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Brief Report

Characterization of glyphosate-resistant *Burkholderia anthina* and *Burkholderia cenocepacia* isolates from a commercial Roundup® solution

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Summary

Roundup® is the brand name for herbicide solutions containing glyphosate, which specifically inhibits the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase of the shikimate pathway. The inhibition of the EPSP synthase causes plant death because EPSP is required for biosynthesis of aromatic amino acids. Glyphosate also inhibits the growth of archaea, bacteria, Apicomplexa, algae and fungi possessing an EPSP synthase. Here, we have characterized two glyphosate-resistant bacteria from a Roundup solution. Taxonomic classification revealed that the isolates 1CH1 and 2CH1 are *Burkholderia anthina* and *Burkholderia cenocepacia* strains respectively. Both isolates cannot utilize glyphosate as a source of phosphorus and synthesize glyphosate-sensitive EPSP synthase variants. *Burkholderia. anthina* 1CH1 and *B. cenocepacia* 2CH1 tolerate high levels of glyphosate because the herbicide is not taken up by the bacteria. Previously, it has been observed that the exposure of soil bacteria to herbicides like glyphosate promotes the development of antibiotic resistances. Antibiotic sensitivity testing revealed that the only the *B. cenocepacia* 2CH1 isolate showed increased resistance to a variety of antibiotics. Thus, the adaptation of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 to glyphosate did not generally increase the antibiotic resistance of both bacteria. However, our study confirms the genomic adaptability of bacteria belonging to the genus *Burkholderia*.

Introduction

Roundup is the brand name for a solution containing the systemic broad-spectrum herbicide glyphosate [*N*-(phosphonomethyl(glycine)] (Franz, 1979; Duke and Powles, 2008). Glyphosate specifically inhibits the 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase of the shikimate pathway (Steinrücken and Amrhein, 1980). The EPSP synthase converts phosphoenolpyruvate (PEP) and shikimate-3-phosphate to EPSP, which is an essential precursor for *de novo* synthesis of phenylalanine, tyrosine and tryptophan (Steinrücken and Amrhein, 1980; Herrmann and Weaver, 1999). The glyphosate-dependent inactivation of the EPSP synthase results in the depletion of the cellular levels of the three amino acids and thus in plant death (Gresshoff, 1979; Fischer *et al*., 1986; Wicke *et al*., 2019). Glyphosate targets the PEP binding site of the EPSP synthase, thereby acting as a competitive

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inhibitor of the enzyme (Schönbrunn *et al*., 2001). As the inhibition of the EPSP synthase impairs the production of aromatic amino acids, it is not surprising that the herbicide has a severe effect on the general physiology of organisms including bacteria. Indeed, a transcriptome analysis revealed that more than 1000 genes were differentially expressed in *Escherichia coli* when the bacteria were exposed to glyphosate (Lu *et al*., 2013). It has also been observed that the exposure of soil bacteria to herbicides like glyphosate promotes the development of antibiotic resistances (Liao *et al*., 2021).

The isolation of glyphosate-insensitive bacterial EPSP synthases and their use to engineer crops tolerating the herbicide allowed to control weed growth by applying Roundup without harming the desired plants. This development paved the way for the massive use of glyphosate in agriculture (Duke and Powles, 2008). The intensive use of glyphosate over the past years has placed a strong selective pressure on plants and microbes to evolve mechanisms of resistance such as mutations in the EPSP synthase gene as well as mutations resulting in increased expression of the target and altered herbicide transport (Chekan *et al*., 2016; Wicke *et al*., 2019; Hertel *et al*., 2021). The Pro106Ser EPSP synthase variant with decreased glyphosate sensitivity but without significant kinetic changes as compared to the wild type enzyme was first identified in the weed goosegrass (Bradshaw *et al*., 1997). In the following years, the Pro106Ser EPSP synthase variant was identified in other glyphosate-resistant plants (Chekan *et al*., 2016). In addition to the evolution of insensitive EPSP synthase variants, plants and bacteria can overcome glyphosate toxicity by acquiring multiple EPSP synthase gene copies by selective gene amplification (Gaines *et al*., 2010; Wicke *et al*., 2019; Hertel *et al*., 2021). The overproduction of a glyphosate-sensitive EPSP synthase enables the organism to titrate the herbicide and a small fraction of the EPSP synthase can function normally. The evolution of glyphosate resistance due to altered transport has also been observed in plants and bacteria (Ge *et al*., 2014; Wicke *et al*., 2019). For instance, the Gram-positive soil bacterium *Bacillus subtilis* rapidly acquires glyphosate resistance by inactivating the genes encoding high- and low-affinity glutamate transporters (Wicke *et al*., 2019). Furthermore, the breakdown of the herbicide by a large number of bacteria can be seen as another mechanism of glyphosate resistance (Hove-Jensen *et al*., 2014). Finally, bacteria can tolerate glyphosate due to covalent modification of the herbicide. For instance, it has been shown that the hygromycin phosphotransferases from *E. coli* and *Burkholderia pseudomallei* detoxify glyphosate by phosphorylating the herbicide (Rao *et al*., 1983; Penaloza-Vazquez *et al*., 1995).

Here, we have characterized the glyphosate-resistant strains *Burkholderia anthina* 1CH1 and *Burkholderia cenocepacia* 2CH1 that were isolated from a commercial Roundup solution. Both strains are resistant to high amounts of glyphosate but do not degrade or grow with the herbicide. Glyphosate is likely not taken up by *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 because the EPSP synthases of the strains are glyphosate sensitive. We have also determined the first closed genome sequence of a *B. anthina* isolate. Surprisingly, antibiotic sensitivity testing uncovered that the *B. cenocepacia* 2CH1 strain is resistant to various antibiotics.

Results

Isolation and microscopic analysis of glyphosate-resistant bacteria from commercial Roundup solutions

Recently, we have demonstrated that the transporter GltT mediates glyphosate uptake in *Bacillus subtilis* (Wicke *et al*., 2019). When carrying out growth experiments with Roundup containing 41 mM (6.9 g L^{-1}) glyphosate, we observed that the glyphosate solution was contaminated with bacteria (see Experimental procedures for more information). Plating experiments with Roundup spray bottles 1 (CH1) and 2 (CH2) uncovered 3.1×10^6 and 1.4×10^6 colony-forming units per ml respectively, present in the solutions (Fig. 1A). Eight colonies with different morphology were selected for the taxonomic classification (16S rRNA gene sequencing, Supporting information). Spray bottle 1 (CH1) contained four bacterial isolates related to *Burkholderia* sp. and one each related to *Pseudomonas* and *Staphylococcus* (Table S4). Spray bottle 2 (CH2) contained two bacterial isolates related to *Burkholderia*. To conclude, the Roundup solutions contained many bacteria, which could be assigned to three different genera.

Previously, C-Glc minimal medium lacking amino acids is suitable to test the susceptibility of bacteria to glyphosate because aromatic amino acids and the amino acids of the glutamate family reduce glyphosate toxicity (see Supporting Information) (Wicke *et al*., 2019). Growth experiments revealed that only the *Burkholderia* isolates 1CH1 and 2CH1 grew in C-Glc minimal medium (data not shown). Therefore, the other bacterial isolates were no longer considered for further experiments. Next, we assessed the potential of the remaining isolates to grow in the presence of glyphosate. The bacteria were propagated on C-Glc plates without and with 10 mM glyphosate. The *E. coli* and *B. subtilis* laboratory strains W3110 and SP1 respectively, served as controls. All bacteria grew on the C-Glc plates and only the *Burkholderia* isolates 1CH1 and 2CH1 grew in the presence of 10 mM glyphosate (Fig. 1B). Thus, the commercial Roundup

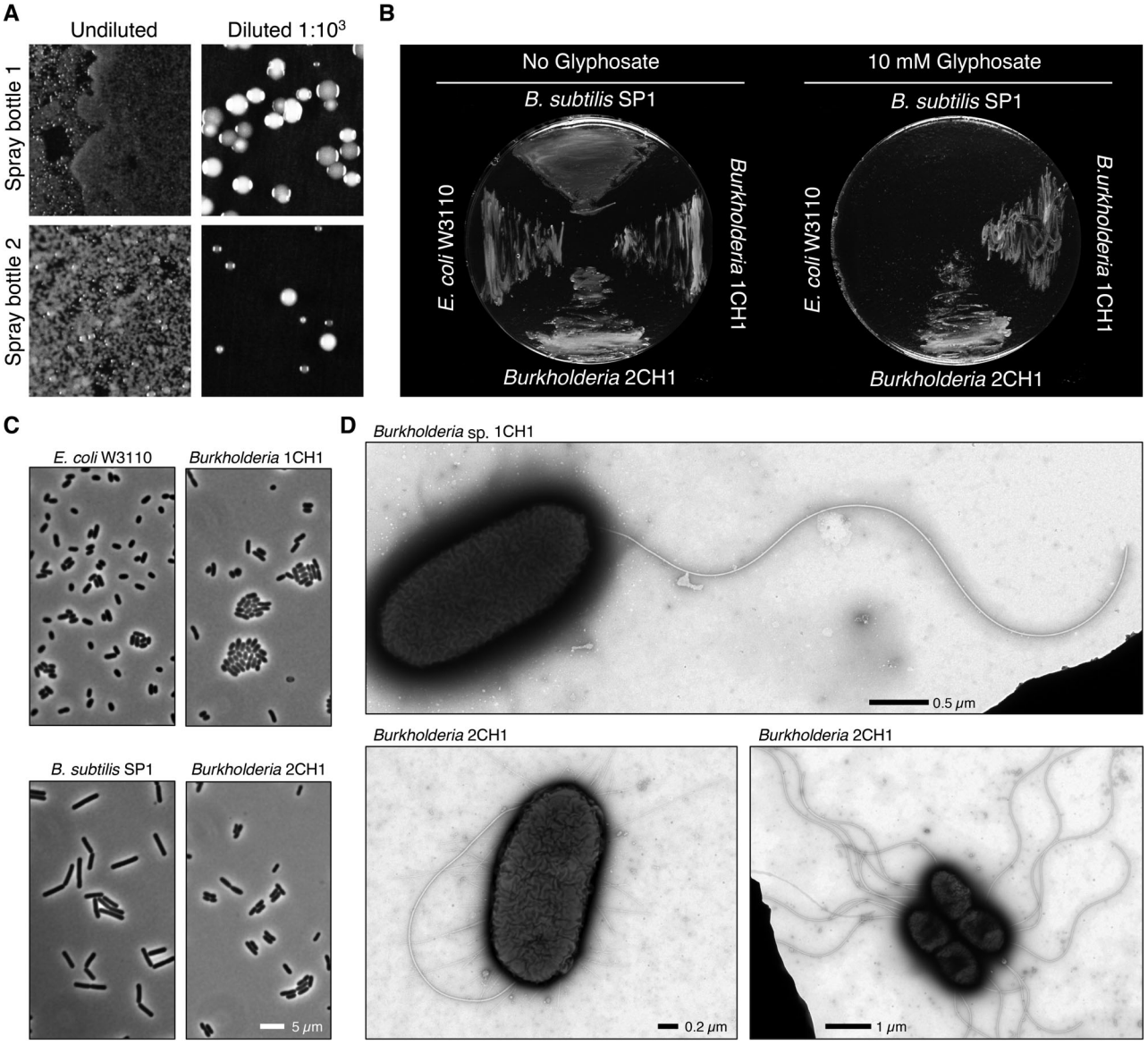

Fig. 1. Isolation and microscopic analysis of glyphosate-resistant *Burkholderia* isolates. A. Bacterial colonies that appeared after propagating 100 μl of the non-diluted and diluted Roundup bottles CH1 and CH2 on BHI plates (incubation for 2 days, 37°C). B. Growth of the *B. subtilis* and *E. coli* strains SP1 and W3110 respectively, and the *Burkholderia* isolates 1CH1 and 2CH1 on C-Glc minimal medium plates without and with 10 mM glyphosate (incubation for 2 days, 37°C). C. Light microscopic analysis of *B. subtilis* SP1, *E. coli* W3110 and the *Burkholderia* isolates 1CH1 and 2CH1. D. TEM analysis of the *Burkholderia* isolates 1CH1 and 2CH1.

contained at least two bacterial isolates that grew in the presence of 10 mM glyphosate.

The assessment of the cell morphologies of the *Burkholderia* isolates 1CH1 and 2CH1 by light microscopy revealed that the *Burkholderia* isolates formed rod-shaped and 2–3 μm long cells, resembling the *E. coli* cells (2–3 μm), which were considerably shorter than *B. subtilis* cells (3–4 μm) (Fig. 1C). The *Burkholderia* isolates 1CH1 and 2CH1 were motile (data not shown), most likely due to flagella as previously shown for *Burkholderia terrae* (Yang *et al*., 2017). The transmission electron microscopy (TEM) analysis indeed uncovered that the *Burkholderia* isolates formed polar flagella and pili surrounding the cells (Fig. 1D).

The Burkholderia *isolates are highly resistant to glyphosate without degrading it*

To examine the glyphosate resistance of the *Burkholderia* isolates 1CH1 and 2CH1 the bacteria were propagated on C-Glc agar plates containing increasing amounts of glyphosate (0–80 mM). The *E. coli* and *B. subtilis* strains W3110 and SP1 respectively, served

as controls. All bacteria grew on C-Glc plates without glyphosate and the *E. coli* and *B. subtilis* laboratory strains did not grow with 10 mM glyphosate (Fig. 2A) (Wicke *et al*., 2019). By contrast, the *Burkholderia* isolates 1CH1 and 2CH1 grew at glyphosate concentrations of up to 60 mM. Both bacteria did not grow with 70 and 80 mM glyphosate (Fig. 2A). To conclude, the high resistance of the *Burkholderia* isolates 1CH1 and 2CH1 to glyphosate could explain why the bacteria survive in commercial Roundup.

To assess the ability of the *Burkholderia* isolates to use glyphosate as a source of phosphorus the bacteria were cultivated in an MOPS-based minimal medium (C-Glc-MOPS) supplemented with different sources of phosphorus (120 mM phosphate or 10 mM glyphosate). The *E. coli* and *B. subtilis* strains W3110 and SP1 respectively, served as controls. The control bacteria, *Burkholderia* isolate 1CH1 and *Burkholderia* isolate 2CH1 were able to grow in C-Glc-MOPS-K medium containing 120 mM phosphate, albeit to a lesser extent (Fig. 2B). None of the bacteria showed significant growth in C-Glc-MOPS medium without a source of phosphorus and with 10 mM glyphosate (Fig. 2B). UV spectrophotometric analysis revealed no traces of $o\text{-}PO_4^{3-}$ in the C-Glc-MOPS-glyphosate medium. Thus, the slight increase of the optical density of the culture containing *Burkholderia* isolate 1CH1 and C-Glc-MOPS-glyphosate medium was probably due to traces of phosphate carried along by the preculture medium.

To ensure that the *Burkholderia* isolates 1CH1 and 2CH1 were experiencing phosphate stress during cultivation without phosphate and with glyphosate, we used alkaline phosphate activity as an indicator of phosphate limitation (Shropshire *et al*., 2021). Both *Burkholderia* isolates showed a significant increase in alkaline phosphatase activity after 2 h of cultivation without phosphate and

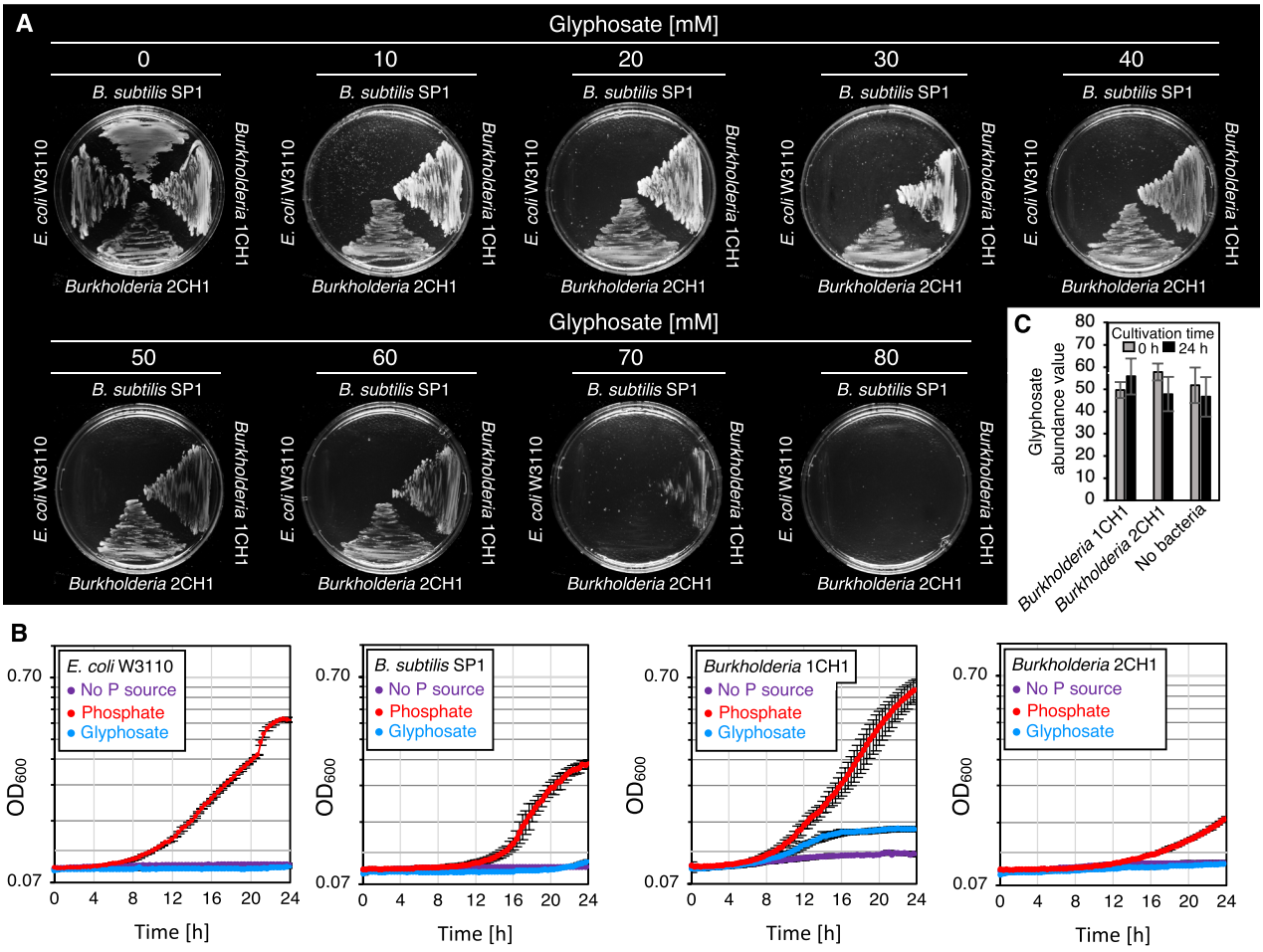

Fig. 2. Assessment glyphosate resistance of the *Burkholderia* isolates and their potential to utilize glyphosate as a source of phosphorus. A. Growth of the *B. subtilis* and *E. coli* strains SP1 and W3110 respectively, and the *Burkholderia* isolates 1CH1 and 2CH1 on C-Glc minimal medium plates containing increasing amounts of glyphosate (incubation for 2 days, 37°C). B. Growth experiments to assess phosphorus utilization of the *Burkholderia* isolates 1CH1 and 2CH1.C. Determination of glyphosate degradation by the *Burkholderia* isolates 1CH1 and 2CH1. The glyphosate concentration in the medium was adjusted to 30 mM. The error bars represent standard errors of means of triplicate cultures.

in medium containing glyphosate (Fig. S1). The alkaline activity further increased after 4 and 24 h of cultivation. The lower phosphatase activity of *Burkholderia* 2CH1 cultures with added glyphosate might derive from a global effect on gene expression due to glyphosate exposure (Kang *et al*., 2011; Lu *et al*., 2013). However, these results confirmed that the *Burkholderia* isolates 1CH1 and 2CH1 were experiencing phosphate starvation, which is a prerequisite for testing the consumption of alternative sources of phosphorus such as glyphosate (Hove-Jensen *et al*., 2014). We determined the stability of glyphosate in cultures containing the *Burkholderia* isolates and C-Glc-MOPS-glyphosate medium by GC–MS analyses. For this purpose, the bacteria were incubated for 24 h in C-Glc-MOPS medium supplemented with 30 mM glyphosate as a phosphorus source. As shown in Fig. 2C, no significant decrease of the glyphosate concentration was detectable in the cultures containing the *Burkholderia* isolates.

Finally, we examined whether the *Burkholderia* isolates and the microbial consortium present in the Roundup spray bottle could grow in the Roundup solution. For this purpose, 10 ml of the Roundup solution from 1CH1 and 2CH1 were centrifuged (10 min, 5000 *g*). Together with the *Burkholderia* isolates, the sedimented cells were used to inoculate shake flasks containing 10 ml of the sterile-filtrated Roundup solution, which were incubated for 7 days (37°C, shaking at 180 rpm). No growth was observed in any of the cultures.

To conclude, the Roundup spray bottles 1 and 2 contain 10^6 viable bacteria ml^{-1} that are resistant to glyphosate. The *Burkholderia* isolates 1CH1 and 2CH1 are highly resistant to glyphosate without degrading it. In addition to the experiments described here, different conditions for glyphosate breakdown were tested, but degradation was not detected in any case.

Genome sequencing and proteomic analyses of the Burkholderia *isolates*

The taxonomic classification revealed that the bacterial isolates 1CH1 and 2CH1 belong to the genus *Burkholderia*. Illumina whole-genome sequencing allowed us to assemble draft genomes and perform a sequence comparison to genome sequences of phylogenetically related species using the Type (Strain) Genome Server (TYGS) (Meier-Kolthoff and Göker, 2019). Results revealed the *Burkholderia* isolates 1CH1 and 2CH1 associate with species belonging to the *B*. *anthina* and *B*. *cenocepacia* complexes respectively. Average nucleotide analysis with genomes retrieved from the TYGS analysis revealed 1CH1 and 2CH1 have 99% nucleotide identity with *B*. *anthina* LMG20980 (DSMZ16086) and *B*. *cenocepacia* J2315 type strains (Fig. 3). Thus, *Burkholderia* isolates 1CH1 and 2CH1 are designated as *B*. *anthina* 1CH1 and *B*. *cenocepacia* strains 2CH1, from now on. Next, we assessed whether high-level glyphosate resistance is a general property of *B*. *anthina* strains. For this purpose, we cultivated the *B*. *anthina* strain DSMZ16086 with 10 mM glyphosate and observed no growth, indicating that *B*. *anthina* species are not generally glyphosate resistant.

At the time of this study, no complete genome sequence was available for the *B*. *anthina* species, and the available draft genomes ranged from 7.2 to 9.3 Mbp. To create a reference genome for this species, we obtained the first complete genome of *B*. *anthina* strain 1CH1. Therefore, this strain was additionally sequenced with Nanopore. Unfortunately, hybrid genome assemblies did not lead to closed replicons, indicating extended repetitive genome regions. Only a pure long read assembly resulted in almost closed replicons with one uncertainty to resolve the sequence data in four or five independent replicons. To determine the replicon number and to estimate their sizes, pulsed-field gel electrophoretic (PFGE) analyses were performed. Results confirmed the presence of two chromosomes (4 and 3 Mbp), a megaplasmid (1 Mbp) and two plasmids (270 and 150 kbp) (Fig. S2). PFGE allowed resolving of the *B*. *anthina* 1CH1 genome, which is the first closed genome of a *B*. *anthina* species.

Finally, we confirmed genome analysis of *B*. *anthina* 1CH1 [NZ_CP071825.1 to NZ_CP071829.1] and *B*. *cenocepacia* 2CH1 [NZ_JAGEMP000000000.1] by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. *Burkholderia anthina*. 1CH1 and *B*. *cenocepacia* 2CH1 were identified with score values of 2.10–2.18 and of 2.37–2.39 respectively. Thus, the genome sequencing and the MS analyses confirmed the identities of two previously undescribed glyphosate-resistant *Burkholderia* isolates.

The EPSP synthases of B. anthina *1CH1 and* B. cenocepacia *2CH1 are glyphosate sensitive*

We assessed the glyphosate sensitivities of the EPSP synthases from *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 through heterologous expression of their *aroA* genes. Both genes were introduced into the plasmid pAC7, allowing their expression in *E*. *coli* (Weinrauch *et al*., 1991). We also cloned the *aroA* and *aroE* genes from *E*. *coli* and *B*. *subtilis* respectively, and the recently described *E*. *coli aroA*C358A allele encoding the AroAR120S variant with slightly reduced glyphosate sensitivity (Wicke *et al*., 2019). The expression of the EPSP synthase genes was driven by the strong constitutively active P_{alf4} promoter (Gundlach *et al*., 2017). The empty plasmid pAC7 and the plasmids carrying the EPSP

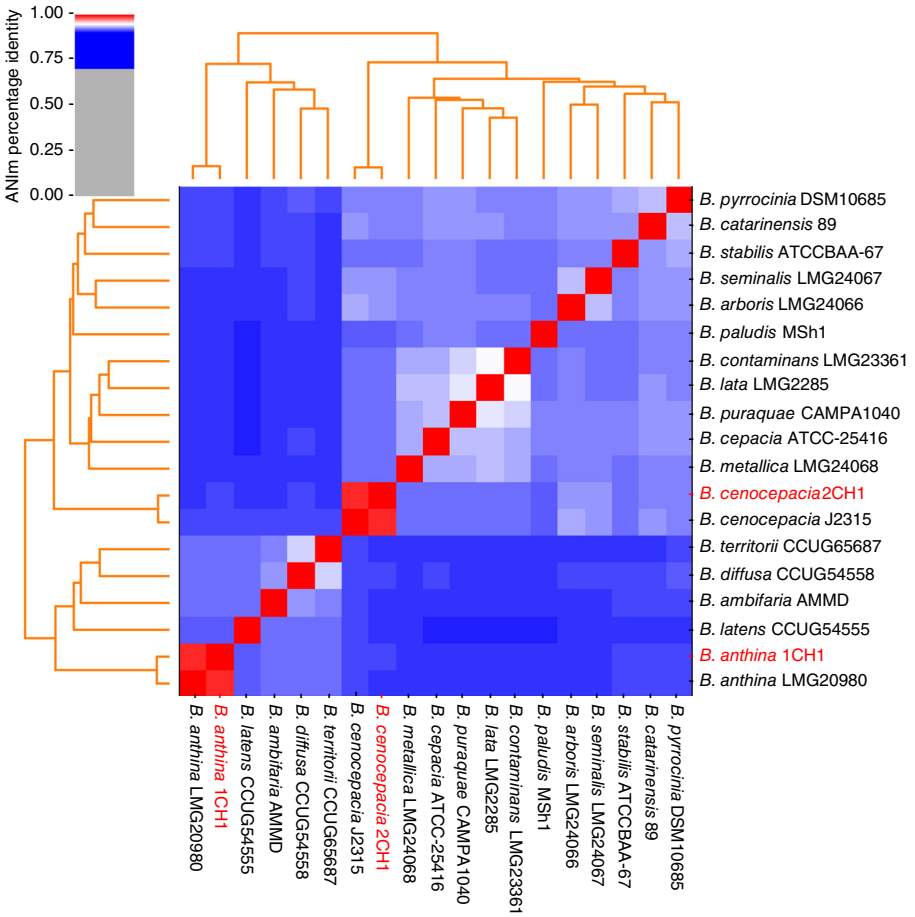

Fig. 3. Phylogenetic analysis of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1. For the phylogenetic analysis, a subset of representative strains from the genus *Burkholderia* was considered. Calculations were done with pyani (https://github.com/widdowquinn/pyani) (Richer and Rosselló-Móra, 2009; Arahal, 2014) using the ANIm method with standard parameters.

synthase genes were used to transform the *E. coli* strain JW0891-1 lacking the native *aroA* gene. The generated *E. coli* strains were propagated on C-Glc minimal medium plates without glyphosate and supplemented with either 20 or 40 mM glyphosate. As expected, the strain carrying the empty plasmid did not grow on the C-Glc minimal medium plates (Fig. 4). By contrast, the derivatives of the *E. coli aroA* mutant carrying the plasmids with the EPSP synthase genes from *B. subtilis*, *E. coli*, *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 grew on plates without aromatic amino acids. Thus, the EPSP synthases from *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 were synthesized and functional in *E. coli*. All *E. coli* strains synthesizing an EPSP synthase also grew with 20 mM glyphosate (Fig. 4). The fact that the plasmid pAC7 is present in multiple copies in *E. coli* and that a strong constitutively active promoter drives the expression of the EPSP synthase genes could explain why no differences in the glyphosate resistance levels were detectable among the strains. None of the *E. coli* strains grew with 40 mM glyphosate (Fig. 4). Thus, at least in *E. coli*, the EPSP synthases from *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 do not confer glyphosate resistance.

Antibiotic susceptibility tests with B. anthina *1CH1 and* B. cenocepacia *2CH1*

Previously, it has also been observed that the exposure of soil bacteria to herbicides like glyphosate promotes the development of antibiotic resistances (Liao *et al*., 2021). Since we observed that *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 are highly resistant to glyphosate (Fig. 2A), we examined whether the exposure of the bacteria to glyphosate could have led to a general increase of antibiotic resistance. For this purpose, we performed antibiotic susceptibility tests (ASTs) and determined the

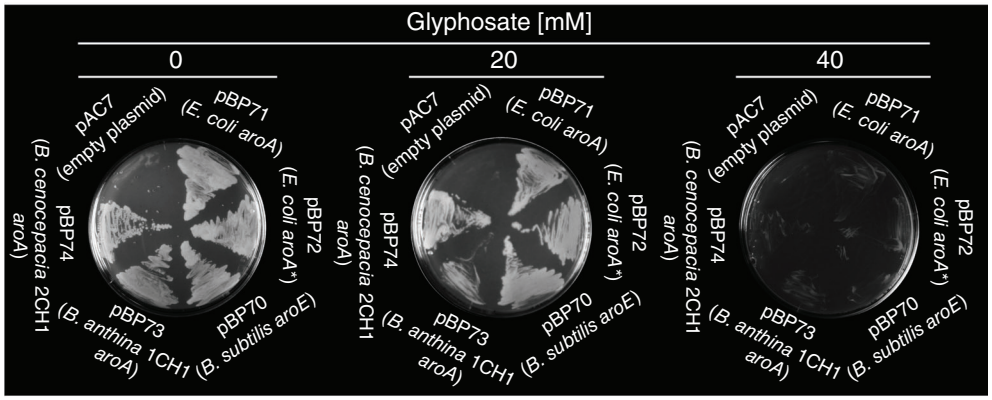

Fig. 4. Expression of the *aroA* genes from the *Burkholderia* isolates to assess the glyphosate sensitivities of the encoded EPSP synthases. Growth of the *E. coli aroA* mutant strain JW0891-1 carrying the plasmids pAC7 (empty plasmid), pBP71 (*E. coli aroA*), pBP72 (*E. coli aroA**), pBP70 (*B. subtilis aroE*), pBP73 (*B. anthina* 1CH1 *aroA*) and pBP74 (*B. anthina* 2CH1 *aroA*) on LB agar plates without and with either 20 or 40 mM glyphosate. The plates were incubated for 1 day at 37°C.

zone of inhibitions around filter discs containing antibiotics targeting the cell envelope, DNA condensation/segregation, translation and cofactor synthesis. The *E. coli* and *B. subtilis* strains W3110 and SP1 respectively, served as controls. *Bacillus subtilis*. SP1 was susceptible to all tested antibiotics (Fig. 5). *Escherichia. coli* W3110 and *B. anthina* 1CH1 were not susceptible to erythromycin, lincomycin and sulfonamide. By contrast, *B. cenocepacia* 2CH1 was only susceptible to five of the 14 tested antibiotics (Fig. 5). Next, we determined MICs of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 using the VITEK-2 fully automated equipment (BioMérieux). The card used in this experiment was created especially for non-fermenting bacteria like *Pseudomonas* spp. including *Burkholderia*. In contrast to *B. anthina* 1CH1, *B. cenocepacia* 2CH1 was less susceptible to 10 of the 12 used antibiotics (Table S6). To conclude, the high glyphosate resistance of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 does not cause a general increase in antibiotic resistance.

Determination of the microbial communities present in commercial Roundup solutions

The 16S rRNA gene sequencing analysis revealed that the Roundup solutions contained several bacteria belonging to different genera. To assess the microbial diversity of the Roundup solutions, we propagated 100 µl of each Roundup solution spray present in bottles 1 (CH1) and 2 (CH2) as well as from a third bottle (CH3), which was ordered later but belonged to the same production batch, on BHI rich medium plates. The cells of the bacterial colonies that appeared on the plates after 24 h of incubation at 37°C were dissolved in 5 ml 0.9% (wt./vol.) NaCl solution and collected by centrifugation (5 min, 5000*g*) for chromosomal DNA isolation. In addition, cells from three Roundup bottles were directly collected and chromosomal DNA was extracted. All six DNA isolations were subjected to 16S rRNA gene amplicon sequencing. The analysis of the relative abundances of all detected bacterial species allowed us to identify 12 taxonomical units (ASVs, see Table S7) (Fig. 6). The genera *Burkholderia* and *Paraburkholderia* were predominant in the Roundup solutions and active as they grew on BHI agar plates. Other bacteria present in the Roundup solutions belong to the genera *Pseudomonas* and *Rhodospirillum*. However, they could not be enriched on BHI agar plates (Fig. 6). To conclude, the amplicon sequencing results confirmed that the three Roundup solutions contained bacteria, most of which belong to the genera *Burkholderia* and *Paraburkholderia*.

Discussion

In the present study, we have characterized two glyphosate-resistant *Burkholderia* strains, namely *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 that were isolated from a commercial Roundup solution. Previously, it has been demonstrated that related species such as the *Burkholderia vietnamiensis* strain AQ5-12 and the *Burkholderia* sp. strain AQ5-13 indeed have the ability to degrade glyphosate (Manogaran *et al*., 2018a, 2018b). Both strains were isolated from glyphosate-contaminated sites in Malaysia (Manogaran *et al*., 2017). Although their ability to degrade glyphosate seems to be clear, the metabolic pathway responsible for glyphosate degradation and the mechanism of phosphate release remains to be identified. Surprisingly, our *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 strains could not use glyphosate as a source of phosphorus, at least under the tested conditions. We could demonstrate that both isolates are highly resistant to glyphosate. The genomes of *B. anthina*

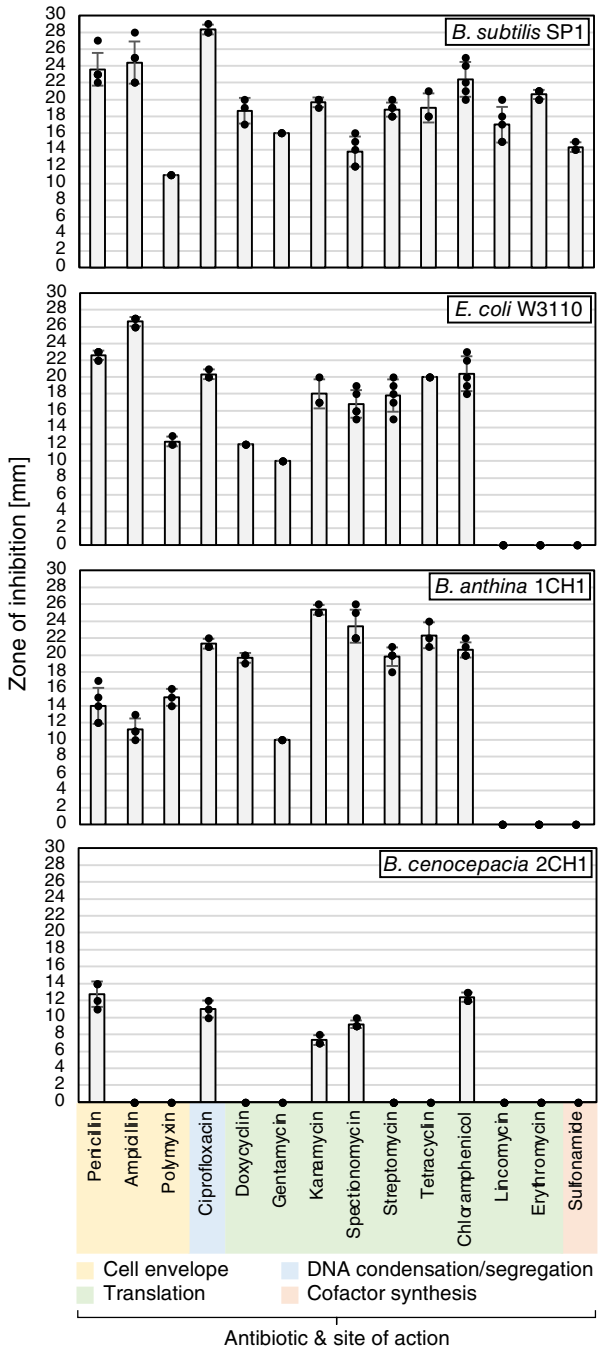

Fig. 5. Antibiotic susceptibility tests. Error bars represent standard errors of means of triplicate plating experiments.

1CH1 and *B*. *cenocepacia* 2CH1 strains contain genes coding for enzymes that are potentially involved in the degradation of amino phosphonates. However, the presence of those genes does not allow the conclusion that these bacteria are *per se* able to break down glyphosate (Hove-Jensen *et al*., 2014). In the past years, several molecular mechanisms conferring glyphosate resistance in bacteria and other organisms have been described (for a recent review, Hertel *et al*., 2021). As described above for the *Burkholderia vietnamiensis* strain AQ5-12 and the *Burkholderia* sp. strain AQ5-13, bacteria can be resistant to glyphosate due to their ability to degrade it (Manogaran *et al*., 2018a, 2018b; Hertel *et al*., 2021). Moreover, bacteria can acquire glyphosate resistance by detoxifying the herbicide (Rao *et al*., 1983; Penaloza-Vazquez *et al*., 1995). Furthermore, a variety of glyphosate-resistant bacteria and plants have been isolated that either synthesize glyphosate-insensitive mutant variants of the EPSP synthase or overproduce the target of glyphosate (Hertel *et al*., 2021). Finally, glyphosate resistance can be achieved by decreasing the uptake or increasing the export of the herbicide (Hertel *et al*., 2021). At a first glance, there seemed to be several possibilities for high glyphosate resistance of the *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 strains. On the one hand, glyphosate resistance could be caused by covalent modification and thus detoxification of the herbicide (Castle *et al*., 2004). On the other hand, the *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 strains could be simply resistant to glyphosate due to reduced uptake of the herbicide. Finally, the isolates could have evolved glyphosate resistance by acquiring mutations in the *aroA* genes rendering the encoded EPSP synthase insensitive to glyphosate (Barry *et al*., 1992; Wicke *et al*., 2019). By performing cultivation experiments in combination with GC–MS analyses, we could exclude the possibilities that the *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 strains are glyphosate resistant due to degradation or covalent modification of the herbicide. Moreover, the expression of *aroA* from *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 did not confer glyphosate resistance to *E*. *coli*. Thus, our *Burkholderia* isolates are also not glyphosate resistant due to the presence of glyphosate-insensitive EPSP synthase variants. Since we observed that the amount of glyphosate in cultures containing *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 seemed to be unaffected, we hypothesise that resistance is caused by hindered entry of glyphosate into the cell. Recently, we could indeed show that soil bacteria like *Bacillus subtilis* can evolve glyphosate resistance by acquiring loss-of-function mutations in glutamate uptake systems (Wicke *et al*., 2019). Thus, the inactivation of promiscuous transport systems could be a common mechanism conferring resistance to this herbicide. Alternatively, the uptake system for glyphosate may simply not be expressed. Thus, the underlying molecular mechanism for high-level glyphosate resistance of *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1 remains to be identified.

The taxonomic classification of our *Burkholderia* isolates revealed that these are two novel strains of *B*. *anthina* and *B*. *cenocepacia*. Given the fact that

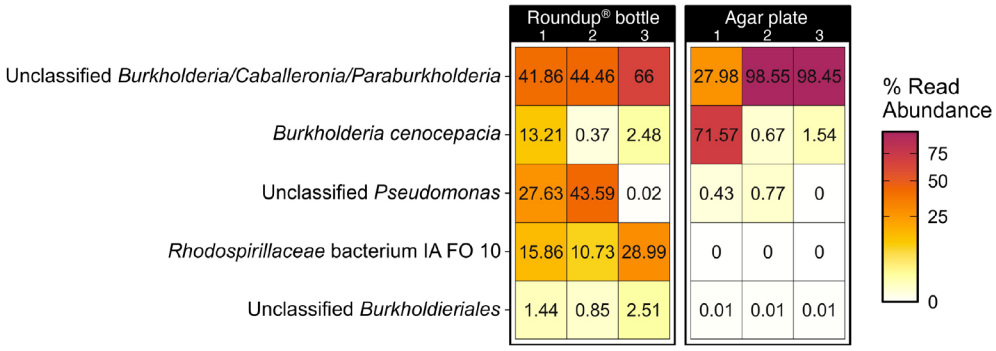

Fig. 6. Determination of the bacterial community compositions in commercial Roundup solutions by 16S rRNA gene amplicon sequencing. Relative abundance of the species detected in three Roundup bottles and of the species grown on BHI rich medium plates.

species belonging to the genus *Burkholderia* have been isolated from remarkably diverse ecological niches like soils, rhizospheres, water, plants, fungi, animals, hospital setting and diseased humans (Coeyne and Vandamme, 2003; Vial *et al*., 2011) it is not surprising that they might also occur in commercial products such as herbicide solutions. However, it remains unclear why the Roundup solutions contained such large amounts of bacteria (10^6 viable bacteria ml^{-1}), which obviously do not degrade glyphosate. Both *B. anthina* and *B. cenocepacia* belong to the so-called *Burkholderia cepacia* complex (Vandamme *et al*., 2002; Drevinek and Mahenthiralingam, 2010). Strains of *B. anthina* and *B. cenocepacia* have been isolated from the environment and from infected human such as cystic fibrosis patients (Vandamme *et al*., 2002; Drevinek and Mahenthiralingam, 2010). Moreover, *B. cenocepacia* belongs to the dominant species of the *B. cepacia* complex and some isolates of *B. cenocepacia* are known to be multiresistant against antibiotics (Drevinek and Mahenthiralingam, 2010; Rhodes and Schweizer, 2016; Scoffone *et al*., 2020). Indeed, the *B. cenocepacia* 2CH1 strain was much less susceptible than *B. anthina* 1CH1 to a variety of antibiotics. In the future, study of the precise molecular mechanism underlying the glyphosate resistance of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 should be carried out. Moreover, the ability of *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 to act as true pathogens should be addressed.

Experimental procedures

Bacterial strains, DNA manipulation and chemicals

All bacteria used in this study are listed in Table S1. Primers were purchased from Sigma-Aldrich (Munich, Germany) and are listed in Table S2. *Bacillus subtilis*. chromosomal DNA was isolated using the peqGOLD Bacterial DNA Kit (Peqlab, Erlangen, Germany). Plasmids were isolated from *E. coli* using the Nucleospin Extract Kit (Macherey and Nagel, Germany). PCR products were purified using the PCR Purification Kit (Qiagen, Germany). Phusion DNA polymerases, restriction enzymes and DNA ligases were purchased from Thermo Scientific (Germany) and used according to the manufacturer's instructions. Other chemicals and media were purchased from Sigma-Aldrich, Carl Roth (Karlsruhe, Germany) and Becton Dickinson (Heidelberg, Germany). DNA sequencing performed the Microsynth-SeqLab Sequence Laboratories (Göttingen, Germany). The DNA sequences were evaluated using the Geneious software package (Kearse *et al*., 2012).

Roundup solution

The glyphosate-containing Roundup solution 'Roundup Unkrautfrei ALPHEE' (1 L) (article number: 827562; production date: C7376 – PROD. DATE 22/11/2017) was purchased from Westfalia (www.westfalia.de, Hagen, Germany). The same solution containing 1% (wt./vol.) glyphosate isopropylamine salt, 0.5% (wt./vol.) surfactant and 98.5% (wt./vol.) H_2O was ordered three times and designated as CH1, CH2 and CH3.

Plasmid construction

The plasmids that were used and constructed in this study are listed in Table S3. The *E. coli* strain XL1-Blue was used for cloning experiments and the transformation was performed using standard procedures (Sambrook *et al*., 1989). For the constitutive expression of *aroA*, *aroAC358A* and *aroE* EPSP synthase genes from *B. anthina* 1CH1, *B. cenocepacia* 2CH1, *E. coli* W3110, *E. coli* BPE1 and *B. subtilis* SP1 (Table S1), the genes were amplified using chromosomal DNAs and the primer pairs LMS9/LMS10, JM3/JM4, JM7/LMS10 and JM8/JM9. The forward primers contain the constitutively active P_{alf4} promoter (Gundlach *et al*., 2017). The PCR

fragments were purified, digested with the restriction enzyme pair BamHI/EcoRI and ligated to pAC7 that was cut with the same enzymes (Weinrauch *et al*., 1991). The EPSP synthase gene sequences of the plasmids pBP70, pBP71, pBP73 and pBP74 (Table S3) were verified by Sanger sequencing.

Bacterial strains, growth conditions and construction of mutant strains

The *B*. *subtilis* and *E*. *coli* strains were grown in lysogeny broth (LB). Agar plates were prepared with 1.5% (wt./vol.) agar (Roth, Germany). Brain heart infusion (BHI) medium (Sigma-Aldrich, Darmstadt, Germany) was used for the cultivation of bacterial isolated from the Roundup solutions. *E*. *coli* transformants were selected on LB plates containing ampicillin (100 μg ml^{-1}). Growth in liquid medium was monitored using 96-well plates (Microtest Plate 96-Well, F, Sarstedt) at 37°C and medium orbital shaking at 237 cpm (4 mm) in an Epoch 2 Microplate Spectrophotometer, equipped with the Gen5 software (02.09.2001; BioTek Instruments) and the OD_{600} was measured in 15 min intervals. C-Glc minimal medium contained glucose and ammonium as sources of carbon and nitrogen respectively (Commichau *et al*., 2007). C-Glc-MOPS minimal medium was used to assess the ability of the *Burkholderia* isolates to utilize glyphosate as a source of phosphorus. 1 l of C-Glc-MOPS minimal medium was composed of 200 ml 5 X MOPS buffer (16.58 g L^{-1} $(NH_4)_2SO_4$, 41.9 g L^{-1} MOPS, 3.6 g L^{-1} Tricine), 10 ml ammonium iron(III) citrate (2.2 mg L^{-1}), 10 ml III' salt solution (Commichau *et al*., 2007) and 10 ml of a 50% (w/v) glucose solution. C-Glc-MOPS-K medium contained 200 ml of a 5 X potassium phosphate solution (20 g L^{-1} KH_2PO_4, 80 g L^{-1} K_2HPO_4). Glyphosate was added as indicated. A modified minimal A medium (Shropshire *et al*., 2021) was used to assess phosphate limitation. The medium was composed of Na-succinate (5.4 g L^{-1}), NaCl (200 mg L^{-1}), NH_4Cl (450 mg L^{-1}), $CaCl_2$ (200 mg L^{-1}), KCl (200 mg L^{-1}), $MgCl_2$ (450 mg L^{-1}) $FeCl_2$ (10 mg L^{-1}), $MnCl_2$ (10 mg L^{-1}) and 10 mM Hepes buffer (pH 7). Na_2HPO_4 or glyphosate was added as a source of phosphorus at a final concentration of 5 mM.

Taxonomic classification of the isolates

Initial taxonomic classification of the bacteria that were isolated from the Roundup solution was realized by sequencing the 16S rRNA genes. The 16S rRNA genes were amplified by PCR using the primer pair 27F/1492R (Frederiksson *et al*., 2013). The sequences obtained by Sanger sequencing (Microsynth-SeqLab Sequence Laboratories) were compared to the rRNA/ITS databases of NCBI via BLASTn (Table S4). Furthermore, both draft genome sequence data sets were uploaded to the TYGS, available at https://tygs.dsmz.de, for a whole genome-based taxonomic analysis (Meier-Kolthoff and Göker, 2019). All genomes of type strains involved in the TYGS analysis were downloaded from GenBank in FASTA format and used for average nucleotide identity analysis using the average_nucleotide_identity.py script (https://github.com/widdowquinn/pyani#script-average_nucleotide_identitypy), employing the ANIm option.

Genome sequencing and assembly

Total nucleic acids were extracted using the MasterPure™ DNA Purification kit (Epicentre, Madison, WI, USA) and directly used for sequence library preparations without further processing. Illumina paired-end shotgun libraries were generated using the Nextera XT DNA Sample Preparation Kit and sequenced using the MiSeq system and reagent kit V.3 (2 × 300 bp) (Illumina, San Diego, CA, USA). For Nanopore sequencing, libraries were prepared using the Ligation Sequencing Kit 1D (SQK-LSK108) and the Native Barcode Expansion kit (EXP-NBD103) (Oxford Nanopore Technologies, Oxford, UK). Sequencing was performed for 72 h on a MinION device Mk1B and a SpotON Flow Cell R9.4 using MinKNOW software v19.06.8 (Oxford Nanopore Technologies). Short read called with the MiSeq Control Software v2.6.2.1 and Nanopore long reads with Guppy v3.2.1.

Illumina paired-end reads were quality-processed, adapter trimmed with Trimmomatic v.0.39 (Bolger *et al*., 2014), which resulted in 2 × 3 292 908 high quality reads for *B*. *anthina* 1CH1, and 2 × 3 658 376 for 2CH1. Long reads of *B*. *anthina* 1CH1 were quality trimmed with Filtlong (https://github.com/rrwick/Filtlong) using all respective quality processed Illumina short reads as a reference and setting the minimum size for the long reads to 1000 bp. The unprocessed long reads were 1 957 773 916 bp with an N50 of 16 134 bp and an N90 of 2885 bp, and the processed 1 762 010 791 bp with an N50 of 17 290 bp and an N90 of 4414 bp.

Genome assembly for *B*. *anthina* 1CH1 was performed with the Flye 2.8.2-b1689 long-read assembler (Lin *et al*., 2016; Kolmogorov *et al*., 2019) using raw and quality processed long reads as input in separate assemblies. The minimum overlap was set to 1500 bp. Assembly results were visualized and compared with each other for consistency with Bandage (Wick *et al*., 2015). Data from a pulsed-field gel electrophoresis (PFGE) analyses (see below) helped to resolve the sizes and number of present replicative units. The final *B*. *anthina* 1CH1 genome was polished using the unicycler_polish.py pipeline-script (https://github.com/rrwick/Unicycler/blob/master/docs/unicycler-polish.md). The replication origins of each replicon were identified

using Ori-Finder 2 (Luo *et al*., 2014) and the individual units aligned manually accordingly.

The genome assembly of *B. cenocepacia* 2CH1 was performed with the SPAdes assembler version 3.14.0 (Bankevich *et al*., 2012). Contigs smaller than 500 bp and with a coverage <10% of the average were removed. For both genomes, assembly quality was verified with QualiMap v.2.2.1 (Okonechnikov *et al*., 2016) and Genome consistency for *B. anthina* 1CH1 with breseq version 0.35.5 (Deatherage and Barrick, 2014). Contigs of the *B. cenocepacia* 2CH1 genome were sorted with Mauve employing *B. cenocepacia* J2315 (NC_011000.1) as reference. Both genomes were annotated with the PGAP pipeline (Tatusova *et al*., 2016) during upload to GenBank.

Pulsed-field gel electrophoresis

PFGE analyses were performed to resolve the sizes and number of present replicative units of the genome that was subjected to Illumina and Nanopore sequencing. Preparation of unbroken genomic DNA *in situ* in agarose blocks was performed as described by .Tynkkynen *et al*.,. (1999) with slight modifications (Tanskanen *et al*., 1990). Briefly, cells of 1.5 ml LB overnight cultures were harvested by centrifugation, washed with 10 mM Tris, 20 mM NaCl, 50 mM EDTA (pH 7.2) and embedded in InCert™-agarose dissolved in the respective running buffer, lysed with 2.5 mg ml^{-1} lysozyme for 4.5 h at 37°C with a subsequent proteinase K treatment at 50°C for about 16 h. Enzymes were removed by washing four times for 1 h with 20 mM Tris, 50 mM EDTA (pH 8.0) and the two solutions for the first washing steps contained 1 mM PMSF. Before loading 1 mm thick plug stripes into the PFGE-gel pockets, plug pieces were equilibrated for 1 h in running buffer. PFGE was performed with the system CHEF-DR® III system (BioRad, Germany) three times under different conditions to visualize the bigger replicative units (5–1 Mbp and 1 Mbp–220 kbp) and the smaller replicative units (300–50 kbp). Electrophoresis parameters were set according to the recommendations of BioRad and NEB for the used DNA markers (Table S5). The resulting Gels were stained with ethidium bromide and the DNA fragments were visualized by UV light.

Identification of isolates by MALDI-TOF analysis

Samples were prepared for analysis by the Bruker Biotyper® Sirius matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry system according to the manufacturer's instructions. Briefly, isolates were propagated on Columbia blood agar plates with 5% sheep blood (Becton Dickinson Microbiology Systems Sparks, USA) and incubated for 24 h at 37°C. Few bacterial materials of single colonies were transferred to a 96-spot polished steel target plate (Bruker Daltonik GmbH) and a saturated solution of 1.0 µl of MALDI matrix (alpha-cyano-4-hydroxycinnamic acid matrix solution; Bruker Daltonik GmbH) was applied to each sample and dried. Spectra ranging from 2000 to 20 000 *m/z* were analysed using the MALDI Biotyper system's automation control and the current Bruker Biotyper software and library. Identification scores of ≥2.000 indicated positive species-level identification. Scores of <2.000 indicated only genus-level identification. Scores below 1.700 indicated no reliable identification.

Determination of glyphosate degradation

100 µl of the internal standard *allo*-inositol (0.05 mg ml^{-1}) and 250 µl extraction solvent [methanol/chloroform/water 32.35:12.5:6.25 (vol./vol./vol.)] were added to 50 µl of the growth medium. 10 µl of the resulting upper aqueous phase were dried under nitrogen steam and derivatized as described previously (Rotsch *et al*., 2017). GC–MS analysis was performed as previously described (Touraine *et al*., 2019). The two obtained derivatives of glyphosate (carrying 3 and 4 trimethylsilyl groups respectively) were quantified using the mass to charge ratios of 385 and 457 respectively and added based on their total ion count.

Determination of ortho-phosphate and total phosphorus

To determinate the amount of potentially free ortho-phosphate (o-PO_4^{3-}) and total phosphorus (i.e. organically bound) in the glyphosate used for the growth experiments (Sigma-Aldrich), o-PO_4^{3-} measurements were carried out according to the European standard procedure EN ISO 6878:2004 using a Shimadzu UV-2450 spectrophotometer (Tokyo, Japan). The stock solution of glyphosate was first analysed for free soluble o-PO_4^{3-}. Chemical digestion was carried out with the same stock solution by adding 200 mg of 'Oxisolv' (Merck, Darmstadt, Germany) into 5 ml sample volume. The samples were treated using the microwave digestion unit MARS 5 (CEM, Kamp-Lintfort, Germany). The samples were linearly heated to 170°C within 3 min and the temperature was maintained for an additional 3 min. The samples were cooled down to room temperature and the total phosphorus was measured as o-PO_4^{3}.

Alkaline phosphate assay

The alkaline phosphate assay to determine whether the bacteria experience phosphate starvation was performed according to Shropshire *et al*., (2021). with the following

modifications. The *B. anthina* 1CH1 and *B. cenocepacia* 2CH1 strains were cultivated overnight in 4 ml LB medium at 37°C at 220 rpm. The cells were washed twice in minimal A medium. For the adaptation of the bacteria to the minimal A medium, the cell pellets were dissolved minimal A medium lacking any source of phosphorus and incubated for 3 h at 37°C. Next, the 6 ml minimal A medium without a source of phosphorus and containing either 5 mM Na_2HPO_4 or 5 mM glyphosate supplemented in 50 ml plastic reaction tubes were inoculated to an OD_{600} of 0.1. The samples for determining the activity of the alkaline phosphatase were taken at 0, 2, 4 and 24 h and the OD_{600} was determined. The total volume of an assay sample was 1 ml and it was composed of 1 mM *p*-nitrophenol phosphate (10 µl of a 100 mM stock solution), Tris buffer (pH 8) (940 µl of a 100 mM stock solution) and 50 µl of the minimal A medium containing the cells. The samples were incubated for 1 h at 37°C in the dark and the cells were removed by centrifugation (10 min at 13 000 rpm). The absorption of the *p*-nitrophenol (*p*NP) was determined at a wavelength of 405 nm. As a negative control minimal A medium was added to the reaction tube. The measurements were compared with a *p*NP standard curve to calculate the amount of *p*NP produced within 1 h, normalized to the OD_{600} and multiplied with the dilution factor of 20.

Antibiotic susceptibility tests

ASTs shown in Fig. 5 were performed via a disc diffusion assay. Briefly, the bacteria to be tested were cultivated overnight in 4 ml LB medium at 28°C. Next day, the bacteria were washed twice in 0.9% (wt./vol.) NaCl solution and 100 µl of the cell suspensions were propagated on LB agar plates. Cotton disks (Roth, Germany) containing 5 µl of the antibiotic solutions [penicillin G (100 mg ml^{-1}), ampicillin (100 mg ml^{-1}), polymyxin (10 mg ml^{-1}), ciprofloxacin (1 mg ml^{-1}), doxycycline (6 mg ml^{-1}), gentamycin (2 mg ml^{-1}), kanamycin (50 mg ml^{-1}), spectinomycin (100 mg ml^{-1}), streptomycin (100 mg ml^{-1}), tetracycline (20 mg ml^{-1}), chloramphenicol (5 mg ml^{-1}), lincomycin (25 mg ml^{-1}), erythromycin (2 mg ml^{-1}), sulfonamide (60 mg ml^{-1})] were placed in the centre of the plates, which were incubated for 24 h at 37°C. For evaluating the antibiotic susceptibilities, the zone of inhibition was measured.

The antibiotic susceptibility was also tested using the VITEK®-2 fully automated equipment (BioMérieux, France) (Table S6). The minimum inhibitory concentrations (MICs) were determined for the following antibiotics: piperacillin, piperacillin/tazobactam, ceftazidim, cefepim, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin and colistin. For the AST, the AST-N248 card and the VITEK-2 equipment were applied as recommended by BioMérieux. The strains were analysed for 15.52 h (*B. anthina* 1CH1) or 16.25 h (*B. cenocepacia* 2CH1).

Microscopic analyses

For negative staining (Hoppert and Holzenburg, 1998), a thin carbon film, evaporated by glow discharge onto freshly cleaved mica, was partly floated off on a sample drop. After 1 min of incubation, the carbon-mica sheet was withdrawn and briefly blotted dry with filter paper. Subsequently, the carbon film was again partially floated off briefly on a drop of de-ionized water, withdrawn and blotted dry. Finally, the carbon film was completely floated off on a drop of negative staining solution and then adsorbed onto a 300 mesh copper grid for TEM (Plano, Wetzlar, Germany). Negative staining solution was phosphotungstic acid (3%, wt./vol., adjusted to pH 7.0 with 0.1 N NaOH). The grid was removed from the drop and the staining solution was completely soaked off with filter paper. Electron microscopy was performed with a Jeol 1011 (Jeol, Eching, Germany) equipped with a Gatan Orius SC1000 CCD camera (Gatan, Munich, Germany).

Processing of 16S rRNA gene amplicon data

Paired-end reads were quality-filtered with fastp 0.20.0 (Chen *et al*., 2018) using default settings with the addition of an increased per base phred score of 20, base pair corrections by overlap (-c), as well as 5′- and 3′-end read trimming with a sliding window of 4, a mean quality of 20 and minimum sequence size length of 50 bp. Quality-controlled reads were merged with PEAR v0.9.11 (Zhang *et al*., 2014) and primer clipping was performed with cutadapt 2.5 (Martin, 2011) with default settings. VSEARCH 2.14.1 (Rognes *et al*., 2016) was used for size sorting, size filtering (≥300 bp) and dereplication. The sequences were denoised with UNOISE3 implemented in VSEARCH using default settings and chimaeras were removed with UCHIME3 (*de novo* followed by reference-based) implemented in VSEARCH leading to the final set of amplicon sequence variants (ASVs). Taxonomy of ASVs was assigned with BLCA v2.1 (Gao *et al*., 2017) against the entire SILVA database v138.1 (Quast *et al*., 2013) using default settings. Further data processing was performed with R v4.0.2 (Team R, 2020) and R Studio v1.3.1056 (Team R, 2019). BLCA-derived lineage entries below 80% confidence were marked as unclassified. To remove potential cross-contamination from the sequencer and spurious ASVs, all ASVs with a relative abundance below 0.25% in all samples were removed from the dataset (Reitmeier *et al*., 2021). Data visualization was performed with ampvis2 package

(Andersen *et al*., 2018). The 16S rRNA gene paired-end raw reads are shown in Table S7.

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Data Availability Statement

The genome sequencing data generated in this study have been submitted to the NCBI [*B*. *anthina* 1CH1 (NZ_CP071825.1 to NZ_CP071829.1) and *B*. *cenocepacia* 2CH1 (NZ_JAGEMP000000000.1)].

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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:

Fig. S1. Alkaline phosphatase assay
Fig. S2. PFGE analyses
Table S1. Primers
Table S2. Plasmids
Table S3. Strains
Table S4. 16S rRNA gene sequences
Table S5. PFGE conditions
Table S6. Determination of MICs of *B*. *anthina* 1CH1 and *B*. *cenocepacia* 2CH1
Table S7. ASV sequences