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Biological Soil Crusts from Different Soil Substrates Harbor Distinct Bacterial Groups with the Potential to Produce Exopolysaccharides and Lipopolysaccharides

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Abstract

Biological soil crusts (biocrusts) play an important role in improving soil stability and resistance to erosion by promoting aggregation of soil particles. During initial development, biocrusts are dominated by bacteria. Some bacterial members of the biocrusts can contribute to the formation of soil aggregates by producing exopolysaccharides and lipopolysaccharides that act as "glue" for soil particles. However, little is known about the dynamics of "soil glue" producers during the initial development of biocrusts. We hypothesized that different types of initial biocrusts harbor distinct producers of adhesive polysaccharides. To investigate this, we performed a microcosm experiment, cultivating biocrusts on two soil substrates. High-throughput shotgun sequencing was used to obtain metagenomic information on microbiomes of bulk soils from the beginning of the experiment, and biocrusts sampled after 4 and 10 months of incubation. We discovered that the relative abundance of genes involved in the biosynthesis of exopolysaccharides and lipopolysaccharides increased in biocrusts compared with bulk soils. At the same time, communities of potential "soil glue" producers that were highly similar in bulk soils underwent differentiation once biocrusts started to develop. In the bulk soils, the investigated genes were harbored mainly by *Betaproteobacteria*, whereas in the biocrusts, the major potential producers of adhesive polysaccharides were, aside from *Alphaproteobacteria*, either *Cyanobacteria* or *Chloroflexi* and *Acidobacteria*. Overall, our results indicate that the potential to form exopolysaccharides and lipopolysaccharides is an important bacterial trait for initial biocrusts and is maintained despite the shifts in bacterial community composition during biocrust development.

Keywords Biological soil crusts · Exopolysaccharides · Lipopolysaccharides · Microbiome · Metagenomics

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Introduction

Biological soil crusts (biocrusts) are important biotic components of many terrestrial ecosystems [1, 2]. They consist of highly specialized and complex communities of algae, mosses, lichens, fungi, cyanobacteria, and other prokaryotes [3]. These organisms live in a close association with soil particles, forming a coherent layer within the uppermost few millimeters of the topsoil, or directly on the soil surface [1]. An important structural element of biocrusts is the extracellular polymeric matrix (EPM) which is composed mostly of polysaccharides and connects organisms and soil particles [4]. EPM ensures biocrust integrity, provides protection from external harmful agents, and alters moisture content as well as nutrient availability [4]. EPM also fosters the stabilization of soil aggregates and protects soils from erosion by wind or water [5–8]. Among organisms forming biocrusts, the best-studied producers of polysaccharides are cyanobacteria and algae [1]. However, although not as thoroughly studied, also non-photosynthetic microbial members of biocrusts, including fungi, proteobacteria, and actinobacteria, are prominent producers of these compounds [9–11].

The composition and chemical properties of polysaccharides in EPM strongly depend on the community of organisms forming biocrusts. For example, it has been demonstrated that non-photosynthetic bacteria primarily produce simple polysaccharides, composed mainly of mannose, galactose, and glucose [12], while cyanobacteria, algae, and fungi produce more complex polysaccharides, which may contain high amounts of non-neutral sugars [13–15]. As it was shown that even slight differences in the sugar composition can result in completely different physical traits of the polysaccharide [16], the properties of EPM could be influenced by any factor that changes the structure of polysaccharide-producing communities. It is known that the composition of organisms forming biocrusts changes depending on (i) the developmental stage of biocrusts [17–19], (ii) environmental factors like radiation, humidity, elevation, and temperature [17–24], and (iii) edaphic factors like soil pH, texture, and nutrient content [1, 17, 20, 21]. However, not all members of biocrusts have the ability to produce polysaccharides, and little is known about the dynamics of polysaccharide-producing organisms during the development of different types of biocrusts.

In this respect, bacterial polysaccharides, specifically exopolysaccharides (EPSs) and lipopolysaccharides (LPSs), are of great interest, as cyanobacteria and non-phototrophic bacteria form biocrusts in the initial stage of biocrust development [25]. EPSs are either synthesized intracellularly and excreted by one of three different pathways: the Wzy-dependent pathway, the ABC transporter-dependent pathway, and the synthase-dependent pathway, or synthesized extracellularly [26, 27]. In contrast, parts of LPSs are initially synthesized inside a cell, then ligated together at the inner membrane and transported to the cell surface as mature molecules [28, 29]. While LPSs are present in most Gram-negative bacteria [30], EPSs are exuded by a wide range of taxa [16]. Among the most-recognized producers of EPSs are cyanobacterial members of *Oscillatoria*, *Nostoc*, *Lyngbya*, and *Schizothrix*, as well as bacterial members of *Microbacterium*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, and *Streptomyces* [31]. These microorganisms are the first colonizers of bare soils, and their EPSs as well as LPSs are considered as essential for the initial consolidation of soil particles and the preparation of conditions for the establishment of cryptogamic surface cover in the later stages of biocrust development [32]. Thus, a better understanding of the dynamics of polysaccharide-producing organisms during the initial development of biocrusts requires more in-depth knowledge on cyanobacteria and other bacteria that initialize biocrust establishment [4].

Many researchers studied polysaccharide-producing bacterial strains that were isolated from biocrusts at different stages of development [33–36]. However, data on the community dynamics of bacterial EPS and LPS producers under natural conditions is missing. Thus, our aim was to investigate polysaccharide-producing bacterial communities during the initial stage of biocrust development. We assumed that the relative abundance of genes related to EPS and LPS formation would increase once biocrust development starts. Moreover, we hypothesized that different types of initial biocrusts would harbor different communities of potential EPS and LPS producers. To test our hypotheses, we performed a microcosm experiment cultivating biocrusts on two different soil substrates. As the soil substrates came from sites with different types of naturally occurring biocrusts, we expected that the biocrusts cultivated in the microcosm experiment would also consist of distinct microbial communities. To address our research questions, we used a high-throughput shotgun sequencing of DNA extracted from bulk soils from the beginning of the experiment, as well as initial biocrusts sampled after 4 and 10 months of incubation. We employed a bioinformatics pipeline described by Cania et al. [37], which targets genes specific for EPS and LPS production, to obtain information on bacteria potentially involved in the production of adhesive polysaccharides.

Materials and Methods

Site Description

Soils for the incubation experiment were collected in 2011 from two sites at the initial stages of ecosystem development. One was the artificial catchment Chicken Creek (51°36′18″ N, 14°15′58″ E) and the other was an initial moving sand dune close to Lieberose (51°55′49″ N, 14°22′22″ E). Both sites are located in the state of Brandenburg in eastern Germany,

approximately 37 km apart. The climate of the region is temperate continental with a mean air temperature of 8.9 °C and mean rainfall of 569 mm a^{-1} [19].

The Chicken Creek catchment was constructed in 2005 in an opencast mine near Cottbus by dumping and contouring sand and loamy sand material originating from Pleistocene sediments. Details on the construction works and site conditions are given by Gerwin et al. [38] and Russell et al. [39]. After construction, no restoration was undertaken and the area was allowed to undergo natural succession. Biocrust development at the site was heterogeneous depending on the appearance of vascular vegetation, which was still dynamic at the time of sampling [40]. For the Lusatian post-mining sites, the cyanobacterial species *Microcoleus vaginatus*, *Nostoc* spec., *Phormidium* spec., *Schizothrix* spec., and *Tolypothrix* spec.; the green algal species *Bracteacoccus* minor, *Chlorococcum* spec., *Cylindrocystis* spec., *Elliptochloris* spec., *Gloeocystis* spec., *Klebsormidium*, *Chlorella* spec., *Zygogonium* spec., *Ulothrix* spec., and *Haematococcus* spec.; and the lichens *Placynthiella oligotropha* and *Cladonia subulata*, as well as the mosses *Polytrichum piliferum* and *Ceratodon purpureus* were reported [18, 41, 42]. The Chicken Creek site heterogeneity was also reflected by high variability of moss coverages, which were recorded on 107 vegetation monitoring plots each having a size of 5 × 5 m^2, which ranged from 0.1 to 95% with a median coverage of 30 ± 25% [40]. Terminal successional stages of cryptogamic surface cover development could not be identified, mainly due to biocrust extinction caused by vascular plant overgrowth.

The moving sand dune occurs near Lieberose as a result of extensive disturbances of the land surface by former military activities (until approximately 1992). The dune is composed of Pleistocene aeolian sand. A detailed description of the site is provided by Dümig et al. [43], and Fischer and Veste [19]. Depending on their position downslope an inland dune catena, three stages of biocrust development could be identified. In microdepressions and at the lee side of tussocks consisting of *Corynephorus canescens* located in the center of the dune slope, dominating sand grains were physically stabilized in their contact zones by accumulated organic matter and by few filamentous algae (biocrust stage one, surface coverage 20%) [44]. At surface patches of the lower dune slope, filamentous algae enmeshed the sand grains and partially filled in the soil pores (biocrust stage two, surface coverage 40%) [44]. Biocrust stage three was characterized by full cover with filamentous and coccoid algae, and by few mosses, the latter covering less than 5% of the surface. The dominating green algal and moss species were *Zygogonium ericetorum* and *Polytrichum piliferum*, respectively [45]. Cyanobacteria were a minor component within the *Zygogonium* crust, which did not form individual patches, whereas lichens could not be observed at the sampling site. The terminal successional stage of cryptogamic surface cover development, which was found in the vicinity of a less disturbed neighboring Scots pine forest (distance to the sampling site of around 500 m), was characterized by co-appearance of *Cladonia* spec. and *P. piliferum*, which formed dense surface covers.

Sampling and Incubation Experiment

Bulk soil from the Chicken Creek catchment and the Lieberose sand dune was used to establish a microcosm experiment. A total amount of 100 kg of soil was taken per site from the top 20 cm. At Lieberose, the soil was collected from five spots on the top of the dune, where no plants were growing, while at Chicken Creek, five plant-free spots were used for the soil sampling. The soil was transported and afterwards stored in the dark at room temperature for approximately 6 months before the incubation experiment. During that time, pre-experiments to adjust the incubation conditions for biocrust growth were performed.

The soil was mixed and passed through a 2-mm sieve, then packed into plastic pots (10 cm × 10 cm × 10 cm) and compacted to the natural soil density of approximately 1.6 g cm^{-3} [43]. In total, the microcosm experiment consisted of 18 pots (9 per site). The water content was set to 50% of the maximum water holding capacity of the soil samples, and adjusted weekly from the bottom, which ensured very low disturbance for biocrust development. Realistic climatic and light conditions were simulated in the sun simulator facility of the Helmholtz Zentrum München (Neuherberg, Germany) by generating the entire spectrum from the ultraviolet (UV, 280–400 nm; UV-B, 280–315 nm; UV-A, 315–400 nm) to the near-infrared (NIR) light with a combination of four types of lamps: metal halide lamps (Osram Powerstar HQI-TS 400W/D), quartz halogen lamps (Osram Haloline 400), blue fluorescent tubes (Philips TL-D 36W/BLUE), and UV-B fluorescent tubes (Philips TL 40W/12). The lamps were arranged in several groups to obtain the natural diurnal variations of solar irradiance by switching appropriate groups of lamps on and off. The short-wave cutoff was achieved by selected borosilicate and soda-lime glass filters as previously described [46, 47]. The pots were exposed to radiation for 16 h per day. Maximum radiation was reached in the middle of the day for 8 h at PAR (photosynthetic active radiation, 400–700 nm) of 940 μmol m^{-2} s^{-1}, UV-A of 17.7 W m^{-2}, and UV-B of 0.37 W m^{-2}. The climatic conditions were adjusted to a night-day cycle from 18 to 25 °C, and a relative air humidity of 95–90%, respectively.

Biocrusts were sampled after 4 (T1) and 10 (T2) months of incubation from three independent pots per soil substrate and sampling time point. Only the upper 2 mm was considered as biocrust. In addition, samples of bulk soil without biocrust development were taken at the beginning of the experiment (T0). In total, 18 samples were collected (3 sampling times × 2 sites × 3 replicates). Samples for DNA analyses were directly

frozen at − 80 °C, while samples for biochemical analyses were stored at 4 °C until further processing. For the determination of water repellency and the computed tomography analysis, undisturbed samples from the end of the experiment were taken using Petri dishes and stored at 4 °C until further analysis.

Physicochemical Measurements

For the analysis of dissolved organic carbon (DOC) and nitrogen (DON), bulk soils and biocrust samples were suspended with a 0.01 M $CaCl_2$ solution in a 1:3 ratio (w/v), and shaken horizontally for 45 min. After passing through a Millex-HV 0.45-μm filter (Merck Millipore, USA), extracts were analyzed for DOC by means of a DIMA-TOC 100 analyzer (Dimatec Analysentechnik GmbH, DE), and for DON using a Skalar Continuous Flow Analyzer SA5100 (Skalar Analytical B.V., NL) [48]. Soil pH of bulk soil samples was measured in 0.01 M $CaCl_2$ solution with a soil:solution ratio of 1:5 (w/v) after 3 h of incubation time. Water repellency of biocrusts was measured as a dimensionless "repellency index" using the ethanol/water microinfiltrometric sorptivity method according to Fischer et al. [49], where a theoretical value of 1 characterizes totally non-repellent soils [50], and may exceed 50 for highly repellent soils [51].

Pre-experiments indicated that only biocrusts from T2 grown on substrate from Chicken Creek developed a thickness sufficient for visualization by computed tomography (CT). Thus, only these samples were used to determine connectivity of the three-dimensional pore system of the biocrusts and the underlying soil as described previously [52]. The structure of the undisturbed samples was analyzed using a micro-computed tomography scanner (X-Tek HMX 225, Nikon Metrology, BE) equipped with a fine-focus X-ray tube (spot size of 5 μm) and a digital flat panel detector with a resolution of 512 by 512 pixels (width by height). The resulting X-ray computed microtomography (XCMT) images were used to calculate Euler characteristics for 26 nearest neighbors of each voxel. So defined Euler numbers were computed as a function of pore size in the range between 15 and 291 μm [53].

DNA Extraction, Library Preparation, and Sequencing

DNA was extracted from bulk soil and biocrust samples using the "Genomic DNA from soil" NucleoSpin Soil Kit (Macherey-Nagel, DE) according to the manufacturer's manual. Based on a performance pretest (data not shown), Buffer SL1 was chosen for sample lysis. DNA purity was verified by means of a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The quantity was also measured using a SpectraMax Gemini EM microplate reader (Molecular Devices, USA) together with a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, USA), and is presented in Table 2. DNA was sheared using an E220 Focused-ultrasonicator (Covaris, USA) with the following conditions: peak incident power = 175 W, duty factor = 10%, cycles per burst = 200, treatment time = 100 s, temperature = 7 °C, water level = 6, sample volume = 50 μL, intensifier = yes. Library preparation was performed using the NEBNext Ultra DNA Library Prep Kit for Illumina and the NEBNext Multiplex Oligos for Illumina (both New England Biolabs, UK) as described in the protocol of the producer. Due to lower DNA concentrations (Table 2), samples from T0 underwent different molecular manipulations during library preparation than samples from T1 and T2. The NEBNext adaptor from Illumina was diluted 10-fold for samples from T1 and T2, and 50-fold for samples from T0, to prevent the occurrence of dimers. Size selection for samples from T1 and T2 was performed with Agencourt AMPure XP beads (Beckman Coulter, USA), using the volumes selecting for libraries with 500–700 bp inserts. No size selection was applied for samples from T0 due to the low DNA concentrations of the libraries. PCR amplification was performed with 15 cycles for samples from T1 and T2, and 18 cycles for samples from T0. Primers used for samples from T1 and T2 were diluted 2-fold. Primers used for samples from T0 were not diluted. Libraries were pooled equimolarily, and 15 pM of the mixture was spiked with 1% PhiX. Sequencing was carried out on a MiSeq sequencer using a MiSeq Reagent Kit v3 for 600 cycles (Illumina, USA). Raw sequencing data obtained from the MiSeq is available at the sequencing read archive (SRA) under the accession number PRJNA509545.

Bioinformatical Analysis of Sequencing Data

The raw sequencing data was processed as described by Vestergaard et al. [54]. Removal of remnant adaptor sequences, trimming of terminal nucleotides with Phred quality scores less than 15, and removal of reads shorter than 50 bp were carried out using AdapterRemoval [55]. Reads containing more than 1% ambiguous bases (N) were removed by means of PRINSEQ-lite (version 0.20.4) [56]. DeconSeq (version 0.4.3) [57] was used to remove PhiX contamination. Sufficient coverage of the metagenomic datasets was confirmed by means of Nonpareil (version 2.4) [58] with default settings (Supplementary Material 1 Fig. S1).

Metagenomes obtained from bulk soils (T0) comprised reads on average 106 bp shorter than metagenomes created from biocrusts (T1 and T2). To test whether the difference in read length affects the accuracy of annotations, T1 and T2 reads were trimmed in silico in a randomized manner to resemble the length distributions of T0 reads. A comparison of the length distributions of exemplary "short" and "long reads" metagenomes, before and after trimming, is presented in Supplementary Material 1 Fig. S2. The metagenomes with

trimmed sequences were analyzed taxonomically together with the original metagenomes. Principal coordinate analysis (PCoA) ordination plots (Supplementary Material 1 Fig. S3) showed that the taxonomic annotations were not notably biased by the difference in read length. Consequently, further analyses were performed on the metagenomes with original read lengths.

For taxonomic classification, metagenomic reads were aligned against the National Center for Biotechnology Information Non-Redundant (NCBI-NR) protein sequences database (January 2017) using Kaiju (version 1.4.4) [59] in Greedy mode with 5 allowed mismatches. Additionally, bacterial 16S rRNA gene sequences were extracted from the metagenomic datasets and annotated using SortMeRNA (version 2.0) [60] with the SILVA SSU database (release 132).

Subsequent functional annotations were performed for bacterial reads identified by Kaiju only. COG (Clusters of Orthologous Groups) functional categories were assigned based on the eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database (version 4.5) [61]. Assignment of genes specific for EPS and LPS biosynthesis and excretion, which were the focus of the current study, was carried out according to Cania et al. [37] by hidden Markov model (HMM) searches combined with blasts against protein sequences derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (October 2016). Briefly, HMMs were obtained from the TIGRFAMs (version 15) [62] and Pfam (version 30) [63] databases. FragGeneScan (version 1.19) [64] was used to predict open-reading frames, which were subsequently scanned with HMMER (version 3) (hmmer.org). Matching reads (*E* value threshold = 10^{-5}) were mapped to KEGG Orthology (KO) numbers. A KO number was assigned to those reads for which the top 25 blast results were consistent. Blasting was carried out using Diamond (version 0.8.38) [65] with more-sensitive parameters. HMMs and KO numbers used for the analysis are listed in Table 1. Genes *algE*, *epsA*, and *epsG* were not included in the analysis due to very low relative abundances ($< 5 \times 10^{7}$). As most reads (> 50%) assigned to the genes of interest using the HMM-KEGG pipeline were classified into the COG category "Function unknown," this study was based mainly on the targeted approach proposed by Cania et al. [37]. The eggNOG pipeline was employed only for a general overview of the data.

Statistical Analysis and Data Visualization

Analyses of the sequencing data were based on relative abundances of reads. These were obtained by dividing the number of reads assigned to a gene, COG functional category or bacterial family, by the total number of bacterial reads per sample, and multiplying by 100.

Statistical analyses and data visualization were conducted using R (version 3.4.4) [66]. Effects of soil substrate, incubation time, and their possible interaction were determined according to Field et al. [67]. Briefly, significant differences were detected by a robust 2-way independent analysis of variance (ANOVA) based on the median as M-estimator, with 2000 bootstrap samples. For this purpose, the pbad2way function from the WRS package [68] was used. The influence was counted as significant if the p value was below 5% ($p < 0.05$). The Benjamini-Hochberg procedure was used to control the false discovery rate in data derived from the metagenomic datasets. Omega squared (ω^2) was calculated as an effect size to estimate the magnitude of observed influences of the analyzed factors. It can be interpreted as the percentage of variation in the dependent variable explained by the independent variable [69].

To detect global differences between samples, principal coordinate analysis (PCoA) ordinations of Bray-Curtis dissimilarity matrices were created using the PCoA function from the ape package [70]. Corrections for negative eigenvalues were performed by means of the Cailliez procedure. Bray-Curtis distances were calculated as an appropriate measure for community abundance data [71] using the vegdist function from the vegan package [72].

Spearman's rank correlation coefficient was used to identify whether the relative abundances of bacterial families and their functional genes were correlated. For this purpose, the function cor.test was used. The correlation was considered to be significant if $p < 0.05$. The average Rho was calculated based on absolute values.

Results

Initial Soil Substrate Parameters

Bulk soils collected from both sites had similar low contents of DOC and DON. DOC values were in the range of 4.57 ± 1.67 μg/g in samples collected from Chicken Creek, and 6.63 ± 0.46 μg/g in those from Lieberose, while DON was below detection limit in samples from both sites. Conversely, pH values differed between soils from both locations. Soil from Chicken Creek was slightly alkaline (7.31 ± 0.30), whereas soil from Lieberose was rather acidic (pH = 5.42 ± 0.39). Initial soil substrate parameters are presented in Table 2.

Biocrust Development

Biocrusts that developed in the microcosm experiment were in the initial stage of development. They consisted mostly of bacterial and algal biofilms, which enmeshed soil particles and formed patches on the soil surface. Mosses were also observed, but they did not form a dense surface cover yet. For biocrusts developed on the Chicken Creek soil, mosses and algae were already visible after the first 4 months of

Table 1 Proteins related to exo- and lipopolysaccharide production with corresponding KO numbers, HMM IDs, and genes

Protein	KO number	HMM ID	Gene
Polysaccharide export outer membrane protein Wza	K01991	PF02563	*wza*
Colanic acid biosynthesis acetyltransferase WcaB	K03819	TIGR04016	*wcaB*
Colanic acid biosynthesis acetyltransferase WcaF	K03818	TIGR04008	*wcaF*
Colanic acid/amylovoran biosynthesis pyruvyl transferase WcaK/AmsJ	K16710	TIGR04006	*wcaK/amsJ*
Capsular polysaccharide export system permease KpsE	K10107	TIGR01010	*kpsE*
Alginate biosynthesis acetyltransferase AlgJ	K19295	PF16822	*algJ*
Levansucrase SacB	K00692	PF02435	*sacB*
Lipopolysaccharide transport system ATP-binding protein Wzt	K09691	PF14524	*wzt*
LptBFGC lipopolysaccharide export complex inner membrane protein LptC	K11719	TIGR04409, PF06835	*lptC*
LptBFGC lipopolysaccharide export complex permease LptF	K07091	TIGR04407	*lptF*
LptBFGC lipopolysaccharide export complex permease LptG	K11720	TIGR04408, PF03739	*lptG*

incubation (T1). For the Lieberose soil, mostly biofilms around single soil particles were visible at T1, whereas mosses and distinct biocrust structures appeared after 10 months of incubation (T2). Representative pictures are presented in Supplementary Material 1 Fig. S4.

ANOVA revealed a significant influence of incubation time on DOC ($p < 0.001$, $\omega^2 = 0.70$) and DON ($p < 0.001$, $\omega^2 = 0.81$). They accumulated over time and increased by one order of magnitude in biocrusts at the end of the experiment compared to the bulk soils at the beginning of the experiment. The water repellency index at T2 was comparable between biocrusts grown on soils taken from both locations. It amounted to 1.12 ± 0.15 for biocrusts originating from Chicken Creek, and 1.16 ± 0.25 for those from Lieberose. Biocrust parameters are summarized in Table 2.

The exemplary CT images (Supplementary Material 1 Fig. S5A-D) of biocrusts from T2 grown on soil from Chicken Creek showed a layer of smaller particles in the crust horizon compared to the underlying soil substrate. Positive Euler numbers (Supplementary Material 1 Fig. S5E) for both biocrusts and the underlying soil indicate more isolated pores than connections in the pore network. The connectivity of the pore space was lower for the biocrusts, especially when small pores (46 μm) were considered (Euler number of 8.6 mm^{-3}). In the underlying soil, the connectivity was the lowest for pore size class of 107 μm (Euler number of 4.6 mm^{-3}). The connectivity then increased towards larger pore sizes as indicated by decreasing Euler numbers.

Major Characteristics of the Shotgun Sequencing Libraries

Shotgun sequencing of 18 libraries made from bulk soils from the beginning of the experiment (T0) and biocrusts from the 4-month (T1) and 10-month (T2) samplings generated 18.3 Gbases of data in total. This corresponded to 59,710,640 filtered reads. The number of filtered reads per sample varied between 2.1 and 5.3 million. Mean lengths of sequences after trimming ranged from 120 to 250 bp. Details of the raw and filtered sequencing data are summarized in Supplementary Material 2 Table S1.

The coverage of the microbial diversity by the metagenomic datasets, which was calculated using Nonpareil, varied from 16.5 to 67.3% (Supplementary Material 1 Fig. S1). As expected, metagenomes from T0 (nonpareil diversity index of 19.24 ± 0.07) had higher coverage (41.9 ± 12.8%) compared with metagenomes obtained from T1 and T2 (nonpareil diversity index of 20.44 ± 0.31, coverage of 25.5 ± 6.0%).

Taxonomic Analysis

42.83% of all metagenomic reads were assigned to *Bacteria*, which could be further differentiated into 366 families. Only these reads were further analyzed, as the main focus of this study was on EPS and LPS producers of bacterial origin, and molecular data on other microbial polysaccharide producers in the employed databases is poor. The principal coordinate analysis (PCoA) ordination plot (Fig. 1(a)) showed that bacterial communities were highly similar at the family level in bulk soils, and underwent differentiation during the development of biocrusts. Dominant families were identified by selecting the five most abundant families from each location at each time point, and sorting them according to their relative abundance of all metagenomes. Relative abundances of the dominant families are shown in Supplementary Material 1 Fig. S6. As confirmed by ANOVA, the most characteristic families for T0 were *Burkholderiaceae*, *Comamonadaceae*, and

Table 2 DNA concentration, dissolved organic carbon (DOC) and nitrogen (DON), pH, and water repellency index values. The repellency index has no dimension

Location	Time	DNA [ng/g]	DOC [μg/g]	DON [μg/g]	pH	Repellency index
Chicken Creek	T0	1.14 ± 0.29	4.57 ± 1.67	bdl	7.31 ± 0.30	–
	T1	28.89 ± 8.58	36.50 ± 4.31	1.32 ± 0.36	–	–
	T2	30.95 ± 7.73	48.02 ± 18.06	1.14 ± 0.36	–	1.12 ± 0.15
Lieberose	T0	2.25 ± 1.04	6.63 ± 0.46	bdl	5.42 ± 0.39	–
	T1	5.79 ± 2.14	42.02 ± 6.97	0.83 ± 0.09	–	–
	T2	24.65 ± 4.63	81.03 ± 26.52	1.11 ± 0.15	–	1.16 ± 0.25

The en dash (–) signifies that the parameter was not measured for the respective samples; *bdl*, below detection limit

Moraxellaceae. *Flavobacteriaceae* were also highly abundant at T0, but showed additional differences between the two substrates, and had generally higher relative abundance in samples from Chicken Creek. Similarly, *Sphingomonadaceae* were typical for samples from Chicken Creek, but their relative abundance did not change significantly between the sampled time points. *Streptomycetaceae* had generally higher relative abundance in samples from Lieberose, and occurred mostly at T1 and T2. *Ktedonobacteraceae* and *Acidobacteriaceae* were typical at T1 and T2 for biocrusts grown on soil substrate from the sand dune near Lieberose. *Bradyrhizobiaceae* were also characteristic for biocrusts originating from Lieberose, but their abundance increased there only at T2. Cyanobacteria, including *Leptolyngbyaceae*, *Tolypothrichaceae*, and *Nostocaceae*, were most abundant in biocrusts grown on soil substrate from Chicken Creek at T1 and T2, while *Oscillatoriaceae* and *Microcoleaceae* dominated there at T1. Significance levels and ω^2 values are presented in Supplementary Material 2 Table S2. Overall, the relative abundances of 13 families were influenced only by location, 125 only by time, 63 by both factors, and 130 by interaction of both factors. The full list of impacted families can be taken from Supplementary Material 2 Table S3.

The results of the taxonomic analysis of the whole metagenomic datasets based on the NCBI-NR database were supported by the 16S rRNA gene annotations with SILVA. Although only 0.0062% of all metagenomic reads were assigned to the bacterial 16S rRNA gene, bacterial community composition did not differ when data from the analysis of the complete metagenomics datasets was compared with the phylogenetic analysis of subsampled 16S rRNA fragments (data not shown).

Functional Annotation of Metagenomic Datasets

General function prediction in the metagenomic datasets was performed by means of the eggNOG database. In total, 73.08% of bacterial reads were assigned to COG functional categories. The "function unknown" category was most abundant (~ 20%), followed by "replication, recombination and repair" as well as "amino acid transport and metabolism" (each ~ 6%). Relatively low abundant (< 0.5%), but with special importance to the initiation of biocrust formation, were the "cell motility" and "extracellular structures" categories. ANOVA showed that these two categories were more abundant in bulk soils compared with biocrusts. COG functional classification is presented in Supplementary Material 1 Fig. S7, and significance levels and ω^2 values are listed in Supplementary Material 2 Table S4.

Genes specific for the biosynthesis and excretion of alginate, colonic acid, levan, and other EPSs as well as LPSs, which were identified using an approach combining HMM searches with blasts against sequences derived from the KEGG database, comprised 0.018% of bacterial reads (Fig. 2). Key genes, with the overall relative abundance in all metagenomes in the range between 0.002% and 0.005%, were *wza*, *wcaB*, and *wcaF* of the Wzy-dependent EPS synthesis pathway, and *lptF* and *lptG* of the LPS synthesis pathway. Moderate abundant (≥ 0.001%) were *kpsE* of the ABC transporter-dependent EPS synthesis pathway and *wzt* of the LPS synthesis pathway. Genes *wcaK*/*amsJ*, *algJ*, *sacB*, and *lptC* were the least abundant (≤ 0.0003%).

ANOVA revealed that the relative abundances of most investigated genes changed mainly between T0 and T1. However, the differences in the relative numbers of gene copies were also driven by the underlying soil substrate (Supplementary Material 2 Table S5). In particular, the genes *wza* and *wcaF* increased at T1, and the increase was more pronounced in samples originating from Chicken Creek compared with that in those from Lieberose. Moreover, *wzt* increased in biocrusts grown on soil substrate from Chicken Creek already at T1, while the increase was observed in biocrusts grown on bulk soil taken from Lieberose only at T2. Conversely, *kpsE* and *lptC* decreased at T1. Additionally, *kpsE* was relatively more abundant in samples from Lieberose, whereas *lptG* was dominating in samples from Chicken Creek. Finally, *wcaK*/amsJ, *algJ*, *sacB*, and *lptF* were not significantly affected by either incubation time or soil substrate.

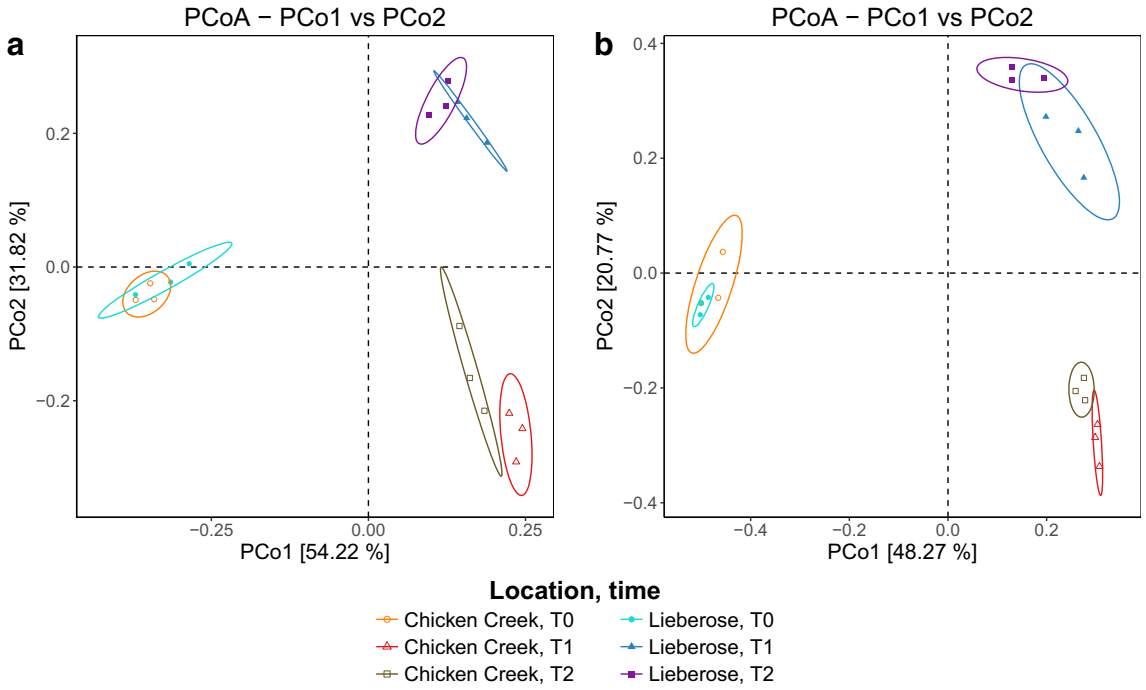

Fig. 1 PCoA plots depicting differences on the family level in (a) bacterial community composition and (b) taxonomic affiliation of genes related to EPS and LPS formation. Ellipses drawn around triplicates represent a 95% confidence level

Investigation of Potential EPS/LPS Producers

The investigated genes were found in 210 different bacterial families, of which 11 families were found harboring the genes in samples originating from both locations, taken at all three sampling time points (Supplementary Material 1 Fig. S8). The number of families harboring genes related to EPS and LPS formation was higher at T1 and T2 compared with T0 (Fig. 3). At T0, the investigated genes were associated with 33 families in bulk soil from Chicken Creek, and in 34 in bulk soil from Lieberose. These numbers increased at T1 to 150 families in samples originating from Chicken Creek, and 100 in samples from Lieberose. At T2, 146 families harbored the investigated genes in samples from Chicken Creek, and 87 in samples from Lieberose.

Taxonomy of bacteria potentially capable of synthesis and excretion of EPSs and LPSs is presented in Fig. 3 at the level of phylum or class (in case of *Proteobacteria*). At T0, the investigated genes were harbored mainly by *Betaproteobacteria*, whereas at T1 and T2, the major potential producers of EPSs and LPSs, were members of *Cyanobacteria*, *Alphaproteobacteria*, and *Chloroflexi*. Interestingly, differences were also found in the diversity pattern of potential EPS and LPS producers in response to the different soil substrates. In particular, *Cyanobacteria* were typical for biocrusts grown on soil taken from Chicken Creek, while *Chloroflexi* and *Acidobacteria* were characteristic for biocrusts originating from Lieberose.

ANOVA identified a significant impact on the overall relative abundance of the investigated genes caused by location alone in three families, by time alone in 14 families, and by interaction of both factors in 23 families. The full list of affected families can be taken from Supplementary Material 2 Table S3. The PCoA plot (Fig. 1(b)) indicated that the distribution pattern of the analyzed genes among bacterial families resembled that of the total bacterial community (Fig. 1(a)). In fact, Spearman's rank correlation analysis revealed a positive correlation between the total abundance of a given family and the amount of sequences related to EPS and LPS formation harbored by that family for 57 families of potential EPS and LPS producers (average Rho = 0.69, minimum Rho = 0.47, maximum Rho = 0.97). Three families showed a negative correlation (average Rho = 0.56, minimum Rho = 0.49, maximum Rho = 0.61), and 150 exhibited no correlation (average Rho = 0.23, minimum Rho = 0.00, maximum Rho = 0.46).

Of the 57 families that showed a positive correlation, 25 exceeded an abundance of 1%, and encompassed altogether 43.26% of all bacterial reads. Both the relative abundance as well as the potential for EPS and LPS synthesis and export of these families were strongly influenced by both incubation time and underlying soil substrate (Fig. 4). In fact, these factors selected the key producers of EPSs and LPSs already at the phylum level. *Betaproteobacteria* (especially *Burkholderiaceae*), as well as *Gammaproteobacteria* (*Moraxellaceae*) and *Bacteroidetes* (*Flavobacteriaceae*), were prevalent at T0, although most of their members were found also at T1 and T2. *Deltaproteobacteria*

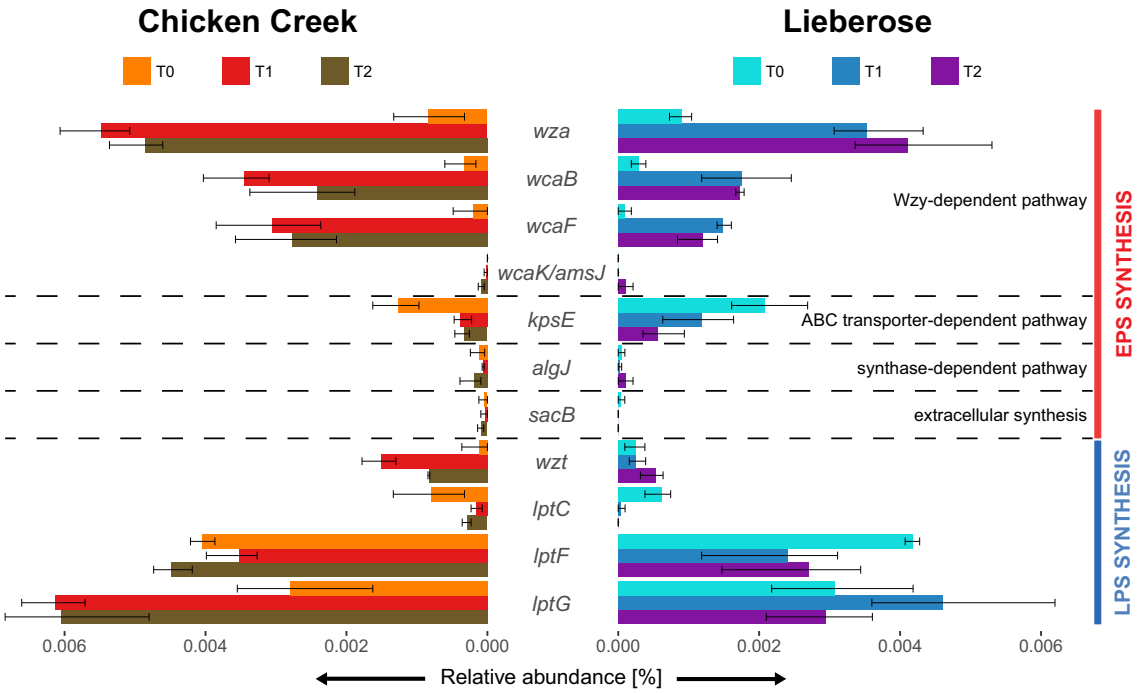

Fig. 2 Relative abundances of genes specific for the formation of EPSs and LPSs. Error bars show standard deviations of triplicates

(*Myxococcaceae* and *Archangiaceae*) and *Planctomycetes* (*Gemmataceae* and *Planctomycetaceae*) occurred mainly at T1 and T2 in biocrusts grown on soil taken from Chicken Creek. However, *Gemmataceae* were relatively abundant also at T2 in biocrusts originating from Lieberose. *Cyanobacteria* were characteristic for Chicken Creek samples from T1 and T2, but some of their members (*Oscillatoriaceae* and *Leptolyngbyaceae*) could also be important for EPS and LPS production in Lieberose samples from T1 and T2. Typical for Lieberose samples from T1 and T2 were *Chloroflexi* (*Ktedonobacteraceae* and *Thermogemmatisporaceae*) and *Acidobacteria* (*Acidobacteriaceae*). *Alphaproteobacteria* were prevalent at T1 and T2 in general, but some of their members were more characteristic for one of the underlying substrates (e.g., *Sphingomonadaceae* for soil from Chicken Creek, and *Acetobacteraceae* for that from Lieberose).

Discussion

Bacterial Communities of Initial Soils

In the present study, initial biocrusts developed from indigenous communities of free-living microbes, which were highly similar in bulk soils from both sites. As carbon and nitrogen availability are one of the most important factors shaping bacterial community structure [73, 74], their low concentrations could be the primary influence selecting only the best-adapted bacteria in nutrient-poor habitats such as the Chicken Creek catchment and the Lieberose sand dune. In fact, the most abundant bacterial families in the bulk soils from our study were *Burkholderiaceae*, *Comamonadaceae*, *Moraxellaceae*, and *Flavobacteriaceae*. These families exhibit oligotrophic traits, as their metabolic versatility and ability to degrade a wide range of compounds, such as various polymers, polycyclic aromatic compounds, phenols, and halogenated aromatics, enable them to thrive even in environments with limited nutritional opportunities [75–78]. Consequently, these groups were isolated from habitats such as crude oil, desert soil, glacier ice, or distilled water. Furthermore, many members of these families possess fimbriae and exhibit motility. This is in line with the higher amount of corresponding reads found in the bulk soils compared with the initial biocrusts. These traits are especially important for free-living bacteria, as they assist in the first steps of cell attachment to a surface and establishment of biofilms [79]. In contrast, genes involved in the formation of EPSs and LPSs, which are particularly relevant in the later stages of biofilm development, were generally more abundant in the initial biocrusts compared with the bulk soils.

Influence of Initial Biocrusts on Soil Stability and Hydrological Properties

EPSs and LPSs have protective functions, bind and mediate penetration of micronutrients into the cell, and function in cell-to-surface and cell-to-cell interactions, which are critical for biofilm development [16, 80, 81]. The prevalence of genes related to EPS and LPS synthesis and export in initial biocrusts was therefore expected. EPSs and LPSs also play

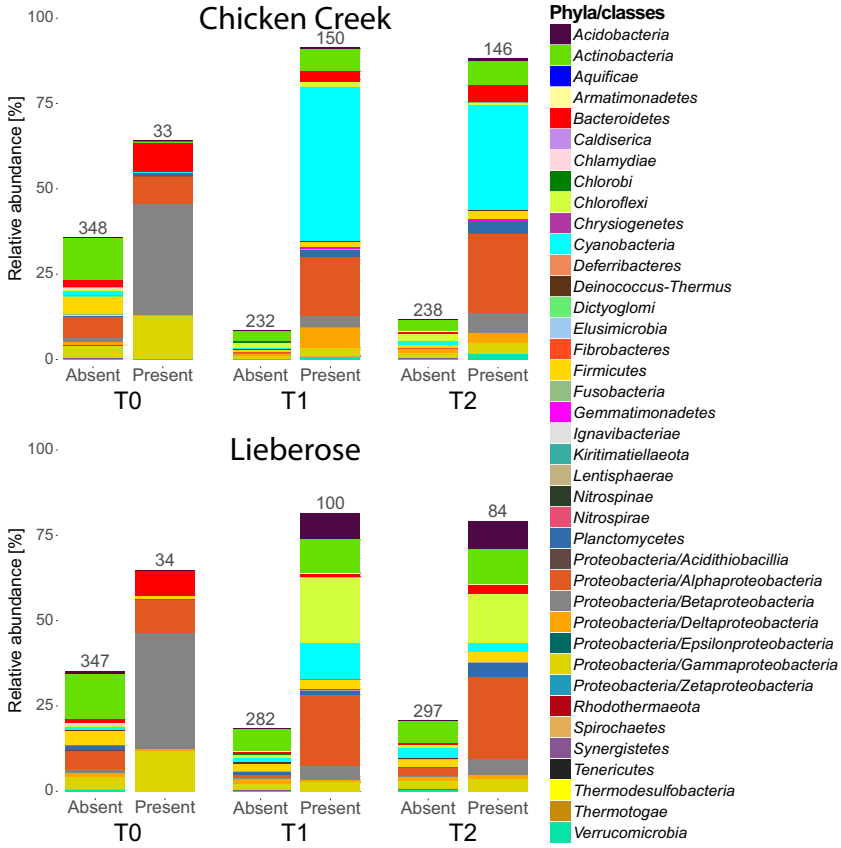

Fig. 3 Comparison of relative abundances of bacteria with and without the potential for EPS and LPS formation (labeled as "Present" and "Absent"). The distinction between the potential producers and non-producers was performed on the level of family. The families were then pooled according to their respective phyla or, in case of *Proteobacteria*, classes. Values above bars represent total numbers of displayed families

an important role in improving soil stability, especially in initial biocrusts that harbor large amounts of bacteria, like in our study. Bacterial polysaccharides adhere around soil particles, connecting them and cementing into larger aggregates [82]. Several studies demonstrated that bacterial polysaccharides increased the amount of stable soil aggregates [83–85] and reduced rainfall-induced erosion up to 98% [35]. Using the exemplary XCMT images of the 10-month-old samples from Chicken Creek, we also confirmed the ability of initial biocrusts to trap surface soil particles. Similar activity of cyanobacterial crusts was captured on XCMT images, for example, by Raanan et al. [86]. Moreover, the increase of the potential for EPS and LPS formation in the initial biocrusts compared with the bulk soils was correlated in our study with an accumulation of dissolved organic carbon (data not shown). Altogether, these point to an increased production of adhesive bacterial polysaccharides in our biocrusts.

Additionally, we measured the influence of biocrusts on soil hydrological properties, as the key role in altering soil moisture dynamics seems to be played by polysaccharides [1]. On one hand, they tend to clog pores through swelling, which may reduce soil infiltrability [32, 34, 49, 87]. On the other hand, they can increase soil porosity, which is known to positively affect water penetration [88, 89]. Some researchers also postulate that polysaccharides alter the hydrophobicity of biocrust surfaces [90]. In our study, the water repellency of biocrusts incubated for 10 months on both substrates was close to ideal wettability. Similar water repellency was reported for very young biocrusts also in other studies [49, 91]. In initial biocrusts, the effect on hydrological processes highly depends on the transient amount and chemical nature of polysaccharides building the bacterial biofilms [92]. For example, water molecules as well as nutrients are bound mainly by the hydrophilic polysaccharide fractions, while the hydrophobic fractions increase the stability of biocrusts and their ability to adhere to solid surfaces [93]. Furthermore, polysaccharides in bacterial biofilms are subjected to constant modification and degradation processes, both enzymatic and abiotic [4]. Colica et al. [94] underlined that polysaccharide content cannot be directly correlated with biocrust age, as the transient amount of polysaccharides in biocrusts depends on the activity of both polysaccharide producers as well as chemoheterotrophic organisms that use polymeric carbohydrates as a carbon source. Thus, the hydrological properties of biocrusts are highly dynamic and may fluctuate during biocrust development, as shown previously [49]. Comparing the structure of bacterial communities in biocrusts with the composition and chemical properties of bacterial polysaccharides throughout the whole development of biocrusts would surely shed more light on this issue. However, more research on the methods of extracting

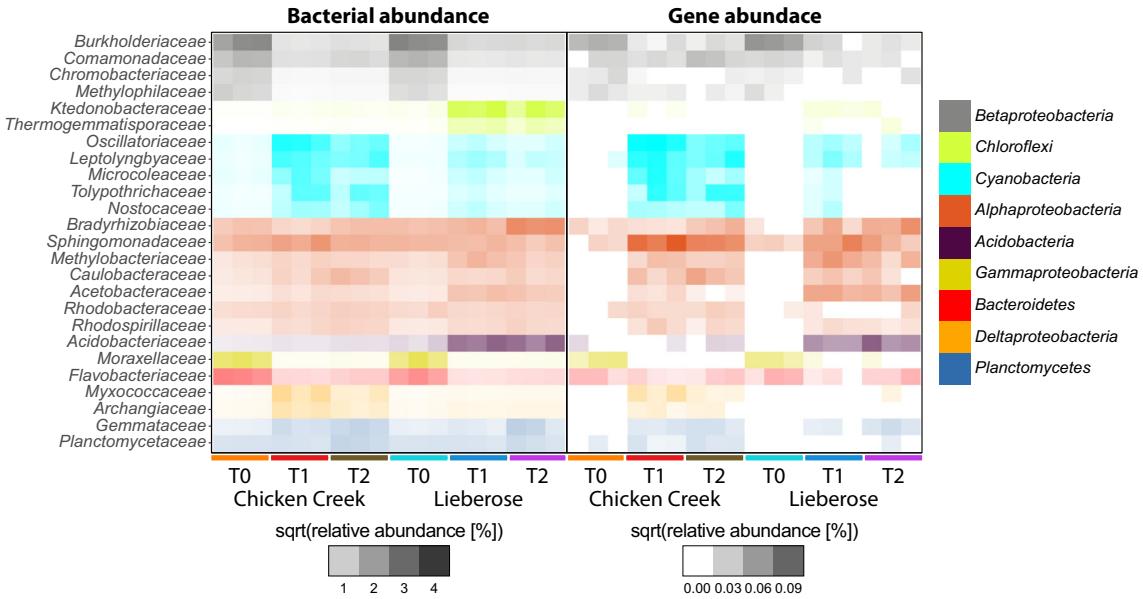

Fig. 4 Potential key families of EPS and LPS formation based on their relative abundance and the relative abundance of their genes related to EPS and LPS biosynthesis. Note the different color intensities between bacterial and gene abundances. The replicates (n = 3) are presented in separate columns

bacterial polysaccharides from biocrust needs to be done before such measurements will be reliable and give additional information compared to the repellency index [4, 95].

Genes Related to EPS and LPS Formation

Although the total relative abundance of genes involved in the formation of EPSs and LPSs increased in the initial biocrusts compared with the bulk soils, the individual genes showed different responses. Especially abundant and showing the strongest increase were genes from the Wzy-dependent EPS synthesis pathway and the LPS synthesis pathway. Most bacterial reads in our study belonged to phyla well-known for LPS production, such as *Proteobacteria* (40%), *Cyanobacteria* (20%), and *Bacteroidetes* (5%) [96]. Moreover, recent evidence shows that LPS producers can be found even in phyla that are commonly considered as lacking LPSs [97, 98]. Therefore, the relatively high abundance of genes from the LPS synthesis pathway in our study was expected. Similarly, the high relative abundance of genes from the Wzy-dependent pathway was expected, as it is the most widely distributed mechanism of EPS assembly and export [99, 100]. In particular, the *wza* gene encodes for an outer membrane protein Wza, which participates in the translocation across the outer membrane of a variety of EPSs in many different taxa [27]. In comparison, genes belonging to the other pathways of EPS assembly and export (ABC-dependent and synthase-dependent), as well as to the extracellular EPS synthesis, were less abundant in our metagenomes. However, these genes are found only in a very limited number of bacteria [101–103].

In contrast to the other investigated genes, the relative abundances of the *kpsE* gene, which is part of the ABC-dependent EPS synthesis pathway, and the *lptC* gene of the LPS synthesis pathway decreased in the biocrusts compared with the bulk soils. The gene *kpsE* is associated with the synthesis of capsular polysaccharides, which enhance survival of bacterial cells in harsh environments [104]. This could explain the high relative abundance of *kpsE* in the low-nutrient bulk soils of the Chicken Creek catchment and the Lieberose sand dune. The LptC protein is part of the LptBFGC LPS export complex together with LptF and LptG. However, unlike LptF and LptG, LptC is not well-conserved among Gram-negative bacteria [105, 106] and may not even be essential for LPS formation [107].

The differences in the relative abundances of genes associated with EPS and LPS formation were observed mainly between the bulk soils and the biocrusts. Conversely, very few differences in the relative abundances of the investigated genes were found between samples originating from Chicken Creek and Lieberose.

Differentiation of Potential Key Producers of EPSs and LPSs During Initial Development of Biocrusts on Different Soil Substrates

Even though the soil substrate had little impact on the relative abundance of the investigated genes, it shaped the composition of bacterial communities in the developing biocrusts. In fact, bacterial communities that were highly similar in the bulk soils underwent differentiation once biocrusts started to develop. Furthermore, the taxonomic affiliation of the investigated

genes reflected the overall composition of the bacterial communities in our study, and thus the differentiation of the overall bacterial communities was accompanied by the differentiation of the communities of potential producers of adhesive polysaccharides. This is in line with the theory about functional redundancy, which states that important functions are preserved by a community even if the community changes its composition [108]. Our results indicate that the potential to form EPSs and LPSs is an important trait for initial biocrusts, as it is maintained despite the different development of bacterial communities on the two investigated substrates.

The importance of the potential to produce "soil glue" in the initial stage of biocrust development is further underlined by the fact that the highest numbers of sequences related to EPS and LPS biosynthesis were harbored by the families dominating the initial biocrusts. The potential key producers of adhesive polysaccharides found in biocrusts grown on soil from the Chicken Creek catchment and the Lieberose sand dune were distinct already at the phylum level. In the Chicken Creek biocrusts, the most abundant potential producers of EPSs and LPSs were *Cyanobacteria*. They are well known for their capability to form external polysaccharidic layers that enable them to survive in extreme environments [13]. In fact, the genetic machinery of the LPS synthesis and the Wzy-dependent pathway of the EPS synthesis were both found in *Cyanobacteria* before [27]. This explains the dominance of these particular polysaccharide biosynthesis pathways in the metagenomes from the Chicken Creek biocrusts. However, *Cyanobacteria* played only a minor role in the community of potential EPS and LPS producers in the Lieberose biocrusts, possibly because they prefer alkaline environments [1]. In the biocrusts grown on the soil from Lieberose, *Cyanobacteria* were replaced by *Chloroflexi* and *Acidobacteria*, which favor acidic habitats [109–113]. While *Chloroflexi* lack the ability to synthesize LPSs, *Acidobacteria* are known LPS producers [96]. Furthermore, even though the information on the proficiency of both phyla in EPS formation is still limited, sequences related to EPS synthesis were previously found in *Acidobacteria*, and a recent report suggests that some members of this phylum produce large amount of EPSs [114]. *Acidobacteria* and *Chloroflexi* are also common members of communities that embed themselves in an EPS matrix, such as biofilms, microbial mats, and biocrusts [115–117]. The low relative abundance of *Cyanobacteria* in biocrusts grown on the soil from Lieberose suggests that, besides *Chloroflexi*, the other major phototrophic organisms there could have been algae, which are also well-known producers of EPSs. Algae dominate acidic soils and are major components of the natural biocrusts found at Lieberose, except for the terminal successional stage that is dominated by mosses and fungi [1, 44, 45, 49]. However, the identification of eukaryotes involved in polysaccharide production is difficult using short-read shotgun sequencing and would require a different approach [118, 119]. In any case, our results show that potential producers of EPSs and LPSs dominate bacterial communities of biocrusts during the initial stage of biocrust development. Consequently, the differentiation of overall bacterial communities leads to the emergence of distinct potential key producers of "soil glue."

The differentiation of bacterial communities in our study could have been on one hand triggered by soil properties. For example, the two soil substrates used in our study differed in pH, which is one of the most important edaphic parameters determining the composition of bacterial communities in soil [120], but usually signifies that other edaphic parameters (e.g., micronutrient availability) also differ [121]. Therefore, the experimental design of the current study prevents us from making any definite conclusions on the influence of edaphic parameters on the community structure of potential "soil glue" producers. On the other hand, the observed differentiation of bacterial communities could have resulted from various rare species that were too low abundant to detect in the bulk soils, and started dominating during the initial development of biocrusts. To identify the main drivers shaping the community composition of potential producers of EPSs and LPSs in initial biocrusts, future experiments should involve multiple sterile soil substrates with diverse edaphic parameters, inoculated with the same initial bacterial community.

Conclusions

Our study indicates that the potential to produce EPSs and LPSs is an important trait for bacterial communities forming biocrusts in the initial stage of biocrust development, as (i) the relative abundance of genes related to the biosynthesis of adhesive polysaccharides increases in the bacterial communities of initial biocrusts compared with the indigenous bacterial communities of bulk soils, (ii) the relative abundances of EPS and LPS genes remain similar in initial biocrusts with different compositions of bacterial communities, and (iii) the highest numbers of sequences related to the "soil glue" production is found in families dominating initial biocrusts. Furthermore, we demonstrate that the community composition of potential producers of EPSs and LPSs reflects the overall structure of bacterial communities in initial biocrusts, and thus, initial biocrusts with different bacterial community compositions harbor distinct potential key producers of adhesive polysaccharides. Whether the ability of biocrusts to improve soil development in the long term is compromised by differences in the efficiency of polysaccharide formation, or the adhesive properties of EPSs and LPSs produced by different taxa, needs further investigation. Similarly, whether the differentiation of bacterial communities during the initial development of biocrusts is primarily triggered by soil

properties, or results from various rare species present in the initial bacterial community of bulk soil, remains to be determined.

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Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare that they have no conflict of interest.

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