

Genus-wide acid tolerance accounts for the biogeographical distribution of soil *Burkholderia* populations

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Summary

Bacteria belonging to the genus *Burkholderia* are highly versatile with respect to their ecological niches and lifestyles, ranging from nodulating tropical plants to causing melioidosis and fatal infections in cystic fibrosis patients. Despite the clinical importance and agronomical relevance of *Burkholderia* species, information about the factors influencing their occurrence, abundance and diversity in the environment is scarce. Recent findings have demonstrated that pH is the main predictor of soil bacterial diversity and community structure, with the highest diversity observed in neutral pH soils. As many *Burkholderia* species have been isolated from low pH environments, we hypothesized that acid tolerance may be a general feature of this genus, and pH a good predictor of their occurrence in soils. Using a combination of environmental surveys at trans-continental and local scales, as well as *in vitro* assays, we show that, unlike most bacteria, *Burkholderia* species have a competitive advantage in acidic soils, but are outcompeted in alkaline soils. Physiological assays and diversity analysis based on 16S rRNA clone libraries demonstrate that pH tolerance is a general phenotypic trait

of the genus *Burkholderia*. Our results provide a basis for building a predictive understanding of the biogeographical patterns exhibited by *Burkholderia* sp.

Introduction

The genus *Burkholderia*, which belongs to the β -Proteobacteria class, currently comprises more than 60 species that are widely distributed and frequently isolated from a large range of natural and clinical environments (Compant *et al.*, 2008). The genus *Burkholderia* can be divided phylogenetically into two main clusters: the first one consists mainly of human, animal and plant pathogens, e.g. the *Burkholderia cepacia* complex (Bcc) and the rice pathogen *B. glumae*. However, it is important to note that some strains belonging to Bcc, such as *B. ambifaria* or *B. lata*, also show plant growth-promoting abilities as well as biocontrol activities against phytopathogenic fungi. The other cluster consists mainly of plant-beneficial-environmental (PBE) *Burkholderia* species (Suárez-Moreno *et al.*, 2012). The members of the first cluster have been extensively studied because of their medical importance, but recently the PBE cluster has been the focus of research efforts with the discovery that various species of this cluster are able to fix nitrogen (Estrada-De Los Santos *et al.*, 2001; Martínez-Aguilar *et al.*, 2008) and to nodulate legumes (Moulin *et al.*, 2001). *Burkholderia* from the PBE cluster have been mainly isolated from plant rhizosphere, but they are also frequently detected in sediment and bulk soil (Salles *et al.*, 2002; Lazzaro *et al.*, 2008; Lim *et al.*, 2008; Lepleux *et al.*, 2012; Štursová *et al.*, 2012). In addition to their nitrogen-fixing and nodulating abilities, their versatile metabolism also enables them to survive in harsh conditions, such as nutrient-limited or polluted environments, and to degrade recalcitrant compounds (Pérez-Pantoja *et al.*, 2012). As such, they have been suggested as good candidates for use in biotechnology, e.g. for bio- or phytoremediation, biocontrol and biofertilization. Despite the high relevance of the *Burkholderia* genus for human health, agronomy and biotechnology, surprisingly little is known about the factors underlying their geographical distribution.

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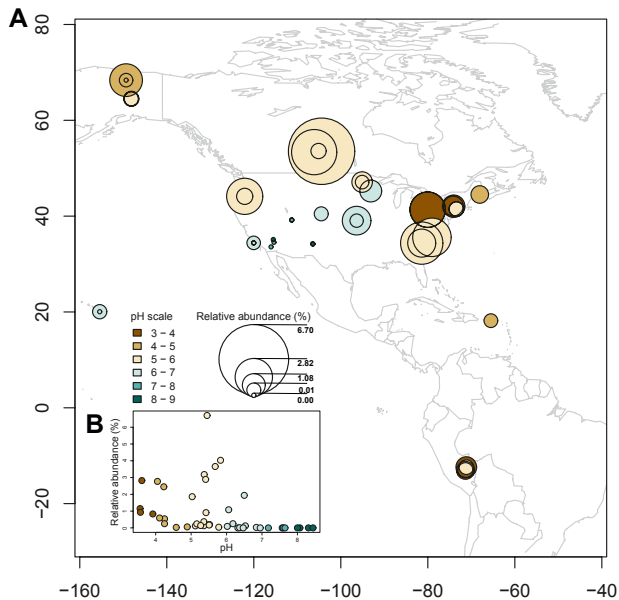


Fig. 1. Relative abundance of *Burkholderia* 16S rRNA genes in 44 soils.

A. Representation of *Burkholderia* 16S rRNA gene relative abundance at the different sites as assessed by qPCR. Relative abundance is represented by the circle size; the colour indicates the pH of the sampled soils.

B. Influence of pH on the relative abundance of *Burkholderia* 16S rRNA genes. Circles represent the average of three replicates for each soil sample.

Soil pH has frequently been shown to be the main predictor of overall soil bacterial community composition, diversity and the relative abundance of many individual taxa (Tiedje *et al.*, 1999; Fierer and Jackson, 2006; Rousk *et al.*, 2010; Griffiths *et al.*, 2011). Although it is not known if *Burkholderia* distributions are related to soil pH, most studies that have reported their presence in soil were investigating acidic environments (Trân Van *et al.*, 2000; Curtis *et al.*, 2002; Salles *et al.*, 2002; 2004; Belova *et al.*, 2006; Garau *et al.*, 2009; Aizawa *et al.*, 2010). For instance, *Burkholderia unamae* could only be isolated from the rhizosphere of plants growing in soils, with a pH ranging from 4.5 to 7.1, but not from soils with a pH higher than 7.5 (Caballero-Mellado *et al.*, 2004). Likewise, a survey of over 800 Australian soil samples revealed that *B. pseudomallei* was specifically associated with low pH soils, but not recovered from higher pH soils (Kaestli *et al.*, 2009). *Burkholderia* species have also been isolated from acidic *Sphagnum* peat bogs (Belova *et al.*, 2006; Opelt *et al.*, 2007a; 2007b), from root tissues of the highly acidifying cluster rooted *Lupinus albus* (Weisskopf *et al.*, 2011) or from soils as acidic as pH 2.9 (Curtis *et al.*, 2002). To the best of our knowledge, only one study reported isolation of *Burkholderia* strains from an alkaline environment (Estrada-de los Santos *et al.*, 2011). While the pH of the rhizosphere soil investigated in this study

was high (8.7), the isolated strains were all able to grow at low pH (4.5). These reports provide anecdotal evidence that *Burkholderia* might be tolerant to low pH conditions, which enables members of this genus to thrive in niches where others would be inhibited. We, therefore, hypothesized (i) that low pH tolerance is an intrinsic phenotypic trait of the *Burkholderia* genus, and (ii) that the relative abundance and diversity of *Burkholderia* populations are highest in low pH soils, with the biogeography of *Burkholderia* predictable from soil pH. To test these hypotheses, we developed a novel quantitative polymerase chain reaction (qPCR) protocol to analyze the relative abundance of *Burkholderia* populations in soils at a trans-continental and a local scale. Intrageneric diversity and community structure were determined by 16S rRNA-based clone libraries constructed from a selected subset of the trans-continental scale soil samples. In addition, *in vitro* physiological assays were used to test the direct effects of pH on *Burkholderia* species.

Results

Low pH tolerance, a genus-wide property of Burkholderia that largely accounts for its relative abundance in soils

To test whether the occurrence of *Burkholderia* species in acidic environments reflects an intrinsic capacity of this genus to tolerate low pH conditions, we tested the ability of 68 strains of *Burkholderia* belonging to 31 different species to grow at a pH range of 3.5–8. All *Burkholderia* strains that were tested in physiological assays grew in pH as low as pH 4.5. Out of 68 tested strains (31 different species), 32 (18 species) were growing also at pH 4 and 15 (8 species) even at pH 3.5, but no species-specific tolerance could be observed under such conditions (Table S1).

Based on this result, we hypothesized that members of the *Burkholderia* genus would be favoured in low pH environments. We tested this hypothesis by analyzing the relative abundance of soil *Burkholderia* in a trans-continental sample collection of 44 soils from a broad array of ecosystem types that represent a wide range of soil and site characteristics (Table S2). The relative abundance of *Burkholderia* species greatly varied between these different soils sampled across North and South America (Fig. 1A). Highest relative abundance was observed in moderately acidic soils (pH 5–pH 6), where up to 6.7% of the total bacterial population was represented by *Burkholderia* species. It is worthwhile to notice that within this pH range a large variability in *Burkholderia* relative abundance was observed (standard deviation = ± 2.02), spanning from 0.04% to 6.25%, whereas in more acidic soils (pH <4) relative abundances were approximately 1% or higher. While high relative abun-

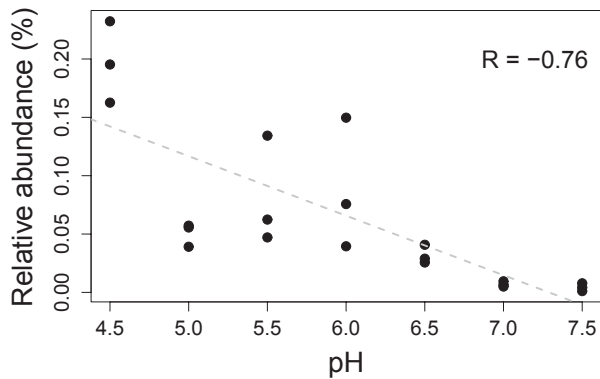


Fig. 2. Relative abundance of *Burkholderia* 16S rRNA genes along a local pH gradient in an agricultural field in Scotland. Relative abundance was quantified by qPCR in three soil samples for each pH along the pH gradient. Abundance of *Burkholderia* is strongly and negatively correlated with pH (Pearson's product-moment correlation: $R = -0.76$, $P < 0.001$).

dance of *Burkholderia* 16S rRNA gene copy numbers was detected in soils with a pH lower than 7, the relative abundance was under the detection limit of our qPCR method (less than 100 copies reaction⁻¹) in neutral and alkaline soils (Fig. 1B). As expected, pH was a significant factor predicting *Burkholderia* relative abundance ($P = 0.03$, $R = -0.33$), although the correlation was weak, probably due to the high variability of *Burkholderia* relative abundance in low pH soils. Moreover, pH is not the only variable that changes across the soils analyzed, and pH often correlates with other soil and site characteristics. The C/N ratio showed the best correlation ($P = 0.0005$, $R = 0.50$) with *Burkholderia* relative abundance, but since pH and C/N ratio correlate ($P = 0.003$, $R = -0.4380$), we tested our hypothesis in a different experimental set-up, in which the effect of pH could be discriminated from that of C/N ratio. To this end, we analyzed the relative abundance of *Burkholderia* in an agricultural field with a pH gradient of 4.5–7.5 but a constant C/N ratio. Relative abundance of *Burkholderia* 16S rRNA was lower in this soil than in soils collected across North and South America. Highest relative abundance was detected at pH 4.5 (0.23–0.16%), and an almost linear decrease with increasing pH was observed, reaching 0.01–0.008% of *Burkholderia* 16S rRNA relative abundance in soil of pH 7.5 ($P < 0.0001$, $R = -0.76$) (Fig. 2).

Intragenetic diversity of Burkholderia soil populations does not depend on pH

To analyze the intragenetic diversity of soil *Burkholderia* populations and to determine whether some groups showed any pH preference, 14 sites varying in pH (from

3.5 to 6.8), C/N ratio, location and relative abundance of *Burkholderia* 16S rRNA genes were selected from the trans-continental scale sampling set for phylogenetic analyses. Clone libraries targeting the 16S rRNA gene were constructed for each of the selected sites, and a total of 675 sequences (590 bp) were obtained, corresponding to 123 operational taxonomical units (OTUs) at a 98% identity threshold between *Burkholderia* 16S rRNA gene sequences (Fig. 3). Diversity and richness of the soil *Burkholderia* communities were highly variable between the sites, e.g. only one phylotype was found in KP2 or PE3, but CL4 and PE5 harboured 33 and 25 phylotypes respectively (Fig. 3). However, there was no significant correlation between pH and *Burkholderia* diversity. To test whether pH or any other of the described environmental parameters could influence *Burkholderia* community composition, a Mantel test was performed (see Table 1). Our data showed that pH had no correlation with community structure ($r_M = 0.110$, $P = 0.204$), while site elevation and spatial parameters did significantly positively correlate with *Burkholderia* communities ($r_M = 0.39$, $P = 0.002$ and $r_M = 0.38$, $P = 0.038$ respectively). *Burkholderia* community structure was also marginally influenced ($P < 0.1$) by climatic factors and soil chemistry ($r_M = 0.295$, $P = 0.077$ and $r_M = 0.260$, $P = 0.094$ respectively). These results indicate that low pH would generally affect the relative abundance of the *Burkholderia* genus, but not the relative abundances of individual species within this genus, which is in line with our observations that low pH tolerance is a genus-wide feature of *Burkholderia* sp. (Table S1).

Burkholderia glathei: a major and widespread soil inhabitant

Within the entire sequence set, those closely related to *B. glathei* were by far the most abundant and most widely distributed of all (approx. 40% of sequences). These sequences comprised four OTUs, which contained 190, 33, 32 and 12 sequences respectively (Fig. 3). The most widespread and abundant OTU (190 sequences) was present at nine sites out of 14. Interestingly OTU 3, the next most abundant OTU of this *B. glathei* group, was present only at one site (HI3) and represented all of the 33 sequences collected at this site. The next most abundant OTU beside the *B. glathei* group was most closely related to *B. terricola* (53 sequences). Unlike the *B. glathei* group, however, this OTU was only found in one site (KP2), where it was the only *Burkholderia* representative. Sequences closely related to *B. phenazinium*, *B. fungorum* and *B. terrae* were very abundant as well, while other OTUs were represented by less than 20 sequences, and a high proportion (103 OTUs) consisted of less than five sequences (Table S3). Despite the high diversity of *Burkholderia* in some of our soil samples,

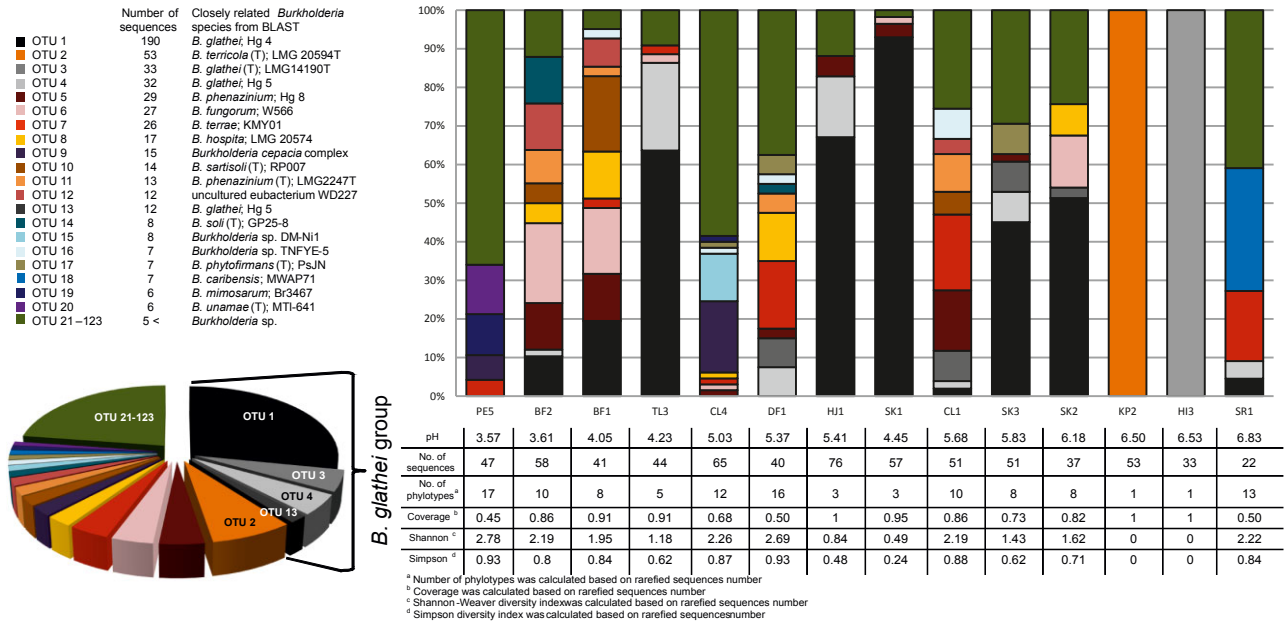


Fig. 3. Intragenetic diversity of soil *Burkholderia*. Pie chart represents the total number of OTUs obtained from 14 sites. Pieces represent OTUs that contain more than five sequences and are ordered by size (number of sequences). Dark green represents OTUs that contain less than five sequences. The largest group of sequences, which is represented by strains of *B. glathei*, is highlighted. The bar chart represents the relative abundance of OTUs per site, ordered by pH. The table contains the diversity indices calculated using rarefied sequence number (22 sequences) per site. All indices were calculated using 98% identity between sequences.

rarefaction curves reached a plateau in almost all soils analyzed (Fig. S1). The least diverse sites were HI3, KP2 and SK1 having less than four OTUs (Fig. 3). In all except for KP2, the majority of sequences were affiliated with *B. glathei* (100% at site HI3 and 93% at site SK1), suggesting a preeminent role of this species in the soil.

Discussion

Low pH tolerance is a general property of the genus Burkholderia

All *Burkholderia* strains that were tested on minimal medium showed tolerance to acid pH (pH 4.5). Similar results were obtained by Estrada-de los Santos and colleagues (2011), who reported that most of the 43 tested *Burkholderia* species were able to grow in a pH range of 5–11, although the medium used in their study was neither buffered nor was growth quantitated. Our results are in agreement with previous reports (Belova et al., 2006; Aizawa et al., 2010; 2011; Schmerk et al., 2011) and suggest that *Burkholderia* are acidotolerant rather than acidophilic. The unveiling of this genus-wide acid tolerance allows conclusions on the lifestyle and environmental adaptation of these bacteria, and also offers new possibilities to select or enrich *Burkholderia* isolates from complex environments.

Burkholderia are relatively more abundant in low pH soils

Our results demonstrated a negative correlation between pH and relative abundance of *Burkholderia* 16S rRNA

Table 1. Relationship of *Burkholderia* community structure to combined and individual environmental parameters revealed by Mantel test.

Environmental parameters	r _M	P
Soil chemistry	0.260	0.094
<i>pH</i>	0.110	0.204
<i>C/N ratio</i>	-0.197	0.916
<i>% organic C</i>	0.226	0.126
Climatic	0.295	0.077
<i>MAT</i>	0.273	0.042*
<i>MAP</i>	0.141	0.231
Soil	0.176	0.170
<i>% silt and clay</i>	0.289	0.030*
<i>Depth of O horizon</i>	-0.119	0.666
<i>SMD</i>	0.125	0.236
Biological	0.227	0.125
<i>C mineralization rate</i>	0.226	0.134
Spatial (longitude, latitude)	0.379	0.038*
Site elevation	0.387	0.002*

Parameters highlighted in bold represent combined matrices that were used and included factors highlighted in italic (used also separately). Bold values represent significant P values (< 0.1). P values < 0.05 are indicated in bold and with an asterisk. r_M, Mantel's correlation coefficient.

genes: *Burkholderia* relative abundance was higher in acidic than in neutral soils and was absent or under the detection limit in alkaline soils. The highest relative abundance was observed in moderately acidic soils (pH 5–6), where *Burkholderia* represented 6.25% of the total bacterial 16S gene copies. Similar relative abundances of *Burkholderia* were previously observed in pyrosequencing studies investigating acidic bulk soils (1.2%), while abundances increased in the rhizosphere (1.96–3.08%) and even more in the mycorrhizosphere environments (3.30–8.33%) (Uroz *et al.*, 2010; 2012). C/N ratio was another environmental parameter that significantly influenced the relative abundance of *Burkholderia* populations in soils, but this parameter was also, as often observed, strongly correlated with pH (Kemmitt *et al.*, 2006). For this reason, we conducted a local-scale study on an agricultural field with a pH gradient where the C/N ratio is fairly constant and where the aboveground plant community is the same. Our data showed that the effect of pH on *Burkholderia* relative abundance was even stronger than what was observed on the trans-continental scale, with an almost linear decrease with increasing pH, which strongly suggests that pH, rather than C/N ratio, influences the distribution of *Burkholderia* populations in soil.

Intragenetic diversity of Burkholderia soil populations does not depend on pH

Previous studies investigating the phylogeny of acidobacteria have shown that their relative abundance and intragenetic diversity are higher in low pH soils. Interestingly, certain subgroups within this genus were identified, which were only found in neutral or even in alkaline soils (Lauber *et al.*, 2008; Jones *et al.*, 2009; Griffiths *et al.*, 2011). Since our results of *Burkholderia* relative abundance are similar to the trends observed for acidobacteria, we investigated whether certain *Burkholderia* lineages would have a preference for soils with a particular pH. However, our diversity analysis showed no correlation between pH and community composition within the genus. While the intragenetic diversity varied greatly between the samples, no OTU was found that was specifically enriched in highly or moderately acidic soils. This is in line with our *in vitro* low pH tolerance assays, which suggested that pH tolerance is a general feature of the genus *Burkholderia*. Interestingly, pathogenic species, such as *B. pseudomallei* or *B. mallei*, or opportunistic pathogens, such as members of the Bcc, were very rarely detected in our soil survey, indicating that while they have been reported to be major inhabitants of maize (Bevivino *et al.*, 2011) or sugar cane (Castro-González *et al.*, 2011) rhizospheres, they are not commonly present in nutrient-limited bulk soil. In contrast,

we observed very high relative abundance of *B. glathei*, which was in this study by far the most abundant and widespread OTU (Fig. 4). *B. glathei* has been previously shown to be widely distributed across soils and rhizospheres (Belova *et al.*, 2006; Uroz *et al.*, 2007; 2012). Here, we show that members of this species are not only very abundant in different soil and ecosystem types but are also extremely widespread over diverse geographical sites. This suggests that *B. glathei* is a preeminent soil inhabitant, which is particularly well adapted to this type of environment, although the specific functions responsible for the success of this species in soil remain undetermined. In addition to site descriptors analyzed in this study, biological factors may have an important role in shaping *Burkholderia* community composition and might be responsible for the highly variable intragenetic diversity observed in the selected soil samples. A good example of such biological factors is the symbiotic association between nitrogen-fixing *Burkholderia* species and plants. For example, *Burkholderia mimosarum* is capable of nodulating *Mimosa* plants, and is therefore only found in areas where the plants are endemic, such as tropical regions of South-eastern Asia and South America (Chen *et al.*, 2006; Elliott *et al.*, 2009). In line with this, we detected *B. mimosarum* only at site PE5, which is located in Peruvian Amazonas.

In summary, this study showed that low pH tolerance is a genus-wide feature of *Burkholderia* species. This explains their presence in acidic soils but not their absence from higher pH environments, especially considering that under laboratory conditions, the majority of *Burkholderia* strains are able to grow in neutral or even alkaline culture media. This suggests that *Burkholderia* have developed pH tolerance mechanisms that enable them to survive and thrive in environmental niches where many other taxa are inhibited, while they are outcompeted by faster growing microorganisms in less harsh conditions. Acid tolerance is a prerequisite for occurrence in low pH soils, but it is tempting to postulate that the preference of *Burkholderia* for such niches is not only the consequence of the ability to tolerate acidity, but the result of a multifaceted strategy involving both tolerance to abiotic stress factors (such as higher toxicity of heavy metals) and to biological constraints (e.g. the predominance of fungi) inherent to such environments.

Experimental procedures

Testing growth of Burkholderia strains in vitro under different pH conditions

To study the effect of pH on *Burkholderia* growth, an *in vitro* approach was used. To this end, 68 *Burkholderia* strains from different isolation origins were used (Table S1). Before spotting 20 µl aliquots of each culture on growth medium, over-

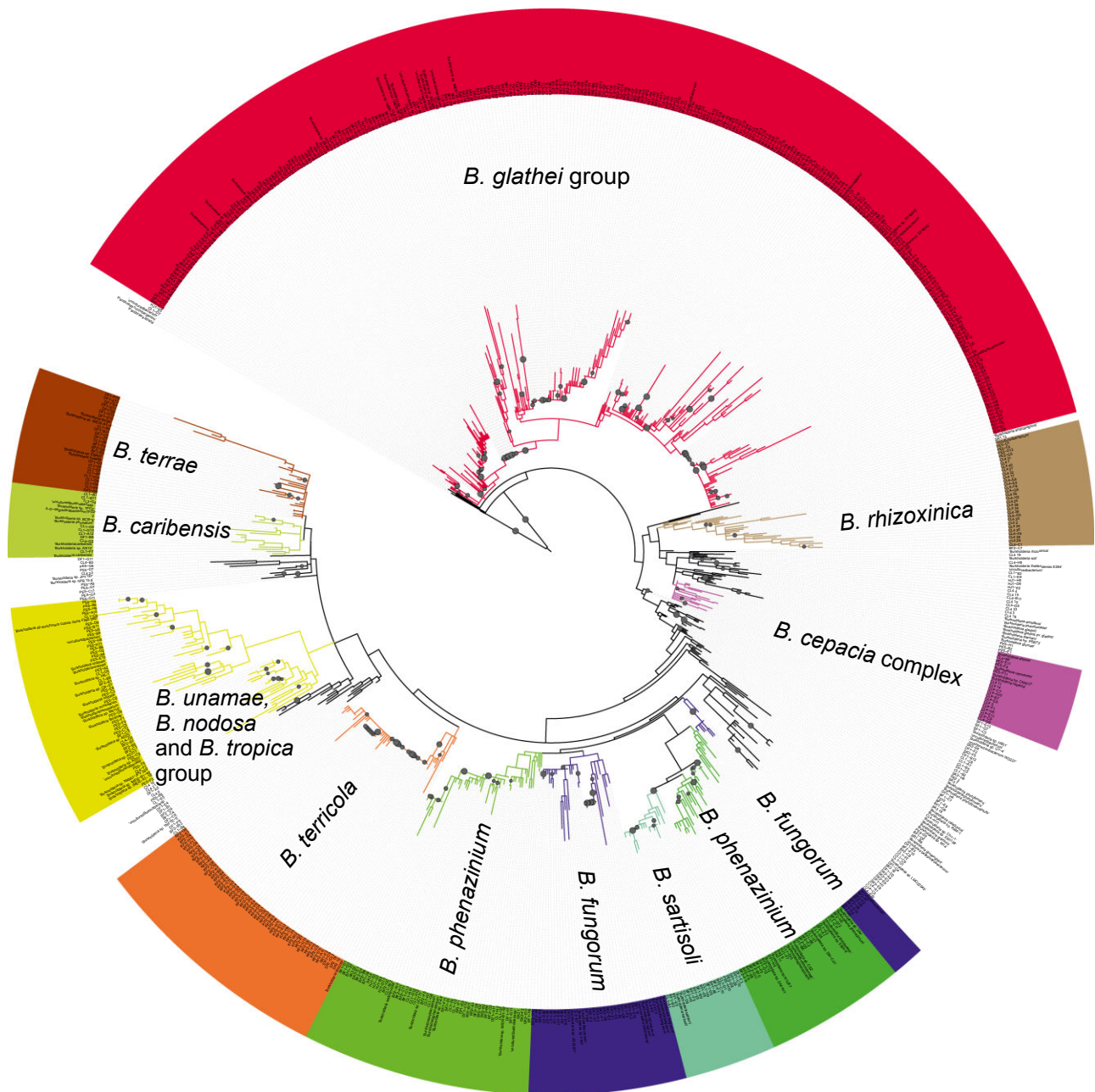


Fig. 4. Phylogenetic analysis of *Burkholderia* 16S rRNA gene sequences from 14 different sites. 675 sequences (590 bp) were aligned with additional reference sequences using ARB. Phylogeny was constructed using a maximum likelihood-based method. Bootstrap values ($n = 1000$) >50% are shown as circles. Colours indicate the affiliation with a given species.

night liquid cultures were washed and resuspended in saline buffer to optical density (OD_{600}) of 1. As growth medium, AB minimal medium (Clark and Maaløe, 1967) supplemented with glucose and agar was used, and the pH of the medium was adjusted to obtain a pH gradient of pH 4–7 in 0.5 unit steps, with an additional medium of pH 8. To test growth under more acidic conditions, liquid AB medium supplemented with glucose was used, adjusted to pH 3, pH 3.5 and pH 4. Liquid AB medium with pH 4 was used as a control to test if the growth patterns were the same between solid and

liquid conditions. Media were buffered with 0.1 M potassium hydrogen phthalate ($C_8H_5KO_4$, $pK_a = 5.4$) and 0.1 M HCl for pH 3, 0.1 M $C_8H_5KO_4$ and water for pH 4, 0.1 M NaOH and 0.1 M $C_8H_5KO_4$ for pH 5–6, and for media higher than pH 7, 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was added. To detect changes in pH caused by bacterial growth, resazurin was added as a pH indicator. Plates were incubated for 4 days at 30°C. Growth was assessed by inspecting the plates for the formation of colonies.

Soil sampling and DNA extraction

The dataset consisted of 44 soil samples distributed across North and South America. The collected soils came from a broad range of ecosystems, climates and soil types (Table S2). Soil collection protocol and methods for edaphic and environmental properties have been described previously (Fierer and Jackson, 2006; Bates *et al.*, 2011). In addition, seven soil samples were collected in March 2011 from an agricultural field divided into several plots with a pH gradient of 4.5–7.5, which has been maintained since 1961 by the addition of either lime or aluminium sulphate, and where plots undergo an 8-year crop rotation cycle (Scottish Agricultural College, Aberdeen, Scotland; grid reference NJ872104). Detailed soil characteristics are provided by Kemp and colleagues (1992). The soils were sampled in triplicates from the upper 20 cm soil layer, homogenized and stored at 4°C prior usage. Total nucleic acids were extracted from 0.5 g of soil, as described by Griffiths and colleagues (2000) with some modifications (Nicol *et al.*, 2005). pH was measured in deionized water using a ratio of 1:2 soil : water (w/v), shaking for 30 min and settling for 30 min before measurement.

qPCR

To quantify *Burkholderia* 16S rRNA genes in soil samples, primers BKH812F (5'-CCC TAA ACG ATG TCA ACT AGT TG-3') and BKH1249R (5'-ACC CTC TGT TCC GAC CAT-3') (Bergmark *et al.*, 2012) were used. In their original publication, Bergmark and colleagues (2012) observed that the designed primers were not specific, suggesting that the most likely explanation for lack of specificity was that the T_m value they used was too low. We, therefore, tested both primers for their specificity using higher T_m values on DNA isolated from soils with different pH. An annealing temperature of 64°C was found to efficiently amplify *Burkholderia* 16S rRNA genes with 100% specificity. This was tested by sequencing 270 clones containing PCR products from soils with three different pH. For both bacterial and *Burkholderia* 16S rRNA gene, high amplification efficiency was obtained by qPCR (93–100% and 91–100%, respectively, and r^2 values between 0.995 and 0.999). Relative abundance of *Burkholderia* was calculated as a ratio between *Burkholderia* gene copy numbers by bacterial gene copy numbers (see supporting information for more details).

Amplification and cloning of *Burkholderia* 16S rRNA gene sequences

To study the diversity of *Burkholderia* in soil, *Burkholderia* 16S rRNA genes were amplified using the modified primers BKH143F (5'- TGGGGGATAGCYCGGCG -3') and BKH1434R (5'- TGCGGTTAGRCTAGCYACT -3') (Schönmann *et al.*, 2009). Cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 60 s, 61.5°C for 60 s, and 72°C for 90 s, final extension at 72°C for 5 min. Reactions were performed in 50 µl volumes containing 1× reaction buffer containing MgCl₂ (1.5 mM) (Sigma-Aldrich, St. Louise, MO, USA), 0.8 µM of each primer (Microsynth, Balgach, Switzerland), 0.2 mM dNTP mixture, 0.25 mg ml⁻¹ of bovine serum albumin, 2U of Taq DNA Polymerase (Sigma-Aldrich, St. Louise, MO,

USA) and 2 µl of template DNA. PCR was carried out in a C1000 Thermal Cycler (Bio-Rad, United Kingdom). PCR products were confirmed by standard 1% agarose gel electrophoresis and gel purified (Gel PCR purification kit, QIAGEN, Hilden, Germany). *Burkholderia* 16S rRNA clone libraries were made from 14 selected locations (Table S2). Purified PCR products were cloned into the pGEM-T Easy vector (Promega, Southampton, United Kingdom). Selected clones from 16S rRNA clone libraries were sequenced using the M13f vector primer.

Sequence analysis

Sequences of chimeric origin were detected by analyzing alignments using Chimera.Slayer and Chimera.UCHIME as implemented by the MOTHRU software (Schloss *et al.*, 2009; Edgar *et al.*, 2011; Haas *et al.*, 2011). Sequences from short or failed reads were excluded from analysis. Sequences were aligned using the SINA web aligner (Pruesse *et al.*, 2007). The alignments were merged into the SILVA SSU reference database release 106 using the ARB software package (Ludwig *et al.*, 2004). Sequences were deposited to the National Center for Biotechnology Information database with accession numbers KC353471 to KC354145. A 50% similarity filter was created for the dataset, based on the alignment, leaving 590 nucleotides for 16S rRNA sequence alignments. The closest cultivated relatives were selected from the reference dataset. Bootstrapped maximum likelihood trees (1000 repetitions) were calculated with sequences affiliated with the groups of interest and close relatives on a dedicated RAXML web server (Stamatakis *et al.*, 2008).

Phylogenetic analysis

Distance matrices were exported to calculate rarefaction curves and diversity indices with the MOTHRU software (Schloss *et al.*, 2009). Sequences were grouped into operational taxonomic units (OTU) using the furthest-neighbour approach, with an OTU defined as containing sequences that are no more than 2% different from each other. This threshold of 98% identity was selected because of the high similarity between *Burkholderia* 16S rRNA sequences over the relatively short read length used in the present study. Richness and diversity were estimated from 16S rRNA gene clone libraries using the Shannon–Weaver diversity index (H) (Shannon and Weaver, 1963) and the Simpson diversity index (D) (Simpson, 1949). Good's coverage (C) was calculated as $C = 1 - (n_i/N)$, where n_i was the number of clones, which occurred only once in a library of N clones (Good, 1953), and relative abundances of major phylogenetic groups were determined.

Statistical analysis

Pearson's product-moment correlations between *Burkholderia* 16S rRNA gene relative abundance and environmental parameters were performed in R 2.12.0 (<http://www.r-project.org/>). For correlating *Burkholderia* trans-continental distribution, we used the following soil and site characteristics: soil pH, organic C content, C/N ratio, C

mineralization rate, elevation, soil moisture deficit (SMD), mean annual temperature (MAT) and mean annual precipitation (MAP). For correlation analysis at the local scale, pH was the only factor used.

We used Spearman's rank correlation to compare estimate of *Burkholderia* composition with site elevation, soil chemistry (matrix including pH, C/N ratio and percentage of organic C) and climatic (MAT, MAP), soil (percentage of silt and clay, depth of O horizon and SMD), biological (C mineralization rate) and spatial (longitude, latitude) parameters. To estimate the pairwise similarity in *Burkholderia* communities, we generated Bray–Curtis dissimilarity matrices, using rarefied abundance table of *Burkholderia* phylotypes (OTUs) as an input (22 sequences per location). We used the Mantel test in R 2.12.0 to compare dissimilarity matrices to pairwise distances in environmental characteristics as estimated using normalized Euclidean distances in the measured soil and site parameters.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Detailed experimental procedure.

Fig. S1. Rarefaction curves for *Burkholderia* 16S rRNA gene libraries from 14 different samples. The OTUs were formed at a 98% identity threshold. Dotted vertical line represents the sequence number threshold used for OTU analyses (22 sequences).

Table S1. *In vitro* tests of acid tolerance. 68 *Burkholderia* strains were tested on AB minimal medium supplemented with glucose and agar. Colony formation was selected as growth criterion. Presence or absence of colony formation under various pH conditions is represented in the table with + and – signs respectively.

Table S2. Site description of selected sampling locations from the trans-continental scale study.

Table S3. OTU table with closest relatives. Sequences from each OTU were compared with the database on NCBI using the BLAST tool, and the best hits were selected as closest relatives.