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The microbiome of the leaf surface of Arabidopsis protects against a fungal pathogen

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Summary

- We have explored the importance of the phyllosphere microbiome in plant resistance in the cuticle mutants *bdg* (BODYGUARD) or *lacs2.3* (LONG CHAIN FATTY ACID SYNTHASE 2) that are strongly resistant to the fungal pathogen *Botrytis cinerea*.
- The study includes infection of plants under sterile conditions, 16S ribosomal DNA sequencing of the phyllosphere microbiome, and isolation and high coverage sequencing of bacteria from the phyllosphere.
- When inoculated under sterile conditions *bdg* became as susceptible as wild-type (WT) plants whereas *lacs2.3* mutants retained the resistance. Adding washes of its phyllosphere microbiome could restore the resistance of *bdg* mutants, whereas the resistance of *lacs2.3* results from endogenous mechanisms. The phyllosphere microbiome showed distinct populations in WT plants compared to cuticle mutants. One species identified as *Pseudomonas* sp isolated from the microbiome of *bdg* provided resistance to *B. cinerea* on *Arabidopsis thaliana* as well as on apple fruits. No direct activity was observed against *B. cinerea* and the action of the bacterium required the plant.
- Thus, microbes present on the plant surface contribute to the resistance to *B. cinerea*. These results open new perspectives on the function of the leaf microbiome in the protection of plants.

Introduction

The nonpathogenic microbiota, often referred to as neutral microbes, turn out to contribute in various ways to the development and to the health of the individuals harbouring them. For example, the microbiome in the human gut has been linked with a healthy function of the brain, the immune system, the digestive system, and with a number of diseases ranging from cancer to metabolic or even psychiatric disorders (Foster & McVey Neufeld, 2013; Biedermann & Rogler, 2015; Dash *et al.*, 2015; Viaud *et al.*, 2015). In animals, the microbiome has been associated with similar functions in development and disease (Engel & Moran, 2013; Kostic *et al.*, 2013; Sabree & Moran, 2014). A lot of interest has also been given to microbes associated with surfaces of roots or leaves (Andrews & Harris, 2000; Lindow & Brandl, 2003; Vorholt, 2012; Humphrey *et al.*, 2014). Such microbes are proposed to be associated with protection and nutrient acquisition (Bulgarelli *et al.*, 2013). Soil types and associated abiotic and biotic parameters, as well as chemicals secreted by roots, create a niche for specific microbes and determine plant-specific rhizosphere communities that help with protection and nutrient acquisition (Walker *et al.*, 2003; Bulgarelli *et al.*, 2013). Bacteria predominantly colonize the phyllosphere (Lindow & Brandl, 2003) where environmental conditions together with surface features determine the composition of the phyllosphere flora (Vorholt, 2012). For instance, single mutations in genes controlling wax biosynthesis in *Arabidopsis thaliana* lead to distinct chemical composition of waxes together with distinct bacterial communities on the phylloplane (Reisberg *et al.*, 2013). The phylloplane of *A. thaliana* plants grown under similar sterile conditions and inoculated initially with a same representative mixture of the most abundant phylloplane bacteria was analysed in a collection of mutants with phenotypes ranging from altered cell walls to impaired defences and secondary metabolism. Interestingly, the strongest difference in bacterial composition and abundance compared to the wild-type plants was observed in *lacs2.3, lacs2.4* and *pec1* mutants affected in the biosynthesis of the cuticle (Bodenhausen *et al.*, 2014). Mutants affected in the structure of the cuticle are characterized by enhanced cuticular permeability and display enhanced resistance to the agriculturally relevant fungal pathogen *Botrytis cinerea*. In addition to resistance, many of these cuticular mutants spontaneously accumulate reactive oxygen species (Serrano *et al.*, 2014). In this study we have tested the hypothesis that the phyllosphere microbes including epiphytes

and endophytes contribute to the resistance of *A. thaliana* to *B. cinerea* with a special emphasis on the cuticle.

Materials and Methods

Plant maintenance

The nonsterile *Arabidopsis thaliana* (L.) Heynh seeds were grown on a pasteurized soil mix of humus and perlite (3 : 1). Seeds were kept at 4°C for 2 d and then transferred to the growth chamber. Plants were grown in a 12 h : 12 h, light : dark cycle with 65% of relative humidity, with a day temperature of 22°C and a night temperature of 19°C. Wild-type (WT) Col-0 plants were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, UK). The mutant referred to as *bdg is bdg2* (Kurdyukov *et al.*, 2006) and *lacs2.3* is described in (Bessire *et al.*, 2007). The *fls2* (SALK_062054C) mutant and the mutant referred to as *efr* was *efr-1* (SALK_044334), both previously described by Zipfel *et al.* (2004, 2006). The *A. thaliana* mutant referred to as *sid2* was *sid2-1*, *etr1* was *etr1-1*, *ein2* was *ein2-1* and *jar1* was *jar1-1*, as described in Chassot *et al.* (2007). Arabidopsis mutant *dde2-2* was previously described (von Malek *et al.*, 2002).

For sterilization, seeds were placed in a tube and shaken in 70% ethanol for 35 min at room temperature. After centrifugation at full speed, the ethanol was removed and absolute ethanol was added to the tube and immediately centrifuged at full speed. The ethanol was then removed and the seeds were placed in a sterile cabinet to dry. The sterile *A. thaliana* seeds of Col-0, *bdg* and *lacs2.3* were grown on 0.5× MS agar plates (2.16 g l^{-1} Murashige and Skoog basal salt mixture, 0.1 g l^{-1} Murashige and Skoog vitamin, 10 g l^{-1} sucrose, pH 5.7 (KOH), 8 g l^{-1} bacteriological agar). The seeds were transferred to a growth chamber for 7 d in a 16 h : 8 h, light : dark cycle with 45% humidity and temperatures of 22°C (day) and 19°C (night). The seedlings were transferred to a sterile box (Model TP1200 + TPD1200, filter code XXL; www.microbox-container.com) and grown on MS agar medium (4.33 g l^{-1} Murashige and Skoog basal salt mixture, 0.1 g l^{-1} Murashige and Skoog vitamin, 30 g l^{-1} sucrose, pH 5.7 (KOH), 7 g l^{-1} bacteriological agar) in the growth chamber under the same conditions as those used for the nonsterile seeds. The sterility of the plant material was assessed by growing plant homogenates on Petri dishes for several weeks.

Culture of *B. cinerea*, inoculation and staining of hyphae

Botrytis cinerea strains BMM, provided by Brigitte Mauch-Mani (University of Neuchâtel, Switzerland), were grown on Difco (Becton Dickinson; http://www.bd.com) 39 g l^{-1} potato dextrose agar. Spores were harvested in water and filtered through glass wool to remove hyphae. Spores were diluted in 0.25× Difco potato dextrose broth (PDB ¼; 6 g l^{-1}) at 5×10^4 spores ml^{-1} for inoculation. Droplets of 6 µl spore suspension at 5×10^4 spores ml^{-1} were deposited on nonsterile leaves of 4-wk-old plants and on sterile leaves of 5-wk-old plants. The nonsterile plants were then incubated in the growth chamber under high humidity in covered trays with water in the dark. The sterile plants were kept under high humidity by adding 2 ml of sterile water in a sterile box. The quantification of lesion size (in mm) was performed after 3 d of infection.

The treatment with microbes of phyllosphere and the infection with *B. cinerea* of nonsterile plants were performed by mixing *B. cinerea* at 5×10^4 spores ml^{-1} to the phyllosphere microbes resuspended in PDB ¼. This protocol was also used for inoculation of *B. cinerea* and strains 1, 2 and 3 on both sterile and nonsterile plants. The infection of sterile plants treated with microbes from leaf surfaces was performed by inoculation of leaves with 6 µl of microbe solution. The plants were incubated for 1 d in the growth chamber then infected with *B. cinerea* at 5×10^4 spores ml^{-1}. Fungal structures and dead plant cells were stained by boiling inoculated leaves for 5 min in a solution of alcoholic lactophenol trypan blue. Stained leaves were extensively cleared in chloral hydrate (2.5 g ml^{-1}) at room temperature by gentle shaking, and then observed using a Leica DMR microscope with bright-field settings.

Sampling of the microbes from the phyllosphere

For each ecotype, one hundred leaf discs (12.6 mm^2) were punched out with a cork borer and 15 µl per disc of 0.1 M potassium phosphate buffer pH 8 were added to the tubes. For the treatment of plants grown under nonsterile conditions, the samples were sonicated in a water bath (47 kHz ± 6%) for 2 min and vortexed for 30 s, this step was repeated two more times. The supernatant was placed in a new tube and centrifuged at 12 000 ***g*** for 20 min at 4°C. The pellet was resuspended with 12 µl of PDB ¼ per leaf disc. This was considered to be the undiluted bacterial concentration of the phyllosphere. For the treatment of sterile plants with microbes from the phyllosphere, the samples were shaken for 15 min at 25 Hz by using a mixer mill MM400 (www.rescht.com) and sonicated in a water bath (47 kHz ± 6%) for 5 min. The pellet was resuspended with 6 µl of H_2O per leaf disc.

For sequencing, the entire leaves were weighed and placed in 10 ml of 0.1 M potassium phosphate buffer pH 8 per gram of leaf material. For the extraction of phyllosphere bacteria, the protocol described above for experiments in nonsterile condition was followed.

Bacterial cultures

Bacterial strains 1, 2 and 3 were spread on Luria Broth (LB) agar medium for 24 h at 28°C, one colony obtained for each strain was spread again on a new LB agar plate and incubated for 24 h at 28°C. Some colonies were used from this plate and diluted in 100 µl of PDB ¼ and mixed. The optical density (OD) at 600 nm was measured. The bacterial suspension was diluted to obtain the approximate value presented in the Table 1. The bacterial suspension was then diluted in a 10-times series dilution until the expected concentration was obtained. For the experiment using boiled bacteria strains, the bacteria were incubated in hot water at 100°C for 25 min.

Table 1 Correspondence between optical density (OD) (600 nm) and the colony-forming units (CFU) μl^{-1} for three bacterial strains isolated from the phyllosphere

Species	OD (600 nm)	CFU μl^{-1}
Strain 1	0.027	4×10^4
Strain 2	0.011	1×10^3
Strain 3	0.015	4×10^4

Apple fruit infection

Apple fruit were disinfected in 2% sodium hypochlorite solution for 2 min and rinsed with sterile tap water. The spore suspension of *B. cinerea* was adjusted at 5×10^4 spores ml^{-1} in PDB ¼ or in a suspension of strain 3. The apples were inoculated with 10 µl of suspension at eight sites that were wounded with a sterile needle and one wounded site was inoculated with PDB ¼. The apples were put in covered trays at very high humidity and incubated 5 d in the growth chamber without direct light. Two diameters of lesions were measured for each inoculated site according to the protocol described in http://www.bio-protocol.org/e1311.

Estimation of bacterial population size

The microbes isolated from Col-0, *bdg*, *lacs2.3* or bacterial strains 1, 2 and 3 were estimated by dilution plating and expressed in colony-forming units (CFU). Twenty-five microlitres of bacterial solution were spread on LB agar plates. The plates were incubated for 24 h at 28°C and the number of colonies were determined.

In vitro toxicity of microbes from the phyllosphere on the growth of *B. cinerea*

Microbes from the phyllosphere (13.5 µl) diluted in PDB ¼ were mixed with 1.5 µl of *B. cinerea* spores in PDB ¼ to a final concentration of 5×10^4 spores ml^{-1} and placed on a glass slide. The glass slide was placed in a humid box and incubated for 16 h in a growth chamber. The growth of fungus was then observed under a microscope.

DNA extraction of samples for sequencing

Entire leaves were collected from 12 different plants per sample and five samples per ecotype were prepared. Twenty millilitres of 0.1 M potassium phosphate buffer pH 8 were added to the tubes. The samples were sonicated in a water bath (47 kHz ± 6%) for 2 min and vortexed for 30 s; this step was repeated two more times. The pellet of microbes was obtained after centrifugation at 12 000 ***g*** for 20 min at 4°C. The pellet of microbes was resuspended with the solution present in the PowerBead tubes; DNA of microbes was extracted using the Power Soil DNA Kit (http://www.mobio.com). The standard protocol described was followed but the volume of elution was 60 µl of water. Three independent experiments were performed. DNA of 250 mg samples of soil where Col-0, *bdg* and *lacs2.3* had grown (respectively) was extracted following the same procedure. A library was prepared using the primers 799F 5′-AACMGGATTAGAT ACCCKG-3′ and 1193R 5′-ACGTCATCCCCACCTTCC-3′, as previously described (Bodenhausen *et al.*, 2013). The samples were then sent to Microsynth (http://www.microsynth.ch) for sequencing using the Illumina kit (http://www.illumina.com).

Microbial community analysis

The software package MOTHUR (v.1.34.4) was used for sequence analysis (Schloss *et al.*, 2009), following the Standard Operating Procedure outlined on http://www.mothur.org/wiki/MiSeq_SOP. Briefly, the two overlapping paired-end reads were combined using make.contig. Then, each unique sequence was aligned with align.seqs to the SILVA reference alignment release 119 (Quast *et al.*, 2013). A distance matrix was calculated allowing for four mismatches. Chimeric sequences were identified using chimera.uchime and removed. Sequences matching 'Chloroplast-Mitochondria-unknown-Archaea-Eukaryota' were also removed. Next, sequences were clustered using the furthest neighbour clustering algorithm to build OTUs (operational taxonomic unit). The resulting file was parsed to separate the data for each sample. OTUs were assigned a taxonomic group with classify.seqs using the RDP reference file (Wang *et al.*, 2007) and a cut-off of 80% of the bootstrap value. For the description of the community, the sequences are split at the order level and OTUs with the same taxonomy were clustered together at a cut-off level of 0.03. METAGENASSIST (Arndt *et al.*, 2012) was used to calculate several statistics on the output of MOTHUR. Briefly, data were filtered by interquartile range (IQR) and normalized row- and column-wise by sum and Pareto scaling. The dendrogram and heat map were calculated at genus level with Spearman distance and Ward clustering algorithm. MEGAN (Huson *et al.*, 2011) was used to calculate the rarefaction curves after converting data to the biom format with MOTHUR.

DNA bacteria extraction for Pacific Bioscience sequencing

Bacterial strains 1 and 3 were spread on LB agar medium for 24 h at 28°C. One colony was resuspended in 3 ml of LB liquid and shaken at 200 min^{-1} at 28°C during 24 h in an incubator. The DNA was extracted by using a GenElute™ bacterial genomic DNA kit (Sigma-Aldrich). The protocol for Gram-negative DNA extraction described was followed, but the wash step was repeated three times and the elution volume was 100 µl of water. The DNA samples were sent to the Center for Integrative Genomic at the University of Lausanne. The libraries for Pacific Bioscience sequencing were prepared as recommended by the manufacturer, and sequenced on two SMRT cells (one for each library), leading to 800–950 Mbp of data. The reads of each SMRT cell were assembled using the SMRT pipeline (v.2.3) with the protocol HGAP3 (Chin *et al.*, 2013) leading to a single contig for genome 3, of 6374 437 bp. The GC content of genome 3 is 59.23%. Similarly, genome 1 led to a single contig of 4835 345 bp and a GC content of 61.7%. No plasmid was detected for both genomes 1 and 3.

Annotation, accession numbers and genome comparison

The genomes of strain 1 and strain 3 were annotated using PROKKA (Seemann, 2014) and deposited in the ENA database (Silvester *et al.*, 2014) under project PRJEB9391 (accession numbers: LN865164 for strain 1 and LN854573 for strain 3; ERR906813-ERR906860 for the 16S rRNA raw reads runs). The genomes of strain1 and strain 3 were compared with MAUVE (Darling *et al.*, 2010) and BRIG (Alikhan *et al.*, 2011).

DNA extraction and qPCR for bacteria quantification

Leaves of 12 different plants from Col0, *bdg* and *lacs2.3* were collected and frozen in liquid nitrogen. The leaves were ground and DNA was extracted from 100 mg of powder by using peqGold Plant DNA mini kit (http://www.peglab.com) following the protocol described, 40 µl of elution water was used. DNA was quantified using nanodrop (http://www.nanodrop.com). Ten microlitres of DNA at 10 ng μl^{-1} were mixed with 12.5 µl of Sensimix SYBR Green kit (http://www.bioline.com), 0.75 µl of primers at 10 µM and 1 µl of H_2O. The specific primers nicA for 5′-GGATCCCT CCATCATCTGG-3′ and nicA Rev 5′-CCCAGACCTTCTAC GGTACG-3′ were used to amplify the nicotinate dehydrogenase subunit A (nicA) gene of bacteria strain 3. The gene expression of nicA was normalized to the expression of the reference plant gene AT5g26751, a SHAGGY-related kinase gene (*AtSK*). The primers used for the reference gene of *A. thaliana* was iASK1 5′-CTTAT CGGATTTCTCTATGTTTGGC-3′ and iASK2 5′-GAGCTCC TGTTTATTTAACTTGTACATACC-3′. This reference gene was previously described and used to quantify *B. cinerea* in leaves (Gachon & Saindrenan, 2004). The qPCR was performed using the program described in the publication of (Gachon & Saindrenan, 2004).

Statistical analyses

One-way ANOVA followed by Tukey's test was performed using the SPSS statistics program (University of Fribourg license; IBM company). For comparison between only two samples, a *t*-test was used. Different letters or asterisks above each bar represent statistically significant differences (Tukey's test and *t*-test; $P < 0.05$). The difference in abundance of bacterial genera was tested pairwise between *bdg* and the other samples (*lacs2.3*, Col-0, soil) using the Metastats website (White *et al.*, 2009). Metastats is based on the metagenomeSeq R package and allows comparison of metagenomic samples on the basis of count data. We used 100 permutations of the data (*bdg* $n = 15$, Col-0 $n = 15$, *lacs2.3* $n = 15$, soil $n = 3$) to estimate the null distribution. *P*-values below 0.05 were considered significant.

Results

The importance of the leaf phyllosphere in the resistance to pathogens

In order to determine the implication of phyllosphere microbes in the resistance of *A. thaliana* to pathogens, seeds of WT Col-0 and cuticular mutants *bdg* and *lacs2.3* were surface-sterilized, grown under sterile conditions and inoculated with *B. cinerea*. Under sterile conditions, the *bdg* mutant lost a large part of its resistance to *B. cinerea* compared to nonsterile conditions. By contrast, the *lacs2.3* mutant was equally resistant and WT was susceptible in both conditions (Fig. 1). *Botrytis cinerea* displayed clear hyphal growth as visualized by Trypan blue staining in Col-0 leaves in sterile and nonsterile conditions, as well as in leaves of *bdg* under sterile conditions (Supporting Information Fig. S1). Thus, the microbes of the phyllosphere might potentially affect the outcome of an inoculation with the fungal pathogen *B. cinerea*.

In order to further test their importance, the phyllosphere microbes were washed off leaves of unsterile plants and reintroduced on leaf surfaces of sterile plants. When *B. cinerea* was inoculated on sterile plants at sites that were pre-treated with the phyllosphere microbes of the corresponding nonsterile plants, *bdg* became significantly more resistant, whereas *lacs2.3* remained resistant and WT plants remained susceptible to *B. cinerea* (Fig. 2). In this experiment, bacteria were extracted from a known total surface area and re-applied to a similar area on the receiver plants. This allowed treatment with a bacterial concentration that remained at best equal but more likely slightly lower than that on donor leaves, given unavoidable losses during extraction. This experiment demonstrates the functional importance of phyllosphere microbes in the resistance of *bdg* to *B. cinerea*.

Is the composition of phyllosphere different in WT and cuticular mutants?

We tested the hypothesis that cuticular mutants harbour different populations of bacteria on the leaf surface. To this end, we used surface washes of leaf material from plants or samples from the soil mixture where the plants were grown. We analysed the bacterial community by sequencing of the variable regions V5, V6 and V7 of the bacterial 16S ribosomal DNA (rDNA) genes. After sequencing we obtained 8191 004 raw paired-end reads spread over 48 samples (min 107 000, max 273 000, median 158 000

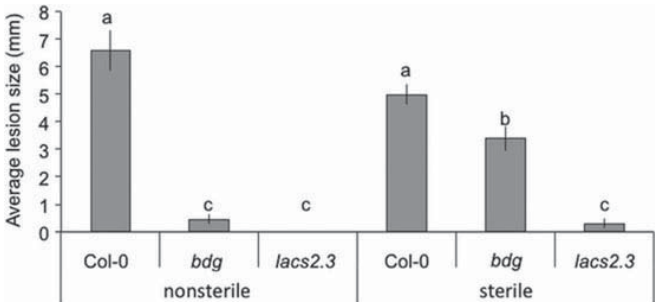

Fig. 1 Resistance of *Arabidopsis thaliana* Col-0 plants and cuticle mutants against *Botrytis cinerea* under sterile conditions. Col-0 plants and cuticle mutants (*bdg*, *lacs2.3*) were inoculated with *B. cinerea* under nonsterile (left) or sterile conditions (right); ($n = 30$; ± SE). The average lesion size was determined 3 d after inoculation with *B. cinerea*. The experiment was repeated three times with similar results. Different letters above each bar represent statistically significant differences (Tukey's test; $P < 0.05$).

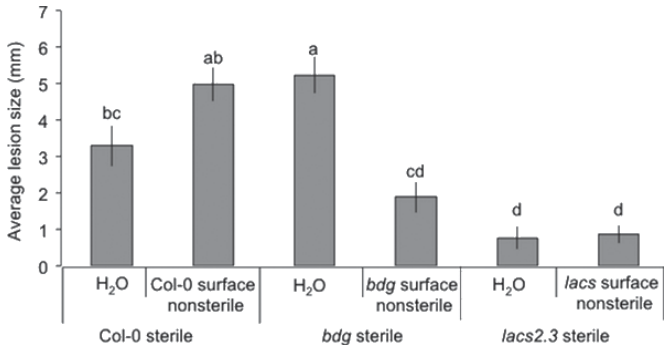

Fig. 2 Resistance of sterile *Arabidopsis thaliana* Col-0 and cuticle mutants against *Botrytis cinerea* after treatment with the microbes of their respective phyllosphere extracted from nonsterile plants. Sterile Col-0, *bdg* and *lacs2.3* were pre-treated for 1 d with their respective phyllosphere microbes from nonsterile plants before infection with *B. cinerea* ($n = 30$; ± SE). The average lesion size was determined 3 d after inoculation with *B. cinerea*. Different letters above each bar represent statistically significant differences (Tukey's test; $P < 0.05$). The experiment was repeated twice with similar results.

reads). Despite the drop in quality of the MiSeq reads after 200 bp, we obtained 5139 594 high quality 413-bp contigs by combining the overlapping forward and reverse reads. Those contigs were further cleaned and analysed with Mothur. The final distribution of 16S rRNA unique contigs for the 48 samples was median 66 131 min 5648 and max 124 272. As shown in the rarefaction curves (Fig. S2), most of the samples (41/48) reached saturation with at least 30 000 contigs, meaning that at least 90% of the diversity was sampled.

The top eight most abundant bacterial genera present in *bdg* are shown in Fig. 3. As a comparison, the same genera were determined in *lacs2.3* and WT plants, as well as in the potting soil (an extension to the 20 most abundant genera in *bdg* is shown in Fig. S3). There was a clear difference between composition of the genera in cuticle mutants where the most abundant genera were *Pseudomonas* and *Rhizobium*. In WT plants and in the soil, *Burkholderia* was the most abundant genus (Fig. 3). A dendrogram and heat map analysis was used to compare the clustering of the genera in all of the samples collected from the potting soils and surface washes from WT, *bdg* and *lacs2.3* (Fig. 4). It should be noted that the abundances shown in the heat map cannot be directly compared with the mean abundances in Fig. 3 because they represent individual abundances. As expected, the samples from the soils used for WT, *bdg* and *lacs2.3* all clustered closely together. The samples of washed surfaces of all plants showed a different clustering than that observed in the soil samples indicating that a selection of bacteria had taken place. Furthermore, the bacteria from the leaf washes of WT clustered together and were closer to the soil samples, but could readily be separated from the samples of the leaf washes of the cuticle mutants. By contrast, the bacterial genera from the *bdg* mutants could not be clearly demarked from those of the *lacs2.3* mutants. This analysis demonstrates that the bacterial community is different on the cuticle mutants compared to WT Col-0 plants. It also highlights the impact of a mutation in a single gene responsible for the cuticular structure on the microbiome of the phyllosphere.

Can the microbes present on the leaf surfaces of cuticular mutants also provide resistance on WT plants?

Fig. 5 shows that microbes extracted from the phyllospheres of both cuticle mutants were most efficient in inducing resistance in Col-0 against an infection by *B. cinerea*. The concentration of the bacteria applied to the surface was estimated to be in the same order of magnitude or slightly smaller than that of the donor leaf, assuming that the extraction is complete and the dispersal of the bacteria is uniform on the leaf surface. Microbes from surface washes of WT plants were effective to a lesser extent (Fig. 5). At

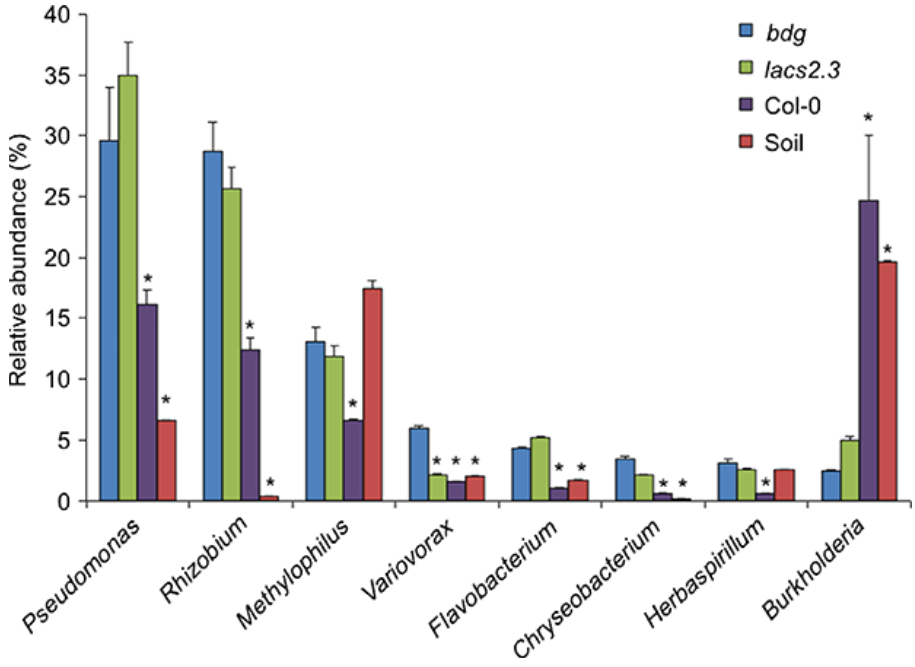

Fig. 3 Top eight most abundant genera in *bdg* and the relative abundance in *Arabidopsis thaliana lacs2.3* mutants, Col-0 plants and soil samples compared to *bdg* (chosen arbitrarily as a reference because it was the most interesting mutant). Asterisks denote significant difference compared to *bdg* ($n = 15$; error bars denote variance within each respective group; $P < 0.05$).

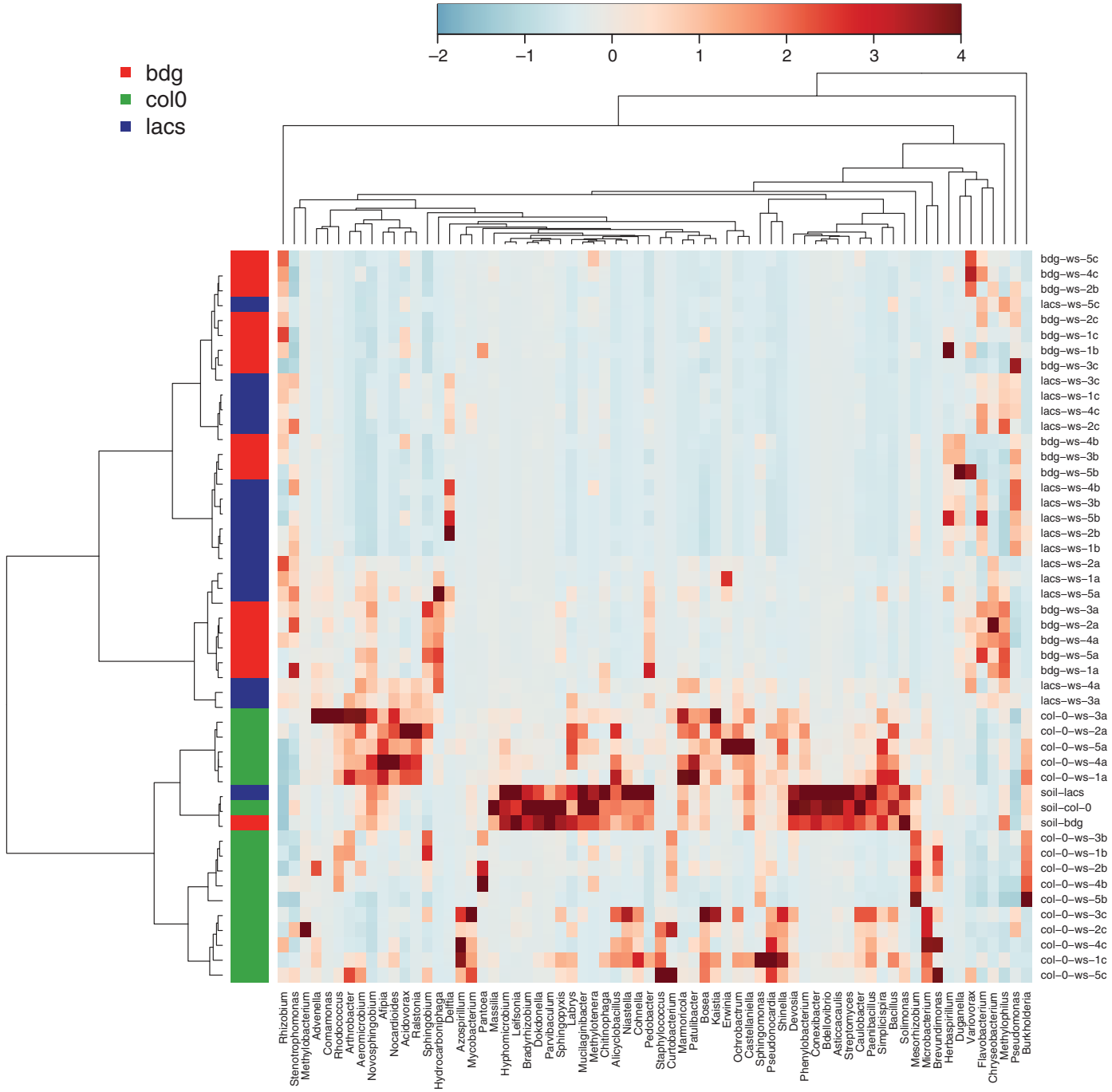

Fig. 4 Dendrogram analysis of the bacterial genera isolated from the soil of pots or from the phyllosphere of *Arabidopsis thaliana* Col-0, *bdg* or *lacs2.3* plants. The analysis shows the relatedness between experiments (left) and the top bacterial genera for each of the five samples in three independent experiments.

1/10 and 1/20 dilutions the effect decreased, although it was still effective with washes from surfaces of *lacs2.3* leaves. A control experiment where the washed surfaces of sterile plants were used to pre-treat nonsterile Col-0 plants gave no protection, indicating that the protective activity derives from the bacteria or their products (Fig. S4). The cultivatable microbes from surface washes did not show any fungitoxic activity when tested *in vitro* against *B. cinerea* (Fig. S5). This implies that the bacteria have an indirect effect via the plants. Thus, phyllosphere bacteria can provide resistance to *B. cinerea* when added to the leaf surface of nonsterile WT Col-0 plants.

Is it possible to isolate single strains of bacteria from surface washes with protective activity against *B. cinerea*?

When surface washes of the phyllosphere of *bdg* leaves were plated out on petri dishes, several colonies had a conspicuous morphology (size, colour, shape). We selected three colonies

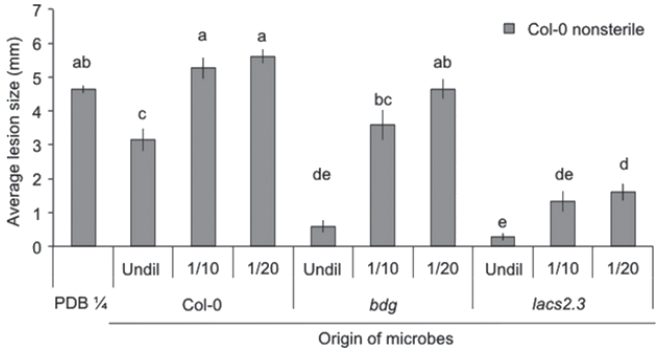

Fig. 5 Effect of microbes extracted from the phyllosphere of *Arabidopsis thaliana* Col-0 plants and cuticle mutants on the resistance of *A. thaliana* Col-0 plants to *Botrytis cinerea*. The microbes were washed off leaf surfaces from Col-0, *bdg* and *lacs2.3*. The Col-0 plants were inoculated using droplets with *B. cinerea* mixed with the microbes undiluted (undil) or diluted to 1/10 and 1/20 in PDB ¼ ($n = 48$; ± SE). The average lesion size was determined 3 d after inoculation with *B. cinerea*. Different letters above each bar represent statistically significant differences (Tukey's test; $P < 0.05$). The experiment was repeated twice with similar results.

(referred to as strains 1, 2 and 3) and tested them individually on WT Col-0 plants against *B. cinerea*. When applied to nonsterile leaf surfaces of WT Col-0 plants, the bacterial strains 1 and 2 gave a partial (*c.* 33%) protection, whereas strain 3 gave *c.* 84% protection to *B. cinerea* (Fig. 6). When plated on King's B medium, strain 3 displayed a fluorescent halo when viewed under UV light, so we presumed that it belongs to the genus *Pseudomonas*. The hyper variable region 16S of these three strains was sequenced: strains 1 and 3 correspond to the genus *Pseudomonas* and strain 2 could be assigned to the genus *Bacillus*. Strains 1 and 3 were studied further and sequenced on the Pacific

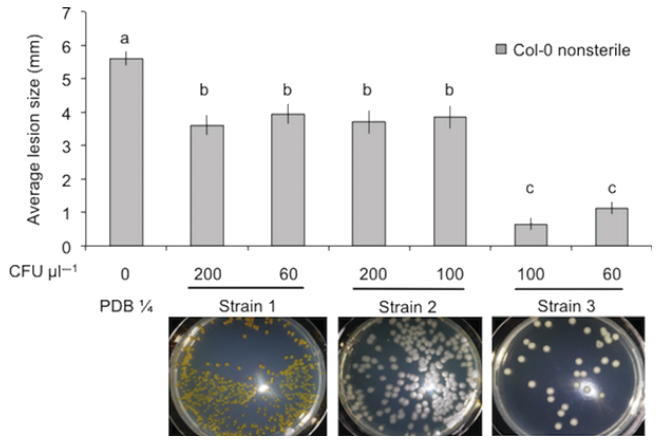

Fig. 6 Effect of individual microbial strains extracted from the phyllosphere of *bdg* mutant on the resistance of *Arabidopsis thaliana* Col-0 plants to *Botrytis cinerea*. The bacterial strains 1, 2 or 3 isolated from surface washes of *bdg* mutants were diluted in PDB ¼ at different concentrations (in colony-forming units (CFU) μl^{-1}) then mixed with *B. cinerea* and inoculated with droplets on Col-0 plants ($n = 24$; ± SE). The average lesion size was determined 3 d after inoculation with *B. cinerea*. A representative picture of the bacterial strains is placed below each histogram. Different letters above each bar represent statistically significant differences (Tukey's test; $P < 0.05$). The experiment was carried out three times with similar results.

Biosciences sequencer of the Lausanne Genomic Technologies Facility. The library was prepared according to the manufacturer's recommendations and sequenced on a single SMRT cell. The coverage of the raw data is *c.* 150×. We assembled the reads using the SMRT Pipeline with the HGAP3 protocol and obtained for strain 3 a single contig of 6374 437 bp with a GC content of 59.23%. No plasmid was detected. With reference to public databases, we found *P.* sp. URMO17WK12:I12 (Genbank AC: GCA_000514395.1) to be a very close draft genome of 33 contigs with a size of 6568 431 bp and GC content of 59.1% (ANI vs strain 3 = 99.56%). The closest finished genome is *P. mandelii* strain JR-1 (genome sequence of cold-adapted *P. mandelii* strain JR-1 (Jang *et al.*, 2012)), but it is more distantly related (ANI = 87.9%) as shown in Fig. S6(a,b). Strain 1 was assembled similarly to a single contig of 4835 345 bp with a GC content of 61.7%. No plasmid was detected. In the public databases, we found *P.* sp. URMO17WK12:I11 (Genbank acc. no. GCA_000514235.1), a very close draft genome of 44 contigs with a size of 4693 207 bp and a GC content of 61.9% (ANI vs strain 1 = 99.59%) as shown in Fig. S6(c). We therefore named strain 3 as *P.* sp. *friburgensis*. *P.* sp. *friburgensis* was active when inoculated on WT plants, showing that its effect is dose dependent and can take place alone without other members of the bacterial community (Fig. S7). *P.* sp. *friburgensis* could act independently of other members of the community because it was also active on WT plants and *bdg* mutants grown under sterile conditions (Fig. S8). Interestingly, PCR analysis using primers specific for *P.* sp. *friburgensis* showed that this strain was most abundant in *bdg* mutant plants whereas strain 3 is present at much lower amounts in WT Col-0 and *lacs2.3* (Fig. 7). This result underlines once more the qualitative differences in the composition of the bacterial population of the phyllosphere. The result in Fig. 3 shows that the *Pseudomonas* genus as a whole is more abundant in both cuticle mutants. Because neither strains 1, 2 nor *P. sp. friburgensis* showed a direct activity against *B. cinerea*, we tested if *P.* sp. *friburgensis* still could protect after boiling. Our results showed that after boiling the biological activity was lost (Fig. S9). The same results were observed with boiled phyllosphere microbes extracted from Col-0, *bdg* and *lacs2.3* (data not shown). Therefore, the effect of *P.* sp. *friburgensis* on

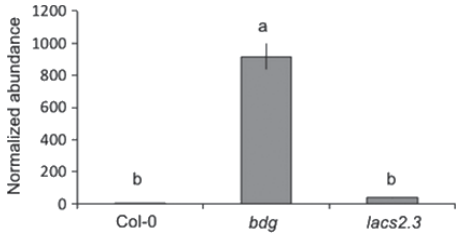

Fig. 7 Relative quantification of DNA from strain 3 (*Pseudomonas sp. friburgensis*) in *Arabidopsis thaliana* Col-0 plants and cuticle mutants. DNA from Col-0, *bdg*, *lacs2.3* leaves were extracted and the relative quantification of strain 3 DNA in the different samples were determined by qPCR ($n = 3$; ± SD). Different letters above each bar represent statistically significant differences (Tukey's test; $P < 0.05$). The experiment was carried out three times with similar results.

the resistance of the plant is heat-labile. In addition, we tested a filtered (0.2 μm) culture of *P.* sp. *friburgensis* in PDB ¼ incubated 3 d in growth chamber (corresponding to the infection timing). In this condition, no active molecules induced the resistance of the plant against *B. cinerea* (data not shown). The effect of *P.* sp. *friburgensis* was still effective in mutants affected in salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling (Fig. S10) and in mutants of receptors for PAMPS such as *fls2* and *efr* compared to WT Col-0 (Fig. S11).

Discussion

The *bdg* and *lacs2.3* mutants of *Arabidopsis thaliana* exhibit alterations in the architecture of the cuticle that typically lead to increased permeability and resistance to *Botrytis cinerea* (Bessire *et al.*, 2007; Serrano *et al.*, 2014). These mutants provided an interesting starting point for the work described here, and the question was asked whether the increased resistance in cuticle mutants could be explained by changes in the microbiome of the phyllosphere. To test this, we determined the resistance of plants under sterile conditions. Under these conditions, the resistance exhibited by the *bdg* mutant was lost to a large extent, whereas *lacs2.3* retained full resistance (Fig. 1). The conditions used for sterile conditions are different than those in nonsterile pots, but the plants can nevertheless be inoculated and show typical grey mould symptoms (Fig. 1). The lesion size correlated with the growth of *B. cinerea* in Col-0 and *bdg* grown in sterile conditions (Fig. S1). Microbes of the phyllosphere were washed off leaves of unsterile plants and applied to leaves of the same plant type grown under sterile conditions before inoculation. The *bdg* mutant pretreated with microbes regained resistance to *B. cinerea* at a comparable level as that observed under nonsterile conditions, demonstrating the importance of microbes of the phyllosphere for resistance (Fig. 2). The resistance of *lacs2.3* is likely to be controlled by endogenous factors because it remained resistant under sterile conditions although an additional effect by phyllosphere microbes cannot be completely ruled out (Figs 1, 2).

This observation led us to explore the microbial diversity of the phyllosphere from these plants and possibly determine if the resistance could be related to any particular resident species. Sequencing of 16S rDNA amplicons indicated that the most abundant bacterial taxa in wild-type (WT) Col-0 plants and in cuticle mutants belonged to the Proteobacteria phylum (*Burkholderia*, *Pseudomonas*, *Rhizobium* and *Methylophilus*) (Figs 3, S3). The relative proportion of these four genera was clearly different between the potting soil and in the plant phyllosphere. Most likely, the microbes are recruited from the soil, but other sources (air, handling) cannot be excluded. Overall, a relative consistency was observed among the genera on the different plants (Figs 3, 4, S3). *Burkholderia*, *Pseudomonas* and *Methylophilus* are among the major genera reported in the phylloplane of *A. thaliana* and other plants (Vorholt, 2012; Reisberg *et al.*, 2013; Bodenhausen *et al.*, 2014). Obviously, the physical and biochemical properties of the plant leaf surface, as well as environmental factors determine the composition of the microbes it harbours (Vorholt, 2012; Copeland *et al.*, 2015). The relative abundance of the four genera in the WT Col-0 was distinct from the cuticle mutants (Figs 3, S3). Because common features characterize both mutants but above all an increased permeability, this feature might possibly affect the hydration, the nutritional properties of the surface or the surface architecture, providing a specific niche for microbes. A dendrogram analysis that takes into account all samples (e.g. five samples per plant type and three replicates) leads to similar conclusions (Fig. 4). The bacterial community present on the washed surfaces of WT Col-0 can be distinguished from those on the cuticular mutants, and the bacterial community of *bdg* and *lacs2.3* are clustering closely together without a specific pattern (Fig. 4).

Can bacteria from the phyllosphere of the *bdg* mutant also protect WT plants grown under nonsterile conditions? This question assumes that the bacterial community of the *bdg* mutant contains one or several unique species that might have the power to interfere with the interaction between *A. thaliana* and *B. cinerea*. Bacteria were washed off a determined surface area from a source plant and applied to a similar surface on the leaf of a recipient plant (see Materials and Methods) allowing for comparisons between leaf washes from different plant types. The undiluted leaf washes originating from *bdg* or *lacs2.3* had a strong protective effect against *B. cinerea*, an effect that diminished upon dilution more so in washes from *bdg* than from *lacs2.3* (Fig. 5). This observation might be explained by the specific composition of the bacterial community on leaves of cuticular mutants and implies the occurrence of species with protective properties in the phyllosphere of cuticular mutants. The resistance observed is not due to a direct activity of the bacterial community on the spore germination (Fig. S5). Besides, a simple increase in the amount bacteria of the phyllosphere could also be involved in the protection, because WT Col-0 plants treated with a supplement of WT Col-0 bacteria also resulted in a minor increase in resistance to *B. cinerea* (Fig. 5). Overall, the colony-forming units (CFU cm^{-2}) were indeed higher in *lacs2.3* compared to WT Col-0, whereas the CFU cm^{-2} of *bdg* was intermediate between *lacs2.3* and Col-0 (Fig. S12).

We isolated 30 colonies from the phyllosphere of *bdg* based on their morphology and colour. Of those we picked out three isolates that appeared conspicuous (yellow colour for isolate 1, matt surface for isolate 2 and shiny surface for isolate 3; see Fig. 6), carried out protection assays and found one strain to be active in protecting *A. thaliana* WT Col-0 against *B. cinerea* whether applied to sterile or nonsterile leaf surfaces (Figs 6, S7, S8). The 16S hyper variable region of these three isolates corresponds to the genus *Pseudomonas* for the strains 1 and 3 and to the genus *Bacillus* for strain 2. The genus *Pseudomonas* is present among the genera isolated from *bdg* phyllosphere, whereas the genus *Bacillus* was not detected (Figs 3, 4, S3). This observation suggests that strain 2 might be present in very low abundance, thus escaping detection. Alternatively, it might be a contamination. Thus, we were able to isolate a species, strain 3, from the leaf washes of the *bdg* phyllosphere that had the power to protect *A. thaliana* WT Col-0 against *B. cinerea* whether applied to sterile or nonsterile leaf surfaces (Figs 6, S7, S8). We referred to

strain 3 as *P.* sp. *friburgensis* and this strain shows near identity (ANI = 99.56%) with an unpublished draft genome for *P.* sp. *URMO17WK12:I12* (Fig. S6). Interestingly this bacterium was also isolated from corn (R. Ley, pers. comm.), but its possible biological function has not been studied. The action of *P.* sp. *friburgensis* is indirect because it has no toxic effect when co-incubated with *B. cinerea in vitro* (data not shown). Antibiosis might, however, still take place on the phyllosphere where nutritional conditions are different. Also, other effects such as competition for iron or other ions or molecules might also take place. The production or release of a putative active compound requires living cells, because treatments with boiled *P.* sp. *friburgensis* (Fig. S9) or phyllosphere microbes from Col-0, *bdg* and *lacs2.3* (data not shown) completely abolished biological activity. However, a degradation of the compound by heat cannot be excluded. The loss of activity of a culture filtrate (filtered through a 0.2-µm pore size) of *P.* sp. *friburgensis* indicates that such a compound is likely to be released *in planta* only (data not shown) or that living cells are required to induce the activity. The effect of *P.* sp. *friburgensis* was still effective in mutants affected in salicylic acid, jasmonic acid and ethylene signalling compared to WT Col-0 (Fig. S10). An effect on the induction of plant resistance mechanisms would have to be sought elsewhere. The response to common bacterial pathogen-associated molecular patterns (PAMPs) such as flg22 and elf18 was also explored. Mutants of the receptors for flg22 or elf18 were still responsive to *P.* sp. *friburgensis*, indicating that this bacterium is detected by other receptors (Fig. S11). These intriguing observations need now to be followed up to understand how *P.* sp. *friburgensis* affects the defences of the plant. Importantly, the activity of *P.* sp. *friburgensis* also extended to other plant species, as shown by our experiments with apple (Fig. S13). Other observations have also documented the action of bacteria from the phyllosphere against foliar pathogens (Innerebner *et al.*, 2011). Specific strains of *P. cepacia* have been shown to control *B. cinerea* and other postharvest diseases, but their mode of action has not always been well documented (Janisiewicz & Korsten, 2002). A number of *P. syringae* strains isolated from the rhizosphere were shown to have an antagonistic activity against *B. cinerea* (Hernandez-Leon *et al.*, 2015). They were found to produce antibiotics, some of which are volatile, as well as plant growth-promoting substances. Thus, the genus *Pseudomonas* appears to harbour interesting potential biocontrol strains with a relatively nonspecific host range that display a wide range of mechanisms of action (Santoyo *et al.*, 2012).

The phyllosphere might be viewed as an external shell providing a first line of defence against mostly nonpathogens, whereas true pathogens have co-evolved to cope with it. Because the microbiomes are different from one plant species to another, changing the original composition of the microbes, for example with phyllosphere microbes from another species (cross-inoculation) would be highly instructional to determine the importance of the microbiome of a plant species as a protective layer. These considerations made us aware that the microbiome of the outer boundary of plants has not really been taken into consideration in our studies on plant disease resistance mechanisms. Future work should be directed at exploring this promising area. To a certain degree our results are in tune with recent data on the function of microbiomes associated with other animals or humans. These data highlight the importance of the phyllosphere as a source of potential biological plant protectants. Efforts in searching for novel active strains against foliar pathogens should definitely target this outer layer of the leaves.

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Author contributions

J-P.M. and F.L. planned and designed the research. U.R., A.V., A.B., L.F. and F.L. performed experiments, conducted fieldwork and analysed data. F.L., L.F., U.R, A.V. and J-P.M. interpreted the data. J-P.M., F.L. and L.F. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The growth of *B. cinerea* on leaves of *A. thaliana* Col-0, *bdg* and *lacs2.3* in sterile and nonsterile conditions determined by trypan blue staining.

Fig. S2 Rarefaction curves.

Fig. S3 Top 20 most abundant genera in *bdg* and the relative abundance in *A. thaliana* Col-0 plants, *lacs2-3* and soil samples compared to *bdg*.

Fig. S4 Effect of surface washes of leaves from sterile *A. thaliana* Col-0 plants and cuticle mutants on the resistance of nonsterile Col-0 plants to *B. cinerea*.

Fig. S5 The activity of phyllosphere microbes from *A. thaliana* Col-0, *bdg* and *lacs2.3* on the spore germination of *B. cinerea in vitro*.

Fig. S6 Genome of strain 3 and strain 1.

Fig. S7 Dose-dependent effect of strain 3 (*P. sp. friburgensis*) on the resistance of *A. thaliana* Col-0 plants to *B. cinerea*.

Fig. S8 Effect of strain 3 (*P. sp. friburgensis*) on the resistance of sterile *A. thaliana* Col-0 plants and cuticle mutants to *B. cinerea*.

Fig. S9 Effect of boiled strain 3 (*P. sp. friburgensis*) on the resistance of *A. thaliana* Col-0 plants to *B. cinerea*.

Fig. S10 Effect of strain 3 (*P. sp. friburgensis*) on the resistance of *A. thaliana* Col-0 plants and SA-, JA- and ET- signalling mutants to *B. cinerea*.

Fig. S11 Effect of strain 3 (*P. sp. friburgensis*) on the resistance of *A. thaliana* Col-0 plants and PAMP receptor mutants to *B. cinerea*.

Fig. S12 Average of colony forming units (CFU) cm^{-2} extracted from the phyllosphere of *A. thaliana* Col-0 plants and cuticle mutants.

Fig. S13 Effect of strain 3 (*P. sp. friburgensis*) on the resistance of apple fruit to *B. cinerea*.