbidity at 600 nm or by ATP quantification using BacTiter-Glo™ according to manufacturer’s protocol (Promega Corporation, Fitchburg, WI, USA).
Mass spectrometry

All mass spectrometry experiments were performed on a 1290 UHPLC system (Agilent, Santa Clara, CA, USA) equipped with DAD, ELSD and maXis II™ (Bruker, Billerica, MA, USA) ESI-QTOF-UHRMS with the gradient: 0 min: 95% A; 0.30 min: 95% A; 18.00 min: 4.75% A; 18.10 min: 0% A; 22.50 min: 0% A; 22.60 min: 95% A; 25.00 min: 95% A (A: H2O, 0.1% formic acid (FA); B: acetonitrile, 0.1% FA; flow: 600 µl min⁻¹). Column oven temperature: 45°C. Column: Acquity UPLC BEH C18 1.7 µm (2.1 x 100 mm) with Acquity UPLC BEH C18 1.7 µm VanGuard Pre-Column (2.1 x 5 mm). Injection volume was either 1 or 2 µl.

Cosine similarity calculations

Data processing was performed with Data Analysis 4.4 (Bruker, Billerica, MA, USA) using recalibration with sodium formate. RecalculateLinespectra with threshold 10 000 and subsequent FindMolecularFeatures (0.5–25 min, S/N = 0, correlation coefficient threshold = 0.7, minimum compound length = 8 spectra, smoothing width = 2) was performed. Bucketing was performed using ProfileAnalysis 2.3 (Bruker, Billerica, MA, USA) (30–1080 s, 100–1600 m/z, Advanced Bucketing with 12 s, 5 ppm, no transformation, Bucketing basis = H⁺). Samples with less than 50 features were excluded from further analysis. The generated bucket table was subsequently used as input for analysis via R (version 3.6.0) (R Core Team, 2020) with libraries readr (https://CRAN.R-project.org/package=readr), coor (https://cran.r-project.org/package=coor), gplots (https://CRAN.R-project.org/package=gplots), data.table (https://CRAN.R-project.org/package=data.table), and parallelDist (https://CRAN.R-project.org/package=parallelDist). The cosine similarities (dot product of vectors) between samples were calculated. Samples were sorted according to clustering results, and pairwise similarities were extracted and subsequently used to define metabolic groups. The script used in this publication was deposited in a GitHub repository (Hartwig, 2020). If the pairwise similarity between two subsequent clustered samples is at the threshold 0.9 or higher, they were assigned to one metabolic group.

Dereplication of bioactive extracts using UHPLC-UHR MS/MS

Crude extracts showing at least 70% growth inhibition were considered as bioactive and were subjected to microfractionation. Five or ten microliter of concentrated methanolic extracts (50-fold relative to the culture volume) were partitioned into 159 fractions (~7 s each) in a 384-well MTP. UHPLC-UHR-MS analysis was performed (settings see Section Mass spectrometry). For microfractionation, 90% of the flow was collected with a custom made fraction collector (Zinsser–Analytic, Eschborn, Germany) while the rest was analysed in MS/MS mode in maXis II™. Collision induced dissociation was performed at 28.0–35.05 eV using argon at 10⁻² mbar. Additionally to the chromatographically separated 159 fractions, crude extract was applied as fraction 160 in the same amount as the injection volume serving as positive control. All extracts were evaporated in a GeneVac HT-12 (SP Industries Warminster, PA, USA) and were rescreened against the same test strain. Correlation between bioactive fractions and the corresponding MS data of analytes detected in those fractions was performed. Dereplication was facilitated by comparison of mass to charge ratios, retention time and fragmentation signatures with our in-house reference database containing ~1700 structurally characterized microbial metabolites at the time of data processing. Molecular formula assignment was done manually for all compounds present in the active fractions, allowing a mass accuracy tolerance of ±2 ppm. Annotation of the MS/MS spectra was performed manually for all the compounds present in active fractions, whenever no hits were found in the in-house compound database. Molecular formula searches were performed on AntiBase 2017 (Laatsch, 2017) and Dictionary of Natural Products (http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml; accessed on Nov 16, 2020).

Molecular networking

Based on published protocols, molecular networking with a cosine similarity cut-off of > 0.7 was performed (Yang et al., 2013; Allard et al., 2016). The tool MSConvert (ProteoWizard package32) was used to convert the raw data (*.d files) into plain text files (*.txt), wherein all detected fragment ions are expressed as a list of mass/intensity value pairs sorted according to their parent ions (peak picking; vendor MS level = 1–2; threshold type = absolute intensity, value = 1000, orientation = most-intense). Sharing at least six fragments (tolerance Δppm 0.05) with at least one partner ion those ions were included in the final network (Riyanti et al., 2020). Known NPs were highlighted by including deposited compounds from the in silico fragmented (Allen et al., 2015) commercial database AntiBase 2017 (Laatsch, 2017) and our in-house reference compound MS/MS database. The data were visualized with CYTOSCAPE v3.6.0 (Shannon, 2003) as described elsewhere (Mamer et al., 2020).

Acknowledgements

The authors would like to thank Dr. Michael Mourez, Dr. Luigi Toti, Dr. Jochen Fracowiak and Mai Ngo for
helpful discussions. We thank Mareike Rothenheber, Christine Wehr, Jennifer Kuhn, Nadine Zucchetto, Sina Abdo, Regina Zweigert, Veronika Eckert, and Kirsten-Susann Bommersheim for their technical support during the experiments.

Authors contributions

MO, SB, JG, and MS conceived and designed the experiments. MO, SB, MS, SM, BL, MM, CH, MP performed the experiments. MO, SB, MS, SM, MM, CH and MP analysed the data. MO, SB, MS and TFS drafted the first manuscript. TFS, JG and MS revised the manuscript. AV and PH initiated the public–private partnership between Fraunhofer and Sanofi (later Evotec). All authors accepted the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest. The authors declare no competing financial interest.

Data availability statement

The Illumina amplicon sequencing data supporting the findings of this study are openly available under the BioProject PRJNA699730 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA699730).

References

Adamek, M., Alanjary, M., and Ziemert, N. (2019) Applied evolution: phylogeny-based approaches in natural products research. Nat Prod Rep 36: 1295–1312.

Akselband, Y., Cabral, C., Castor, T.P., Chikarmane, H.M., and McGrath, P. (2006) Enrichment of slow-growing marine microorganisms from mixed cultures using gel microdrop (GMD) growth assay and fluorescence-activated cell sorting. J Exp Mar Biol Ecol 329: 196–205.

Allard, P.-M., Pérèsse, T., Bisson, J., Gindro, K., Marcourt, L., van Pham, C., et al. (2016) Integration of molecular networking and in-silico MS/MS fragmentation for natural products dereplication. Anal Chem 88: 3317–3323.

Allen, F., Greiner, R., and Wishart, D. (2015) Competitive fragmentation modeling of ESI-MS/MS spectra for putative metabolite identification. Metabolomics 11: 98–110.

Baret, J.-C., Miller, O.J., Taly, V., Ryckelynck, M., El-Harrak, A., Frenz, L., et al. (2009) Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip 9: 1850.

Barra Caracciolo, A., Gremini, P., Cupo, C., and Rossetti, S. (2005) In situ analysis of native microbial communities in complex samples with high particulate loads. FEMS Microbiol Lett 253: 55–58.

Belknap, K.C., Park, C.J., Barth, B.M., and Andam, C.P. (2020) Genome mining of biosynthetic and chemotherapeutic gene clusters in Streptomyces bacteria. Sci Rep 10: 1–9.
Parte, A.C. (2018) LPSN - List of Prokaryotic names with Standing in Nomenclature (bacterio.net), 20 years on. *Int J Syst Evol Microbiol* 68: 1825–1829.

Payne, E.M., Holland-Moritz, D.A., Sun, S., and Kennedy, R.T. (2020) High-throughput screening by droplet microfluidics: perspective into key challenges and future prospects. *Lab Chip* 20: 2247–2262.

Pillies, G. (1975) Nonactin, monactin, dinactin, trinactin, and tetractin. A Raman Spectroscopic Study. *Biopolymers* 14: 2311–2327.

Pöppel, A.-K., Koch, A., Kogel, K.-H., Vogel, H., Kollew, C., Wiesner, J., and Vilcinskas, A. (2014) Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly Lucilia sericata. *Biol Chem 395*: 649–656.

R Core Team (2020) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Rezanka, T., Prell, A., Spízek, J., and Sigler, K. (2010) Pilot-plant cultivation of Streptomyces griseus producing homologues of nonactin by precursor-directed biosynthesis and their identification by LC/MS-ESI. *J Antibi 63*: 524–529.

Riyanti, Marner, M., Hartwig, C., Patras, M., Wodi, S., Rieuppassa, F., et al. (2020) Sustainable low-volume analysis of environmental samples by Semi-Automated Prioritization of Extracts for Natural Product Research (SeaPEPR). *Mar Drugs* 18: 649.

Schärer, T.F., and Hack, I.M. (2014) Overcoming the current dead-end in antibiotic research. *Trends Microbiol* 22: 165–167.

Shannon, P. (2003) Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* 13: 2498–2504.

Shapiro, H.M. (2000) Membrane potential estimation by flow cytometry. *Methods* 21: 271–279.

Stewart, E.J. (2012) Growing unculturable bacteria. *J Bacteri*o 194: 4151–4160.

Strobelt, G.A., Morrison, S.L., and Cassella, M. (2005) Protecting plants from oomycete pathogens by treatment with compositions containing serratamolide and oocydin a from Serratia marcescens. Patent No.: US6926892B2.

Taconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., et al. (2018) Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis* 18: 318–327.

Tamehori, N., Okamoto-Hosoya, Y., Okamoto, S., Ubu kata, M., Hamada, M., Naganawa, H., and Ochi, K. (2002) Bacilyscin, a Novel phospholipid antibiotic produced by Bacillus subtilis 168. *Antimicrob Agents Chemother* 46: 315–320.

Terekhov, S.S., Smirnov, I.V., Stepanova, A.V., Bobik, T.V., Mokrushina, Y.A., Ponomarenko, N.A., et al. (2017) Microfluidic droplet platform for ultrahigh-throughput single-cell screening of biodiversity. *Proc Natl Acad Sci USA* 114: 2550–2555.

Theberge, A.B., Courtot, F., Schaerli, Y., Fischlechner, M., Abell, C., Hollfelder, F., and Huck, W.T. (2010) Microdroplets in microfluidics: an evolving platform for discoveries in chemistry and biology. *Angew Chem Int Ed Engl* 49: 5846–5868.

Theuretzbacher, U., Outterson, K., Engel, A., and Karlén, A. (2020) The global preclinical antibacterial pipeline. *Nat Rev Microbiol* 18: 275–285.

Thies, S., Santiago-Schübel, B., Kovačić, F., Rosenau, F., Hausmann, R., and Jaeger, K.-E. (2014) Heterologous production of the lipopeptide biosurfactant serrawettin W1 in Escherichia coli. *J Biotechnol* 181: 27–30.

Tovar, M., Mahler, L., Buchheim, S., Roth, M., and Rosenberg, M.A. (2020) Monitoring and external control of pH in microfluidic droplets during microbial culturing. *Microb Cell Fact* 19: 16.

Tracanna, V., de Jong, A., Medema, M.H., and Kuipers, O.P. (2017) Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiol Rev* 41: 417–429.

Umetsu, N., and Shirai, Y. (2020) Development of novel pesticides in the 21st century. *J Pestic Sci* 45: 54–74.

Volpatti, L.R., and Yetisen, A.K. (2014) Commercialization of microfluidic devices. *Trends Biotechnol* 32: 347–350.

Ward, J.H. (1963) Hierarchical grouping to optimize an objective function. *J Am Stat Assoc* 58: 236–244.

Wasserman, H.H., Keggi, J.J., and McKeon, J.E. (1961) Serratamolide, a metabolic product of Serratia. *J Am Chem Soc* 83: 4107–4108.

Wiegand, S., Jogler, M., Boedecker, C., Pinto, D., Vollmers, J., Rivas-Marin, E., et al. (2019) Cultivation and functional characterization of 79 plantcomycetes uncovers their unique biology. *Nat Microbiol* 5: 126–140.

Yang, J.Y., Sanchez, L.M., Rath, C.M., Liu, X., Boudreau, P.D., Bruns, N., et al. (2013) Molecular networking as a dereplication strategy. *J Nat Prod* 76: 1866–1699.

Zengler, K., Toledo, G., Rapp, M., Elkins, J., Mathur, E.J., Short, J.M., and Keller, M. (2002) Cultivating the uncultured. *Proc Natl Acad USA* 99: 15681–15686.

Zincheno, A., Devenish, S.R., Kintses, B., Colin, P.Y., Fischlechner, M., and Hollfelder, F. (2014) One in a million: flow cytometric sorting of single cell-lysate assays in monodisperse picolitre double emulsion droplets for directed evolution. *Anal Chem* 86: 2526–2533.

Zizka, Z. (1998) Biological effects of macrotetrolide antibiotics and nonacetic acids. *Folia Microbiol* 43: 7–14.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Poisson distribution of λ0.1 and λ1.0 showing the probability of the amount of cells encapsulated per droplet.

**Fig. S2.** Work scheme of this study showing the microfluidics work.

**Fig. S3.** Live/dead staining using the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (L7007, Invitrogen). Manufacturer’s protocol was applied on the cells retrieved by nycodenz density gradient centrifugation (A: SYTO 9, B: propidium iodide, C: merged) directly after bacterial isolation. Exemplary pictures are shown, ten independent stains were done and considered for the calculation. Live:dead ratio was estimated resulting in ~ 70:30 ± 6.7%.
Fig. S4. Phylogenetic classification of FHG110511 within the phylum Acidobacteria clustering into subgroup 1. The tree is based on a ClustalW alignment of available 16S rRNA gene sequences from the ref_seq database between positions 113 and 1357 [based on Escherichia coli 16S rRNA gene numbering (Brosius et al., 1978)] from the most similar sequences to the isolated strains, and also includes representatives of Acidobacteria subgroups 1, 3, 4, 6, 7, 8, and 10. The tree was calculated using MEGA v7.0.26 with the maximum-likelihood method and GTR-Gamma model. Circles on the tree branches indicate values of 1000 bootstrap replicates with a bootstrap support of more than 50%. Subgroup affiliations are indicated by colors. The new isolate is indicated by a black arrow. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Fig. S5. (A) Assay read-out of μ-fractionation plates of strain FHG110488 against M. smegmatis ATCC 607. Fractions are numbered and those causing at least 70% rel. growth inhibition were considered “active” and marked red. Column 1: medium control; Column 2-3: antibiotic standard (isoniazid); Column 4: growth control. Area AH05-AH24: 5 μl injection volume; Area AH05-AH24 bottom: 10 μl injection; Crude: crude extract as a control. (B) Overlaid Base peak Chromatograms (grey), fraction collector signal analogs (light blue bars) and extracted ion chromatogam of m/z 515.3329±0.005 [M+H]^+ (1, red) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (C) UV and MS spectrum of fractions 84-87. (D) MS/MS fragmentation of the precursor ion at m/z 454.2931 ± 0.005 [M+H]^+ (11, red), m/z 690.5073 ± 0.005 [M+H]^+ (12, blue) and m/z 716.5237 ± 0.005 [M+H]^+ (13, green) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (E) Structures of all three dereplicated compounds 8–10. 

Fig. S6. (A) MS2-network of “active” extract of FHG110488 against Septoria tritici MCUL45407 focusing on the cluster representing all seven detected serratamolide derivatives and their literature known structures (dots of parent ions found as hits in our internal database or AntiBase are marked in gold). (B) Overlaid Base peak Chromatogram (grey) and extracted ion chromatogams of serratamolides 1–7 (1 μl 5323.327 [M+H]^+, C_{26}H_{47}N_{2}O_{8} (red); 2 m/z 541.3483 [M+H]^+, C_{23}H_{34}N_{2}O_{5} (blue); 3 m/z 543.3460 [M+H]^+, C_{23}H_{34}N_{2}O_{5} (black); 4 m/z 533.3433 [M+H]^+, C_{23}H_{34}N_{2}O_{5} (cyan); 5 m/z 547.3598 [M+H]^+, C_{26}H_{34}N_{2}O_{5} (yellow); 6 m/z 575.3902 [M+H]^+, C_{24}H_{33}N_{2}O_{5} (light green); 7 m/z 573.3746 [M+H]^+, C_{23}H_{32}N_{2}O_{5} (dark green)) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (C) MS/MS fragmentation of the precursor ions 1-7.

Fig. S7. (A) Assay read-out of μ-fractionation plate of strain FHG110502 against Mycobacterium smegmatis ATCC 607. Fractions are numbered and those causing at least 70% rel. growth inhibition were considered “active” and marked red. Column 1: medium control; Column 2-3: antibiotic standard (isoniazid); Column 4: growth control. Area AH05-AH24: 2 μl injection volume; Area IP05-IP24: 5 μl injection; Crude: crude extract as a control. (B) Overlaid Base peak Chromatograms (grey), Fraction collector signal analogs (light blue bars) and extracted ion chromatogram of m/z 454.2931 ± 0.005 [M+H]^+ (11, red), m/z 690.5073 ± 0.005 [M+H]^+ (12, blue) and m/z 716.5237 ± 0.005 [M+H]^+ (13, green) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (C) UV and MS spectrum of fractions 93–94 (left) and 130–136 (right). (D) MS/MS fragmentation of the precursor ion at m/z 454.2931 [M+H]^+, m/z 690.5073 [M+H]^+, and m/z 716.5237 [M+H]^+ (dereplicated as lyso-palmitoyl-phosphoethanolamine, palmitoyl-palmitoyl-phosphoethanolamine and palmitoleoyl-oleoyl-phosphoethanolamine, respectively), manual annotation of the neutral losses and proposed structures of the fragment ions. (E) Putative structures of all three dereplicated compounds 11–13.

Fig. S8. (A) Assay read-out of μ-fractionation plate of strain FHG110508 against Staphylococcus aureus ATCC 25923. Fractions are numbered and those causing at least 70% rel. growth inhibition were considered “active” and marked red. Column 1: medium control; Column 2-3: antibiotic standard (gentamycin); Column 4: growth control. Area AH05-AH24: 2 μl injection volume; Area IP05-IP24: 5 μl injection; Crude: crude extract as a control. (B) Overlaid Base peak Chromatograms (grey), Fraction collector signal analogs (light blue bars) and extracted ion chromatogram of m/z 737.4475±0.005 [M+H]^+ (14, red), m/z 751.4363±0.005 [M+H]^+ (15, green), m/z 765.4794±0.005 [M+H]^+ (16, blue), and m/z 779.4956±0.005 [M+H]^+ (17, yellow) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (C) UV and MS spectrum of fractions 120, 124, 128 and 131-132 (from left to right). (D) MS/MS fragmentation of the precursor ion at m/z 737.4462 [M+H]^+, m/z 751.4618 [M+H]^+, m/z 765.4779 [M+H]^+, and m/z 779.4933 [M+H]^+ (dereplicated as nonactin, monactin, dinactin and macrotrolide G, respectively) with manual annotation of the neutral loss and proposed structure of the fragment ion at m/z 213.1482 of parent ion at m/z 779.4933 indicating the presents of macrotrolide G instead of trinactin. (E) Structures of all four dereplicated macrotrolides. Me: methy1; Et: ethyl; iPr: isopropyl.

Fig. S9. (A) MS2-network of “active” extract of FHG110508 against Staphylococcus aureus ATCC 25923 with focus on the cluster representing all five detected macrotrolide derivatives and their adduct ions (dots of parent ions found as hits in our internal database or AntiBase

© 2021 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd.
are marked in gold). (B) Overlaid Base peak Chromatogram (grey) and extracted ion chromatograms of macrotetrolide 14-18 (14 m/z 737.4462 [M+H]⁺, C₄₀H₆₅O₁₂⁻ (red); 15 m/z 751.4618 [M+H]⁺, C₄₁H₆₇O₁₂⁻ (green); 16 m/z 765.4779 [M+H]⁺, C₄₂H₆₉O₁₂⁻ (blue); 17 m/z 779.4933 [M+H]⁺, C₄₃H₇₁O₁₂⁻ (yellow); 18 m/z 793.5104 [M+H]⁺, C₄₄H₇₃O₁₂⁻ (black)) of the 50 fold concentrated extract (in MeOH) with 5 μl injection volume. (C) MS/MS fragmentation of the precursor ions 14-18 dereplicated as nonactin, monactin, dinactin, macrotetrolide G, and macrotetrolide D, respectively, with manual annotation of the neutral loss and proposed structure of the fragment ion at m/z 213.1482 of parent ion at m/z 779.4933 indicating the presents of macrotetrolide G instead of trinactin and fragment ion at m/z 213.1487 of parent ion at m/z 793.5104 indicating the presents of macrotetrolide D instead of tetranactin.

Table S1. Overview of the cultured genera in miSEM² pH 7.2, pH 5.5 and VL55-xyl pH 5.5. 16S rRNA gene sequencing was applied using the primer 1492R on mechanically disrupted culture broth grown for 7 days.

Table S2. Cosine Similarity table – Data for Fig. affiliation.

Video S1. Video showing the production of droplets with the μEncapsulator from Dolomite.