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Running Title of even pages: M. HERNÁNDEZ *et al*.

Running Title of odd pages: MICROBIAL COMMUNITIES IN VOLCANIC SOILS

**Analysis of the Microbial Communities in Soils of Different Ages Following Volcanic Eruptions**

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ABSTRACT

Volcanism is a primary process of land formation. It provides a model for understanding soil-forming processes and the role of pioneer bacteria and/or archaea as early colonizers in those new environments. The objective of this study was to identify the microbial communities involved in soil formation. DNA was extracted from soil samples from the Llaima volcano in Chile at sites destroyed by lava in different centuries (1640, 1751, and 1957). Bacterial and archaeal 16S rRNA genes were analyzed using quantitative polymerase chain reaction (qPCR) and Illumina MiSeq sequencing. Results showed that microbial diversity increased with soil age, particularly between the 1751 and 1640 soils. For archaeal communities, Thaumarchaeota was detected in similar abundances in all soils, but Euryarchaeota was rare in the older soils. The analysis of bacterial 16S rRNA genes showed high abundances of Chloroflexi (37%), Planctomycetes (18%), and Verrucomicrobia (10%) in the youngest soil. Proteobacteria and Acidobacteria were highly abundant in the older soils (16% in 1640 and 15% in 1751 for Acidobacteria; 38% in 1640 and 27% in 1751 for Proteobacteria). The microbial profiles in the youngest soils were unusual, with a high abundance of bacteria belonging to the order Ktedonobacterales (Chloroflexi) in the 1957 soil (37%) compared with the 1751 (18%) and 1640 soils (7%). In this study, we show that there is a gradual establishment of the microbial community in volcanic soils following an eruption and that specific microbial groups can colonize during the early stages of recovery.

*Key Words*:16S rRNA gene, extreme environment, high-throughput sequencing, Ktedonobacterales, microbial diversity, pioneer microbe, recolonization, soil formation, volcanic deposit

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INTRODUCTION

Volcanic eruptions provide a model for understanding soil-forming processes, including microbiological components. After lava solidifies, microbes begin colonizing the rock surface, presumably by photosynthesis or from the chemical energy in reduced gases such as methane (CH4), hydrogen sulfide (H2S), hydrogen (H2), and carbon monoxide (CO). A number of studies have shown that microorganisms are among the first colonizers on volcanic deposits, contributing to early ecosystem development (King, 2003a; Ohta *et al*., 2003; Sato *et al*., 2004; Gomez-Alvarez *et al*., 2007; Fujimura *et al*., 2012; Guo *et al*., 2014; Kim *et al*., 2018). As volcanic environments are widely distributed on Earth (Cockell *et al*., 2011), it is important to understand the microbial communities that play important roles in colonizing these extreme environments. Volcanic environments can be used as models to understand the pioneer colonizing species.

The pioneer microbes colonizing early volcanic deposits include those able to fix nitrogen (N) and carbon (C) from the atmosphere and contribute to the organic matter addition into the deposits (Crews *et al*., 2000; Kurina and Vitousek, 2001; Dunfield and King, 2004; Sato *et al*., 2006; King and Weber, 2008; King *et al*., 2008; Sato *et al*., 2009; Weber and King, 2010b, 2017; King and King, 2014). Previous studies have shown that the class Ktedonobacteria, which belongs to the phylum Chloroflexi, is predominant in non-vegetated volcanic soils (Gomez-Alvarez *et al*., 2007; Weber and King, 2010b), while Proteobacteria dominate vegetated sites (Weber and King, 2010b). Some of these Ktdenobacteria, among other microbial groups, have been associated with CO and H2 consumption in both young, organic matter-poor volcanic deposits, and older (more mature) deposits with well-developed plant communities (King, 2003a; King and Weber, 2008; Weber and King, 2010b).

The colonization of volcanic deposits by plants also plays an important role in soil formation, as their roots enhance rock weathering and plant material contributes to soil organic matter accumulation. Some studies have shown that relatively young volcanic deposits harbor distinct microbial communities when plants are present (Ohta *et al*., 2003; Dunfield and King, 2004; Guo *et al*., 2014). The interaction, and potential co-dependence, of plants and microbes in volcanic soils has not been specifically investigated, but plant diversity is known to affect microbial diversity, function, and soil carbon accumulation (Leff *et al*., 2018). The soil community, including bacteria, fungi, and invertebrates, is also important for the establishment and growth of plants (Philippot *et al*., 2013; Lange *et al*., 2015; Wubs *et al*., 2016). These types of interactions have been demonstrated in some mature soils but have not been investigated in the early stages of soil formation, for example in volcanic soils.

The Llaima volcano is one of the largest and most active volcanoes in Chile. It has had three detailed lava eruptions at different sites of the volcano and plants have colonized these zones. The 1640 and 1751 lava flow sites are now mostly colonized by understory. In contrast, only 5% of the soil sites from the 1957 eruption have been colonized by lichens and consist of soil in the early stage of formation (Fig. S1) (Hernández *et al*., 2014). These sites have very low levels of organic matter (especially sites 1640 and 1751), C/N ratios, and phosphorus (P) (Hernández *et al*., 2014).

Previously, we sampled soils from the Llaima volcano at sites destroyed by lava in different centuries (1640, 1751, and 1957). We hypothesized that autotrophic microorganisms were important in soil formation and indeed found large numbers and high activities of autotrophic ammonia-oxidizing bacteria and archaea in those soils, the values being particularly high in young soils (Hernández *et al*., 2014). In the present study, we aim to evaluate the structure of the total microbial community composition at these three sites with different defined age and soil characteristics, to obtain information regarding recolonization by bacteria and archaea after a lava event.

MATERIAL AND METHODS

*DNA extraction and quantitative polymerase chain reaction (qPCR)*

DNA was extracted from soil samples from the Llaima volcano (38.697407° S, 71.730445° W) in Chile. Sampling and DNA extraction have been previously described (Hernández *et al*., 2014). Briefly, samples were taken from three sites according to lava eruptions (Naranjo and Moreno, 2005), named 1640, 1751, and 1957 (Fig. S1), in February 2011. Samples were taken using a triangle pattern (Gomez-Alvarez *et al*., 2007) and triplicates were taken at each point, with a total of nine replicates per site. All soil samples were stored in polyethylene bags at 4 °C until their transport to Germany. The abundance of bacterial and archaeal 16S rRNA genes was determined by qPCR using an iCycler instrument (BioRad, Germanycountry here please]). For all assays, standards containing a known number of DNA copies of the target gene were used. The qPCR conditions were based on dual-labeled probes described by Yu *et al*. (2005). Primer sets Bac338F/Bac805R/Bac516P and Arc787F/Arc1059R/Arc915P were used for bacterial- and archaeal-16S rRNA genes, respectively. The conditions for both runs were as follows: 0.5 μmol L-1 of each primer, 0.2 μmol L-1 of the dual-labeled probe, 3 µL template, 4 mmol L-1 MgCl2 (Sigma-Aldrich, Germanycountry here please]), and 12.5 µL of JumpStart Ready Mix (Sigma-Aldrich, Germanycountry here please]). Also, 1 µL bovine serum albumin (BSA) (0.8 µg µL-1) was added to archaeal 16S rRNA gene reactions. The program used for both assays: 94 °C for 5 min, 35 cycles of 95 °C for 30 s and 62 °C for 60 s, extension, and signal reading. Efficiencies of 91.2% and *R*2 of 0.994 for bacterial 16S rRNA genes and 93.1% and *R*2 of 0.994 for archaeal 16S rRNA genes were obtained.

*Illumina library preparation and sequencing*

MySeq Illumina sequencing was performed for the total bacterial 16S rRNA gene. The PCR primers 515F/806R were used (Bates *et al*., 2011). The PCR conditions for bacterial 16S rRNA gene amplification were as follows: 94 °C for 5 min, followed by 28 cycles of 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 30 s, and a final elongation at 68 °C for 10 min (Hernández *et al*., 2015). Individual PCRs contained a 6-bp molecular barcode integrated into the forward primer. Amplicons were purified using a PCR cleanup kit (Sigma-Aldrich, Germany[country here please]) and quantified using a Qubit 2.0 fluorometer (Invitrogen, Germanycountry here please]). Equimolar concentrations of the samples were pooled and sequenced on MiSeq using a 2 × 300 bp paired-end protocol. Library preparation and sequencing was performed at the Max Planck Genome Centre, Cologne, Germany.

*Bioinformatics and data processing*

Quality filtering and trimming forward and reverse adaptors from the sequences were carried out using cutadapt (Martin, 2011). Forward and reverse reads were merged using the usearch fastq\_mergepairs command (Edgar, 2013). The downstream processing was performed using UPARSE (Edgar, 2013) and UCHIME pipelines (Edgar *et al*., 2011), following the steps detailed in Reim *et al*. (2017).

*Taxonomy analysis and data accession*

A representative sequence of each operational taxonomic unit (OTU) was classified with the naïve Bayesian classifier using the SILVA-132 16S rRNA gene database (bootstrap confidence threshold of 80%) in mothur (Schloss *et al*., 2009). Sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number PRJNA496066.

*Statistical analyses*

Multivariate statistics for bacterial 16S rRNA Illumina data was mostly performed with phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen *et al*., 2018) packages in R software version 3.5.1. Alpha diversity indexes were determined using the estimate\_richness command in the phyloseq package. Evenness indexes were determined using the vegan package. The relative abundance was calculated by removing taxa not seen more than three times in at least 20% of the samples by using the filter\_taxa command, and figures were constructed using the ggplot2 package (Wickham, 2016). For beta diversity analyses, non-metric multidimensional scaling (NMDS) analysis was carried out using the decostand function for the ordination of Hellinger distances in the vegan package. Heatmaps were constructed with the gplots (Warnes *et al*., 2016), vegetarian (Charney and Record, 2012), and vegan (Oksanen *et al*., 2018) packages. Principal components analysis (PCA) of the Hellinger transformed data was performed using the prcomp function. The OTUs explaining most of the differences between samples were defined as the 20 OTUs contributing to the largest absolute loadings in the first and second dimensions of the PCA, obtained from the rotation output file (Hernández *et al*., 2017). Hierarchical clustering of the distance matrix used the “ward.D2” method and was performed with the hclust function. The heatmap was constructed using the heatmap.2 function in the gplots package.

RESULTS

*Microbial abundance*

The abundance of bacterial 16S rRNA genes was higher than that of archaeal 16S rRNA genes (Fig. 1). Copy numbers of bacterial 16S rRNA genes were in the range of 108 copies per gram of soil for all three sites, and no significant differences were found between the sites (*P* = 0.184). Copy numbers of archaeal 16S rRNA genes were in the range of 106 copies per gram of soil in site 1957 and 105 copies per gram of soil in the sites 1640 and 1751, and no significant differences were found between the sites (*P* = 0.479).

Fig. 1

Fig. 1 Copy numbers of bacterial and archaeal 16S rRNA genes in three soils (1640, 1751, and 1957) sampled from the Llaima volcano in Chile at three sites according to lava eruptions in different centuries. The error bars are standard deviations (*n* = 9).

*Diversity of bacterial communities*

Overall, significant differences were found between soils for all the diversity indexes, *i.e.*, observed species, and Shannon and evenness indexes (*P* < 0.01). Tukey post hoc tests indicated that the youngest soil (1957) is significantly different when compared with the other soils (Table S1). On average, the observed species were 7 575 OTUs for site 1640, 7 116 for site 1751, and 4 593 for site 1957 (Fig. S2a and Table S1). The Shannon diversity indexes were between 5.21 (youngest soil: 1957) and 6.99 (older soils: 1640 and 1751) (Fig. S2b and Table S1). Species evenness had the same behavior as the Shannon indexes, being higher in the older sites (0.71 in site 1640 and 0.76 in site 1751) than in the newest site 1957 (0.62) (Fig. S2c and Table S1).

For beta diversity, NMDS analysis showed that the composition of the bacterial 16S rRNA genes changed across soil age (*P* < 0.05) (Fig. 2). The youngest soils (1957) clustered separately from the two older soils (1640 and 1751) (Fig. 2).

Fig. 2

Fig. 2 Non-metric multidimensional scaling (NMDS) analysis of bacterial community structure in the three soils (1640, 1751, and 1957) sampled from the Llaima volcano in Chile at three sites according to lava eruptions in different centuries.

*Diversity of archaeal communities*

Significant differences were found only for observed species (*P* < 0.01) between soils. Tukey post hoc tests indicated that the youngest soil (1957) was significantly different when compared with the other soils (Table S2). On average, the observed species were 17 OTUs for site 1640, 15 for site 1751, and 5 for site 1957. The Shannon and evenness diversities did not change significantly between soil ages (Table S2).

*Analysis of microbial communities*

The archaeal community encompassed mostly the phylum Thaumarchaeota in all the sites, with relative abundances of 82.6%, 95.8%, and 97.7% for the sites 1640, 1751, and 1957, respectively. Site 1640 had the highest abundance of the phylum Euryarchaeota (16.4%) (data not shown). Within the archaeal classes, Nitrososphaera and soil group 1.1c mainly dominated sites 1751 and 1957, but was also found in site 1640 (Fig. S3). A higher relative abundance of the class Thermoplasmata was found in site 1640 (12.6%) than in the other sites (2.4% in 1751 and 0.4% in 1957) (Fig. S3).

The bacterial community at the different sites included Acidobacteria, Actinobacteria, Chloroflexi, Planctomycetes, Proteobacteria, and Verrucomicrobia (Fig. 3). Site 1640 had a high relative abundance of Acidobacteria (16.2%), Actinobacteria (11.4%), Planctomycetes (12.4%), and Proteobacteria (38.5%). The relative abundances of the phyla Acidobacteria, Actinobacteria, and Planctomycetes were similar in the sites 1751 and 1640 (15.4%, 11.7%, and 13.2%, respectively).. For site 1751, high abundance of Chloroflexi (18.0%) and lower abundance of Proteobacteria (27.4%) were recorded when compared with site 1640. On the other hand, site 1957 had the highest relative abundance of the phyla Chloroflexi (37.8%), Planctomycetes (18.3%), and Verrucomicrobia (10.7%). Within the phylum Chloroflexi, abundance of the class Ktedonobacteria was higher in site 1957 (97.0%) than in the other sites (23.9% in 1640 and 55.9% in 1751) (Fig. 4).

Fig. 3

Fig. 3 Mean relative abundances of bacterial phyla based on bacterial 16S rRNA genes in the three soils (1640, 1751, and 1957) sampled from the Llaima volcano in Chile at three sites according to lava eruptions in different centuries. Taxa not seen more than three times in at least 20% of the samples were removed using the phyloseq package in R.

Fig. 4

Fig. 4 Mean relative abundances of classes from the phylum Chloroflexi in the three soils (1640, 1751, and 1957) sampled from the Llaima volcano in Chile at three sites according to lava eruptions in different centuries.. Taxa not seen more than three times in at least 20% of the samples were removed using the phyloseq package in R.

*Phylogenetic analysis of bacterial communities*

Clustering analyses show distinct differences in bacterial 16S rRNA genes among the sites, with fewer differences between the older soils (1640 and 1751) (Fig. 5). Many OTUs were highly abundant in the youngest soil (site 1957) and belong mostly to the family Ktedonobacteraceae in the phylum Chloroflexi (specially OTU-62, OTU-176, OTU-370, OTU-478, OTU-84, OTU-549, OTU-385, and OTU-697), and two genera from the phylum Acidobacteria: *Bryobacter* (OTU-175) and *Acidipila* (OTU-893).

Fig. 5

Fig. 5 Heatmap showing the relative abundance of the most relevant operational taxonomic units (OTUs) derived from bacteria in the three soils (1640, 1751, and 1957) sampled from the Llaima volcano in Chile at three sites according to lava eruptions in different centuries. The samples and OTUs were clustered according to Euclidean distances between all Hellinger transformed data.

Several OTUs belonging to Ktedonobacteraceae (OTU-597, OTU-2070, OTU-697, OTU-2608, OTU-6103, OTU-352, OTU-3584, OTU-5162154, and OTU-1418) were also abundant in the soil 1751. The site 1751 also had a high abundance of Actinobacteria. The heatmap also shows that some OTUs were abundant in all the soils, especially OTUs belonging to the phylum Proteobacteria, such as OTU-150 which belongs to Xanthobacteracea (Alphaproteobacteria). Proteobacteria was, in general, more abundant in the old site 1640. Finally, we also identified OTUs belonging to the phylum Verrumomicrobia, which were abundant in all the sites (Fig. 5).

DISCUSSION

*Microbial abundance* *in volcanic soils*

Soil coverage differed at each of the study sites, which is consistent with the difference in microbial diversity observed in each of the soils. The youngest soil (1957) accumulated mostly between rocks and was covered by moss. In contrast, the oldest soil was more extensive and colonized by trees (Hernández *et al*., 2014). Despite these differences, the absolute abundance of bacterial 16S rRNA genes was constant across the samples of different ages, suggesting that the abundance of bacteria was high even in the youngest soil. The bacterial 16S rRNA abundance in the range of 108 copies per gram of soil (Fig. 1) is similar to earlier studies from volcanic deposits. Weber and King (2010a) reported 2.6 × 108 bacterial 16S rRNA gene copy number per gram of dry soil in unvegetated patches on a 1959 Hawaiian volcanic deposits in the Kilauea volcano. In another study, by doing total direct microscopic counts of bacteria, abundances of bacteria in the range of (1--4) × 108 cells per gram of dry soil were found at volcanic deposits in Miyake-jima volcano in Japan, which was affected by a lava eruption in the year 2000 (Guo *et al*., 2014). Similar cell numbers (in the range 107--108) were found in poorly to fully vegetated volcanic deposits of different ages (32-, 35-, and 39-year old) in Iceland (Byloos *et al*., 2018).

In contrast to bacteria, the archaeal 16S rRNA genes decreased with soil age (Fig. 1). Very little is known about the colonization of volcanic deposits by archaea. The majority of the sequences belonged to the Thaumarchaeota. Nitrosphaera were the dominant Thaumarchaeota in our soil samples and were most abundant in the youngest soil. These organisms are able to grow autotrophically by ammonia oxidation, suggesting that this process is important in the early formation of volcanic soils (Hernández *et al*., 2014). This is consistent with a previous study on lava-formed forest soils in Korea, where archaeal clones affiliated to the Thaumarchaeota group were more abundant (96.2%) than the clones affiliated to the Euryarchaeota group (3.8%) (Kim *et al*., 2014). The oldest soil (1640) had a higher relative abundance of Euryarchaeota, particularly the Thermoplasmatales (between 3%--30% of archaeal 16S rRNA genes, Fig. S3). Whereas methanogenic members of the Euryarchaeota are abundant in wetland soil (Reim *et al*., 2017), the Thermoplasmatales have been previously reported in upland soils (Kemnitz *et al*., 2007; Hu *et al*., 2013, Hernández *et al*., 2017). Most characterized Thermoplasmata are acidophilic and/or thermophilic (Golyshina *et al*., 2016). However, those in upland soils are poorly characterized and we assumed them to be mesophilic.

*Diversity of bacterial microbial communities*

Higher numbers of OTUs were present in the older soils than in the 1957 soil (Fig. S2a). In general, Shannon diversity and evenness indexes were higher at older sites (1640 and 1751) (Fig. S2b and c). The results presented here support those already published, indicating that as the soil recovers, diversity increases. A study by King (2003a) indicated that the diversity of substrates utilized in Biolog plate assays increased with soil age in Hawaiian volcanic deposits (18--300 years old), indicating changes in the microbial communities. Analyzing the same deposits, Gomez-Alvarez and colleagues (2007) showed that the diversity is higher in older, moderately vegetated deposits (Shannon index *H* = 3.87 in site 1790) than in the youngest soil (Shannon index *H* = 2.62 in site 1921). The diversity indexes calculated in our study in the Llaima volcano are higher than those in the Kilauea volcano; however, it is important to note that the results of Gomez-Alvarez and colleagues (2007) were from cloning-based analyses and thus sampling intensity was lower. The diversity in the vegetated areas of the Llaima volcano (in sites 1640 and 1751) (Fig. S2b and Table S1) is also higher than those in lava-formed Gotjawal forest soils in South Korea (Kim *et al*., 2018), in which the authors found indexes below 5.5 for bacterial 16S rRNA genes based on Illumina sequencing analyses. The diversity was also higher than in vegetated areas from Iceland volcanic deposits, in which diversity increased as the area became fully vegetated (Byloos *et al*., 2018). The microbial communities in the vegetated areas (sites 1640 and 1751) clustered together, and in a separate cluster we can identify members of the unvegetated site (1957) (Fig. 2). This is corroborated by the diversity indexes being similar for the vegetated areas.

*Phylogenetic analysis of bacterial communities*

The abundance of Acidobacteria, Actinobacteria, and Proteobacteria increased with soil age (Fig. 3). Our study found that Acidobacteria and Actinobacteria are stable in the vegetated sites 1640 and 1751. Acidobacteria and Actinobacteria, among others, have been found to be abundant in soils from the Kilauea volcano (Gomez-Alvarez *et al*., 2007). Actinobacteria has been identified as an abundant phylum from Icelandic volcanic rocks of different compositions (Cockell *et al*., 2013). Within the Proteobacteria, a study looking at microbial communities from lava-formed Gotjawal forest soils in South Korea indicated that members of the class Rhizobiales are abundant in these sites (Kim *et al*., 2015). Proteobacteria was also found to be the most abundant phylum in young deposits of volcanic ash from Miyake-jima in Japan (Guo *et al*., 2014), from Icelandic volcanic deposits of different ages and vegetation (Byloos *et al*., 2018), and from vegetated deposits from the Kilauea volcano (Weber and King, 2010b). In our study, within Alphaproteobacteria, the abundance of Rhizobiales was high in the vegetated old sites compared with the youngest soil (Fig. S4). Members of the class Rhizobiales, known for their ability to fix N among other traits, have been identified in old volcanic deposits in the Kilauea Volcano (Nüsslein and Tiedje, 1998).

*Ecological significance*

Carbon monoxide-oxidizers, and N2- and H2-fixers are present in volcanic deposits of different ages and vegetation conditions. For example, *coxL* genes, which encode the large subunit of CO dehydrogenase (CODH), were found in members of Proteobacteria in vegetated sites in the Kilauea (Gomez-Alvarez *et al*., 2007; Weber and King, 2010b) and Miyake-jima volcanoes (King *et al*., 2008). *Burkholderia*, *Paraburkholderia* (Weber and King, 2017), *Stenotrophomonas*, and *Pseudomonas* among others encode *coxL* genes associated with the CODH enzyme responsible for CO-oxidation (King, 2003b). Hydrogen-oxidizing members of Proteobacteria were isolated from volcanic mudflow deposits from Mt. Pinatubo, Philippines (Sato *et al*., 2006). Although we did not study the abundance and distribution of *coxL* genes in this study, we detected many of these organisms in our soils. In particular, we obtained exceptionally high abundances (37%) of bacteria belonging to the order Ktedonobacterales (Chloroflexi) in the youngest soil (Figs. 4 and 5). Relatively high abundances of Ktedonobacteria were also found in a 1959 cinder deposit (Weber and King, 2010b). In our study we observed a decrease in the relative abundance of Ktedonobacterales with soil age, suggesting that they play an important role in early soil formation.

The capacity of Ktedonobacteria (Chloroflexi) for CO uptake has been previously proposed. Only about 14 strains able to either consume CO and/or harbor *coxL* genes have been characterized (King and King, 2014). So far, the class Ktedonobacteria contains only six species (Yabe *et al*., 2017). The order Ktedonobacterales contains the species *Ktedonobacter racemifer* SOSP1-21T (Chang *et al*., 2011), which has the largest bacterial genome reported and contains a *cox* operon conferring it potential for CO oxidation (King and King, 2014). Future studies should be conducted to establish the importance of this group of bacteria as pioneer organisms, possibly utilizing atmospheric trace gases as energy sources for the colonization of new environments in volcanic deposits.

CONCLUSIONS

In the present study, we show that there is a gradual reestablishment of the microbial community in volcanic soils following an eruption, with specific microbial groups playing important roles in the early stages of recovery. Some Ktedonobacterales are carboxydotrophs and hydrogenotrophs (*i.e.*, CO and H2 oxidizers), which provides intriguing evidence that CO and H2 might be important energy sources for the microbial community during the reestablishment of these soils. To our knowledge, this study is the first to examine the recovery of microbial diversity in volcanic soils of defined age and suggests that specific groups of prokaryotes play important functional roles in early soil formation.

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Fig. 1

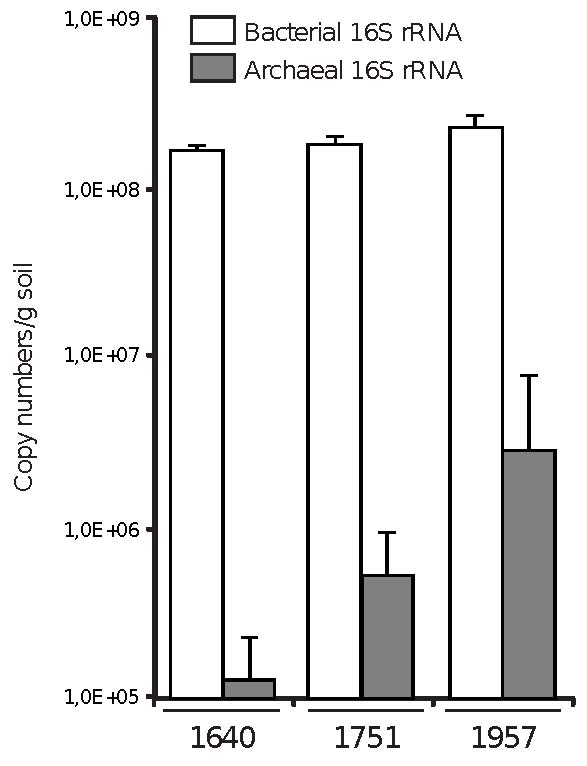


Fig. 2

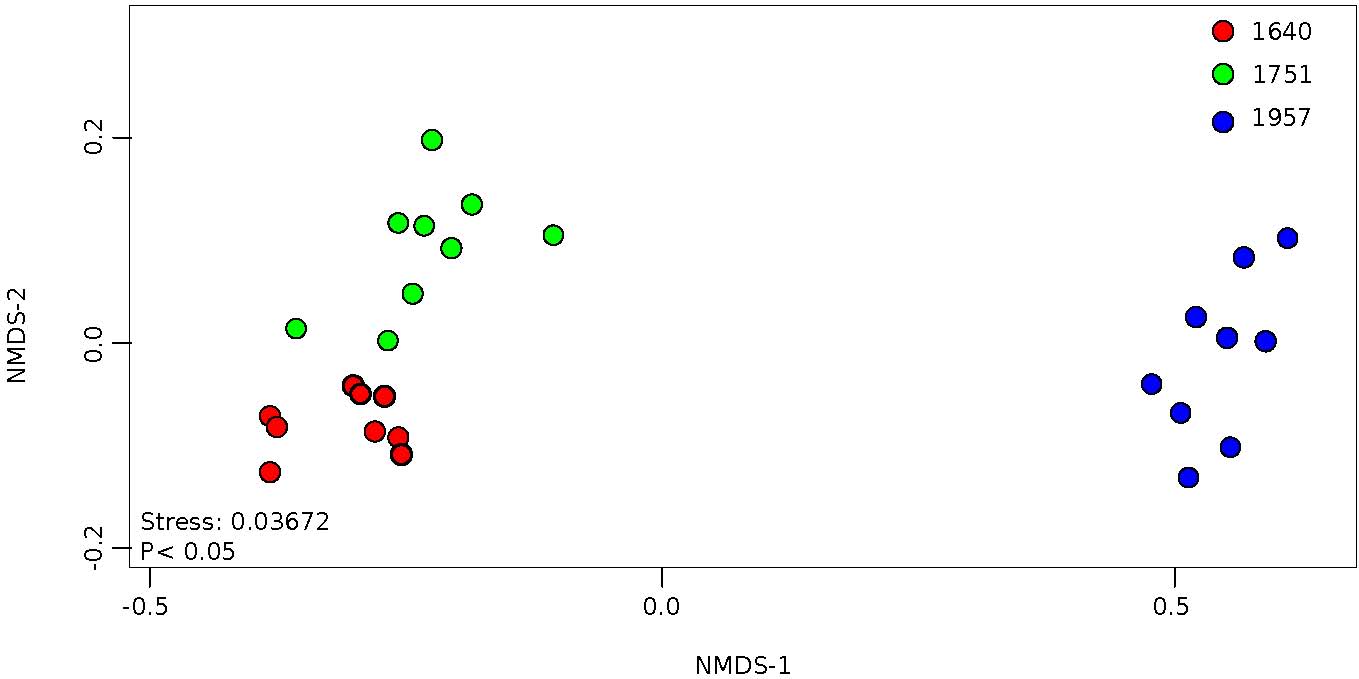


Fig. 3

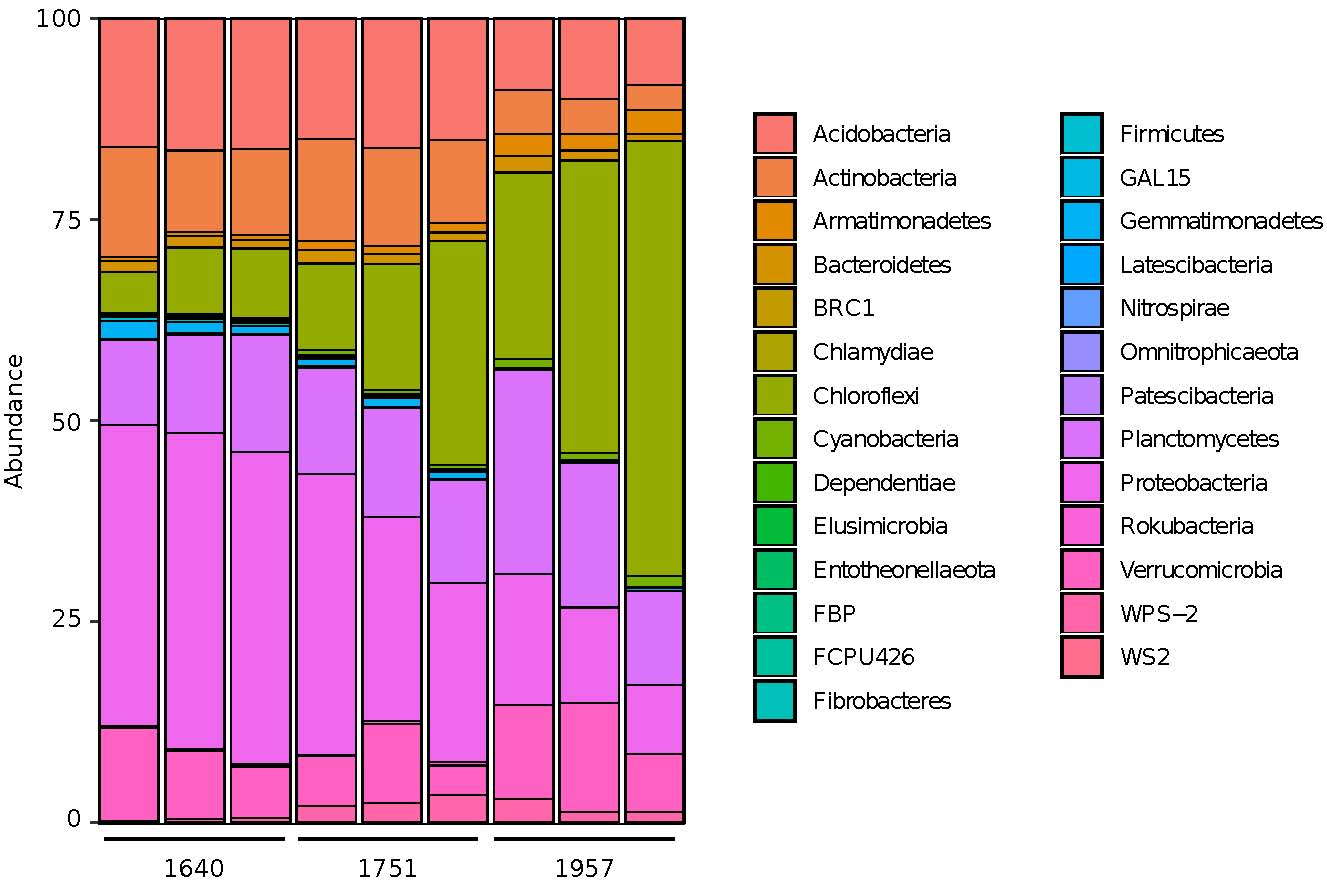


Fig. 4

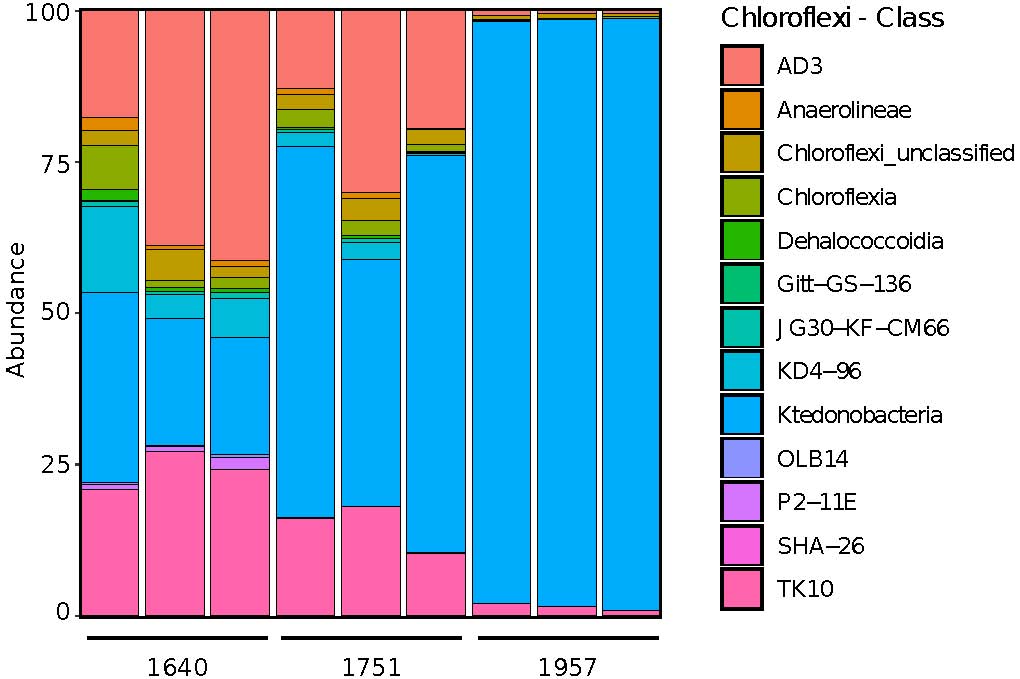
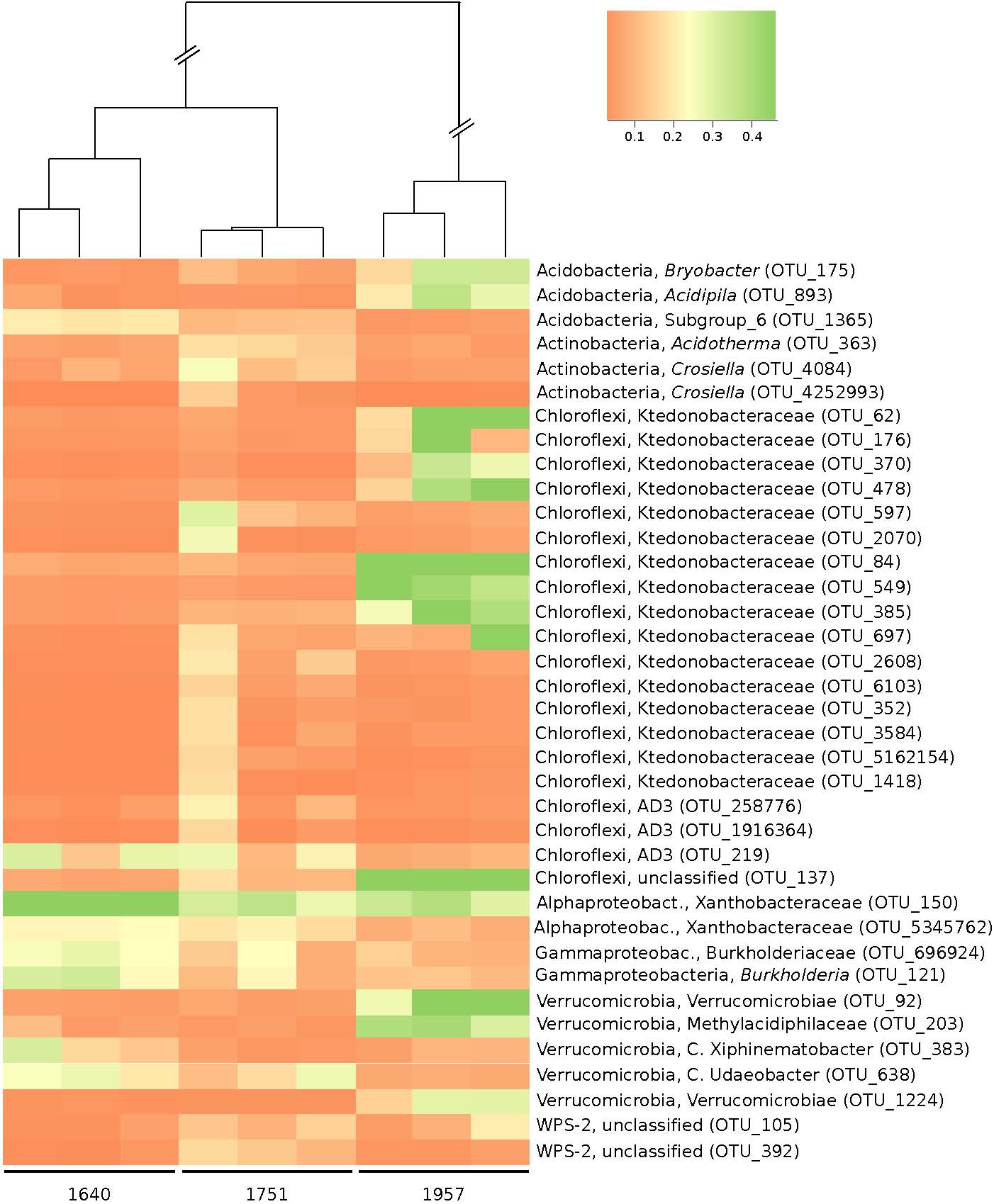


Fig. 5



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